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The Zinc-finger protein ASCIZ regulates B cell development via DYNLL1 and Bim

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Developing B lymphocytes expressing defective or autoreactive pre-B or B cell receptors (BCRs) are eliminated by programmed cell death, but how the balance between death and survival signals is regulated to prevent immunodeficiency and autoimmunity remains incompletely understood. In this study, we show that absence of the essential ATM (ataxia telangiectasia mutated) substrate Chk2-interacting Zn^{2+}-finger protein (ASCIZ; also known as ATMIN/ZNF822), a protein with dual functions in the DNA damage response and as a transcription factor, leads to progressive cell loss from the pre-B stage onwards and severely diminished splenic B cell numbers in mice. This lymphopenia cannot be suppressed by deletion of p53 or complementation with a prearranged BCR, indicating that it is not caused by impaired DNA damage responses or defective V(D)J recombination. Instead, ASCIZ-deficient B cell precursors contain highly reduced levels of DYNLL1 (dynein light chain 1; LC8), a recently identified transcriptional target of ASCIZ, and normal B cell development can be restored by ectopic *Dynll1* expression. Remarkably, the B cell lymphopenia in the absence of ASCIZ can also be fully suppressed by deletion of the proapoptotic DYNLL1 target Bim. Our findings demonstrate a key role for ASCIZ in regulating the survival of developing B cells by activating DYNLL1 expression, which may then modulate Bim-dependent apoptosis.

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Abbreviations used: AID, activation-induced cytidine deaminase; ASCIZ, ATM substrate Chk2-interacting Zn^{2+}-finger protein; HEL, hen egg lysozyme; poly(I:C), polyinosinic:polycytidylic acid.

Developing B cells undergo a series of positive and negative selection steps to allow the generation of a repertoire of peripheral B cells that are able to respond efficiently to foreign antigens but are tolerant to self-antigens (LeBien and Tedder, 2008). One key event during B cell development in the bone marrow is the targeted rearrangement of Ig genes. At the pro-B cell stage, V(D)J recombination of IgH allows production of the pre-BCR, which then sustains proliferation and survival of pre-B cells. V, D, and J coding segments are fused by site-specific recombination, where double-strand breaks generated by RAG1 and RAG2 (Oettinger et al., 1990) are repaired by the NHEJ (nonhomologous end-joining) pathway. Accordingly, RAG-null mice and NHEJ-deficient SCID mice, which have a mutation in DNA-dependent protein kinase, lack mature B lymphocytes (Mombaerts et al., 1992; Shinkai et al., 1992), highlighting the importance of these proteins for early B cell development. At the pre-B cell stage, IgL genes undergo VJ recombination, and upon productive *IgL* rearrangement, heavy and light chains associate to form the BCR, marking the beginning of the immature B cell stage. Immature and transitional B cells expressing an autoreactive BCR are then eliminated by apoptosis to prevent autoimmunity, a process which depends on the proapoptotic BH3-only protein Bim (Enders et al., 2003). It is widely believed that pre-BCR/BCR signals that are too weak or too strong result in programmed cell death (Strasser, 2005), but how apoptotic signaling thresholds are set during these developmental stages and to what extent Bim regulates cell survival also at the pre-B stage remain unclear.

The initial Ig diversity within the B cell repertoire is refined through class switch recombination and somatic hypermutation, both of which are initiated by activation-induced

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cytidine deaminase (AID) upon antigen stimulation in splenic or lymph node germinal centers (Muramatsu et al., 2000). During class switch recombination (Stavnezer et al., 2008), AID mutagenesis can lead to the formation of double-strand breaks in switch regions upstream of the IgH constant region exons and recombination-dependent isotype switching. During somatic hypermutation (Peled et al., 2008), processing of AID-dependent base damage in the Ig variable region leads to the formation of abasic sites whose mutagenic repair may improve antibody affinity to the antigen.

The ATM (ataxia telangiectasia mutated) substrate Chk2-interacting Zn^{2+}-finger protein (ASCIZ; also known as ATMIN/ZNF822) was originally identified as a DNA damage response protein (McNees et al., 2005) specifically involved in the BER (base excision repair) pathway, including oxidative stress responses in vivo (Jurado et al., 2010; Kanu et al., 2010). In the chicken DT40 B cell line, in which AID is constitutively active, ASCIZ deficiency leads to increased rates of Ig diversification, suggesting that under normal conditions ASCIZ channels the repair of AID-dependent base damage into the error-free nonmutagenic BER pathway (Oka et al., 2008). In addition, ASCIZ also has DNA damage–independent functions as an essential transcription factor with crucial roles during the earliest stages of lung organogenesis (Jurado et al., 2010; Heierhorst et al., 2011). A major transcriptional target of ASCIZ is DYNLL1 (dynein light chain 1; Jurado et al., 2012), a multifunctional protein originally identified as a component of the dynein motor complex (King and Patel-King, 1995) with emerging roles as a regulator of hundreds of likely targets (Barbar, 2008; King, 2008; Rapali et al., 2011), including Bim (Puthalakath et al., 1999; Day et al., 2004).

We show in this study that absence of ASCIZ leads to severe peripheral B lymphopenia in mice caused by progressively impaired B cell development in the bone marrow from the pre-B cell stage onwards. This defect appears to be DNA damage independent and mainly caused by unrestrained Bim activity, likely as a consequence of substantially lower levels of its negative regulator DYNLL1 in ASCIZ-deficient B cells. The results highlight the importance of ASCIZ and DYNLL1 in setting homeostatic survival levels during B cell development.

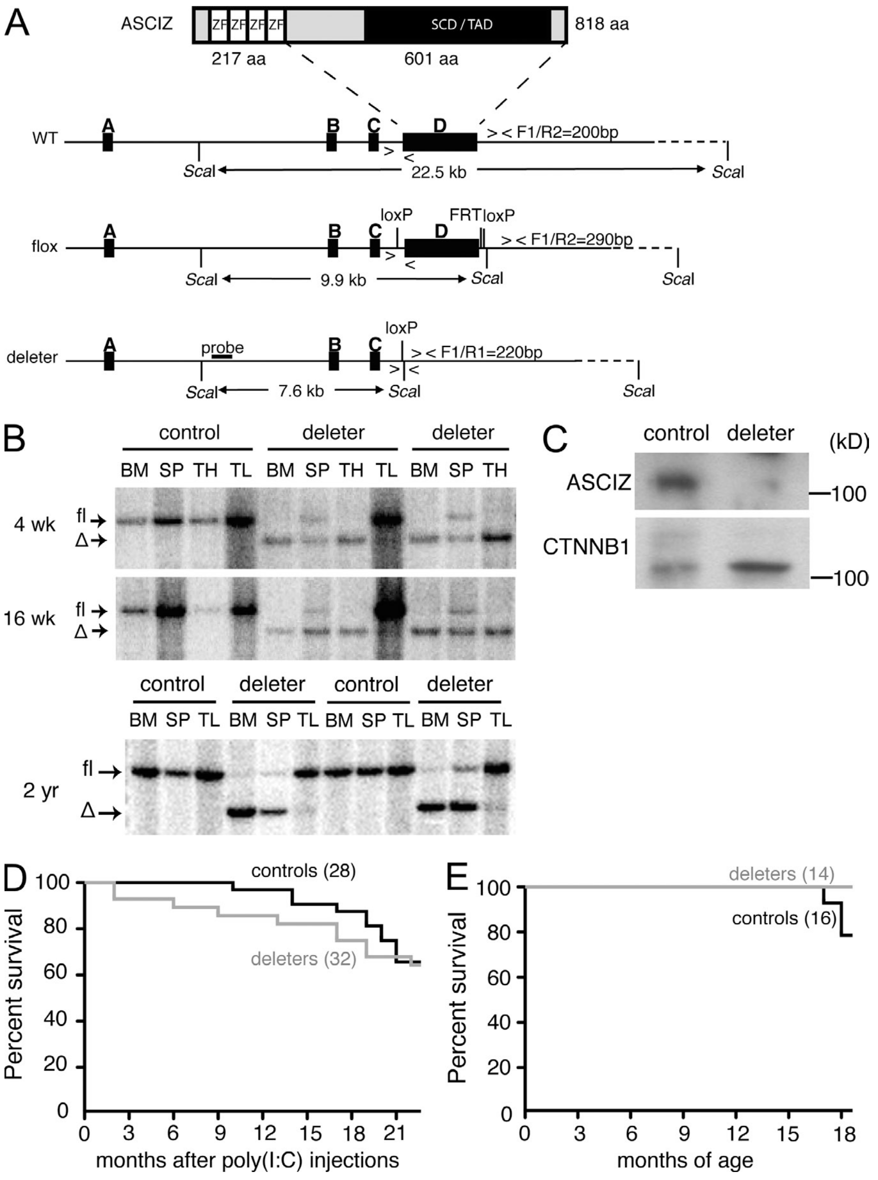

Figure 1. Conditional deletion of *Asciz*. (A) ASCIZ domain organization and conditional deletion strategy. The probe and fragment sizes for Southern blot analysis and primer locations (F1, R1, and R2) and product sizes for PCR genotyping are indicated. SCD, SQ/TQ cluster domain; TAD, transcription activation domain; ZF, Zinc finger. (B) Southern blot analysis of *ASCIZ-Mx* control and deleter tissues 4 wk, 16 wk, and 2 yr after poly(I:C) injection. Samples from four different controls (*Mx1-Cre*$^{-}$*Asciz*$^{fl/fl}$) and six different deleters (*Mx1-Cre*$^{+}$*Asciz*$^{fl/fl}$) are shown. SP, spleen; TH, thymus; TL, tail. (C) Western blot analysis of MACS-purified splenic B cells from single *ASCIZ-Mb* control and deleter mice. β-Catenin (CTNNB1) is used as loading control. Molecular mass standards are indicated on the right. (D) Kaplan-Meyer survival curve of *ASCIZ-Mx* deleters and controls for the indicated times after poly(I:C) injection. n = 28 for controls, which included a mixture of *Mx1-Cre*$^{+}$*Asciz*$^{fl/+}$ and *Mx1-Cre*$^{-}$*Asciz*$^{fl/fl}$ mice, and n = 32 for *Mx1-Cre*$^{+}$*Asciz*$^{fl/fl}$ deleters. Note that there was no statistical difference between the curves and that all nonsurviving mice were sacrificed because of general well-being issues unrelated to neoplasia (seizures, malocclusions, eye infections, etc.). (E) Kaplan-Meyer survival curve of *ASCIZ-Mb* deleters (n = 14) and controls (n = 16; 10 *Mb1/Mb1-Cre Asciz*$^{+/+}$ plus 6 *Mb1-Cre*$^{-}$*Asciz*$^{fl/fl}$).

RESULTS

Pan-hematopoietic deletion of *Asciz* leads to B lymphopenia

To study ASCIZ function in vivo in a wide range of cell types while avoiding the embryonic lethality resulting from its germline deletion (Jurado et al., 2010), we conditionally deleted *Asciz* using interferon-inducible *Mx1*-Cre (Kühn et al., 1995; in the following we refer to this strain as *ASCIZ-Mx*). In the floxed allele, Cre recombinase deletes the entire exon D, which codes for the vast majority of the protein (Fig. 1 A); this is similar to the strategy used for generation of the germline *Asciz* KO (Jurado et al., 2010). After Cre induction by polyinosinic:polycytidylic acid (poly(I:C)) injection, *Asciz* was efficiently deleted in the bone marrow and thymus but not the tail, which was used as a nonresponsive control tissue (Fig. 1 B). Deletion was incomplete in the spleen, which could reflect lack of recombination in interferon nonresponsive stromal cells or reduced Cre activation in the periphery. Recombination patterns in deleters did not change between 4 and 16 wk, and even up to 2 yr, after the last poly(I:C) injection (Fig. 1 B), indicating that *ASCIZ-Mx* deletion in hematopoietic precursors was efficient and stable without selective growth advantages for nondeleted stem cells.

Overall, *ASCIZ-Mx* deletion was well tolerated with a modest (albeit significant) reduction in red blood cell numbers (Fig. 2 A, left) and similar long-term survival rates compared with littermate controls (Fig. 1 D). However, *ASCIZ-Mx* deletion led to a persistent greater than twofold reduction in the number of circulating lymphocytes compared with poly(I:C)-injected *Mx1-Cre*$^+$*Asciz*$^{fl/+}$ and flox-only controls ($P < 0.0001$; Fig. 2 A, right). Cell surface marker analysis revealed that this lymphopenia was specific for B cells (Fig. 2 B), with no gross effect on circulating T cell levels (Fig. 3 B). The B cell defect in *ASCIZ-Mx* deleters was also present in the spleen, where the absolute numbers of B220$^+$IgM$^+$ positive mature B cells were reduced more than twofold compared with controls ($P < 0.0001$; Fig. 2 C) and already evident from the pre-B cell stage (B220$^+$IgM$^-$CD43$^-$CD19$^+$) onwards in the bone marrow ($P < 0.001$; Fig. 2 D). In contrast, despite the complete absence of ASCIZ protein in the thymus (Fig. 3 A), comparable stages of T cell development appeared unimpaired (Fig. 3 C). Altogether, these results demonstrate that ASCIZ is required for normal B cell development.

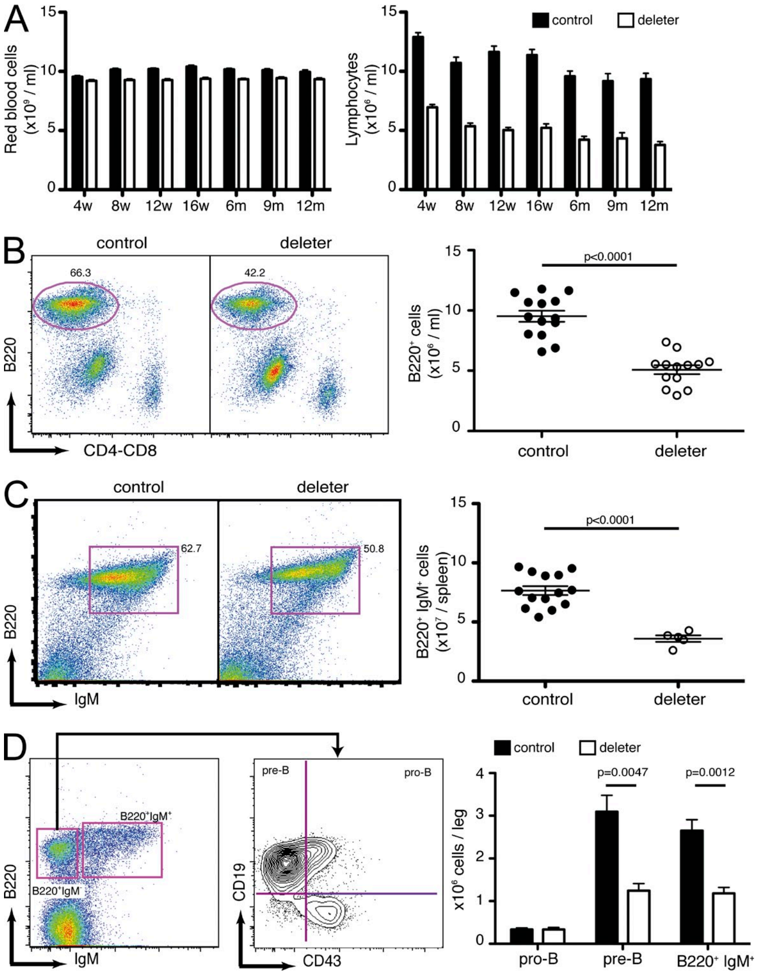

Figure 2. ***Mx1*-Cre deletion of *Asciz* leads to B lymphopenia.** (A) Blood cell content in *ASCIZ-Mx* controls and deleters, showing red blood cells (left) and lymphocytes (right) from 4 wk (w) to 12 mo (m) after poly(I:C) injection. Animal numbers: 4 wk, 31 controls/26 deleters; 8 wk, 31/26; 12 wk, 29/24; 16 wk, 30/22; 6 mo, 30/21; 9 mo, 28/23; and 12 mo, 27/22. Animals are from the same cohorts shown in Fig. 1 D with the control group comprised of *Mx1-Cre*$^+$*Asciz*$^{fl/+}$ and *Mx1-Cre*$^-$*Asciz*$^{fl/fl}$ mice, with no differences between subgroups. Note that at all time points, differences between controls and deleters are $P < 0.0001$, except for red blood cells at 12 mo ($P < 0.01$). (B) Total blood B lymphocyte numbers (right) of *ASCIZ-Mx* controls and deleters and representative FACS plots (left) using B220 as a B cell marker and costaining for CD4 and CD8 conjugated to the same fluorophore as T cell markers. Results shown here were obtained at 4 wk after poly(I:C) injection. $n = 13$ for *Mx1-Cre*$^+$*Asciz*$^{fl/fl}$ deleters, and $n = 14$ for pooled controls ($n = 4$ *Mx1-Cre*$^+$*Asciz*$^{fl/+}$ and $n = 10$ *Mx1-Cre*$^-$*Asciz*$^{fl/fl}$) with no difference between subgroups. (C) Representative FACS profiles for splenic B220$^+$IgM$^+$ B cell populations (boxed gates; left) and absolute numbers (right) of splenic B220$^+$IgM$^+$ cells in *ASCIZ-Mx* controls ($n = 14$; $n = 8$ *Mx1-Cre*$^+$*Asciz*$^{fl/+}$ and $n = 6$ *Mx1-Cre Asciz*$^{fl/fl}$) and *Mx1-Cre*$^+$*Asciz*$^{fl/fl}$ deleters ($n = 5$) at 4 wk after poly(I:C) injection. (D) Gating strategy (left) and total numbers of bone marrow B cell populations (right). The boxed B220$^+$IgM$^+$ population includes immature, transitional, and recirculating B cells, and the boxed B220$^+$IgM$^-$ population is further gated for CD43 and CD19 to distinguish pro-B cells (CD43$^+$CD19$^+$) and pre-B cells (CD43$^-$CD19$^+$). Total numbers represent B cell populations in one hind limb of *ASCIZ-Mx* controls ($n = 15$; $n = 9$ *Mx1-Cre*$^+$*Asciz*$^{fl/+}$ and $n = 6$ *Mx1-Cre*$^-$*Asciz*$^{fl/fl}$) and *Mx1-Cre*$^+$*Asciz*$^{fl/fl}$ deleters ($n = 7$) at 4 wk after poly(I:C) injection. (A–D) All data represent mean ± SEM. Each symbol in B and C represents one animal. Closed bars and circles indicate controls; open bars and circles indicate deleters. Data in B–D were obtained in at least two independent experiments. Similar results were obtained in mice sacrificed at 16 wk after poly(I:C) injection (not depicted).

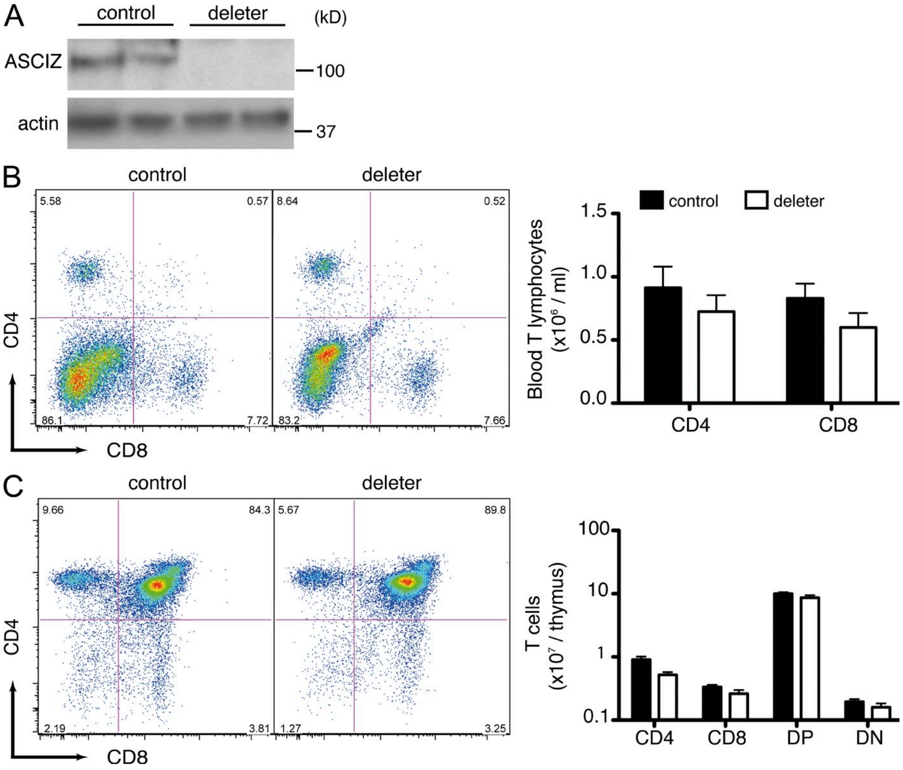

Figure 3. Normal T cell development in *Mx1-Cre Asciz* deleters. (A) Western blot of thymic protein levels of two different *ASCIZ-Mx* deleters and two controls probed for ASCIZ and actin as a loading control at 4 wk after poly(I:C) injection. Molecular mass standards are indicated on the right. (B) Representative FACS plots of blood isolated from *ASCIZ-Mx* controls and deleters (left) stained for CD4 and CD8 and absolute blood T cell numbers at 4 wk after poly(I:C) injection (right). $n = 11$ for controls ($n = 5$ *Mx1-Cre*$^+$*Asciz*$^{fl/+}$ and $n = 6$ *Mx1-Cre*$^-$*Asciz*$^{fl/fl}$), and $n = 8$ for *Mx1-Cre*$^+$*Asciz*$^{fl/fl}$ deleters. (C) Representative FACS plots of a thymus isolated from an *ASCIZ-Mx* control and a deleter thymus (left) and absolute thymic T cell numbers (right) at 4 wk after poly(I:C) injection. $n = 15$ for controls ($n = 9$ *Mx1-Cre*$^+$*Asciz*$^{fl/+}$ and $n = 6$ *Mx1-Cre*$^-$*Asciz*$^{fl/fl}$), and $n = 8$ for *Mx1-Cre*$^+$*Asciz*$^{fl/fl}$ deleters. (B and C) All grouped data represent mean ± SEM. Similar results were obtained at 16 wk after poly(I:C) injection.

B cell lineage–specific *Asciz* deletion leads to severe B cell deficiency

To examine whether the *ASCIZ-Mx* phenotype was indeed caused by a defect in B cell precursors, we used the B lineage–specific *Mb1*-Cre knockin, which efficiently deletes floxed alleles from the pro-B cell stage onward (Hobeika et al., 2006). Efficient *Asciz* deletion by *Mb1-Cre* (in the following simplified as *ASCIZ-Mb*) was confirmed by Western blot analysis of enriched splenic B cells (Fig. 1 C). Strikingly, spleens of *ASCIZ-Mb* deleters were approximately twofold smaller than those of Cre-only and flox-only littermate controls ($P < 0.0001$; Fig. 4 A), concomitant with a more than sevenfold decrease in B220$^+$IgM$^+$ positive mature splenic B cell numbers ($P < 0.0001$; Fig. 4 B). Bone marrow phenotyping of *ASCIZ-Mb* deleters revealed a similar greater than sevenfold reduction of B220^{low}IgM$^+$-gated immature B cell numbers compared with controls ($P < 0.0001$; Fig. 4 C), as well as an approximately threefold decrease ($P < 0.0001$) in the pre-B cell compartment (B220$^+$IgM$^-$CD43$^-$CD19$^+$) but no difference at the pro-B cell stage (Fig. 4 D). Similar results were obtained in reconstitution experiments of lethally irradiated Ly5.1-expressing recipient mice with Ly5.2-positive *ASCIZ-Mb* donor bone marrow. In these experiments, the percentage of donor-derived B220$^+$IgM$^+$ B cells in recipient spleens was 4.6-fold lower for deleters compared with *Mb1*-Cre–only or Cre-negative *Asciz*$^{fl/fl}$ control donors ($P < 0.0001$; Fig. 5 A) and >10-fold lower for B220^{low}IgM$^+$ immature B cells ($P < 0.0001$; Fig. 5 B) and 3.3-fold lower for B220$^+$IgM$^-$CD43$^-$CD19$^+$ pre-B cells ($P < 0.0001$; Fig. 5 C) in the bone marrow. Altogether, these data demonstrate that ASCIZ regulates B cell development in a cell-intrinsic manner.

To identify possible causes of this B lymphopenia, we monitored cell death levels in the bone marrow. Interestingly, in B220$^+$IgM$^+$IgD$^{-/low}$-gated immature and transitional B cells, there was an approximately threefold increase in Annexin V/7-AAD double-positive cells (Fig. 4 E) in *ASCIZ-Mb* deleters compared with controls. These data indicate that the main reason for the peripheral B lymphopenia in *ASCIZ-Mb* deleters may be reduced cell survival during B cell development before exiting the bone marrow.

Loss of ASCIZ-deficient B cells is largely independent of its DNA damage response functions

As ASCIZ was originally identified as a DNA damage response protein, we examined whether the B lymphopenia could be caused by impaired DNA damage responses. DNA base damage–induced apoptosis is typically p53 dependent, and related B lymphopoiesis defects can be prevented by *p53* deletion, for example in case of apurinic endonuclease deficiency (Guikema et al., 2011). We therefore crossed *ASCIZ-Mb* mice with *p53* floxed mice (Jonkers et al., 2001); however, additional *p53* deletion only marginally increased splenic B cell levels (albeit in a statistically significant manner; Fig. 6 A) or bone marrow immature B cell levels (Fig. 6 B) in *ASCIZ-Mb* deleters.

Another possible explanation for the gradual decline of cells at both the pre-B and immature B cell stages could be inefficient V(D)J recombination of the *IgH* and *IgL* loci to generate functional pre-BCRs and BCRs, respectively, defects which would be suppressible by complementation with prearranged BCR genes (Spanopoulou et al., 1994;

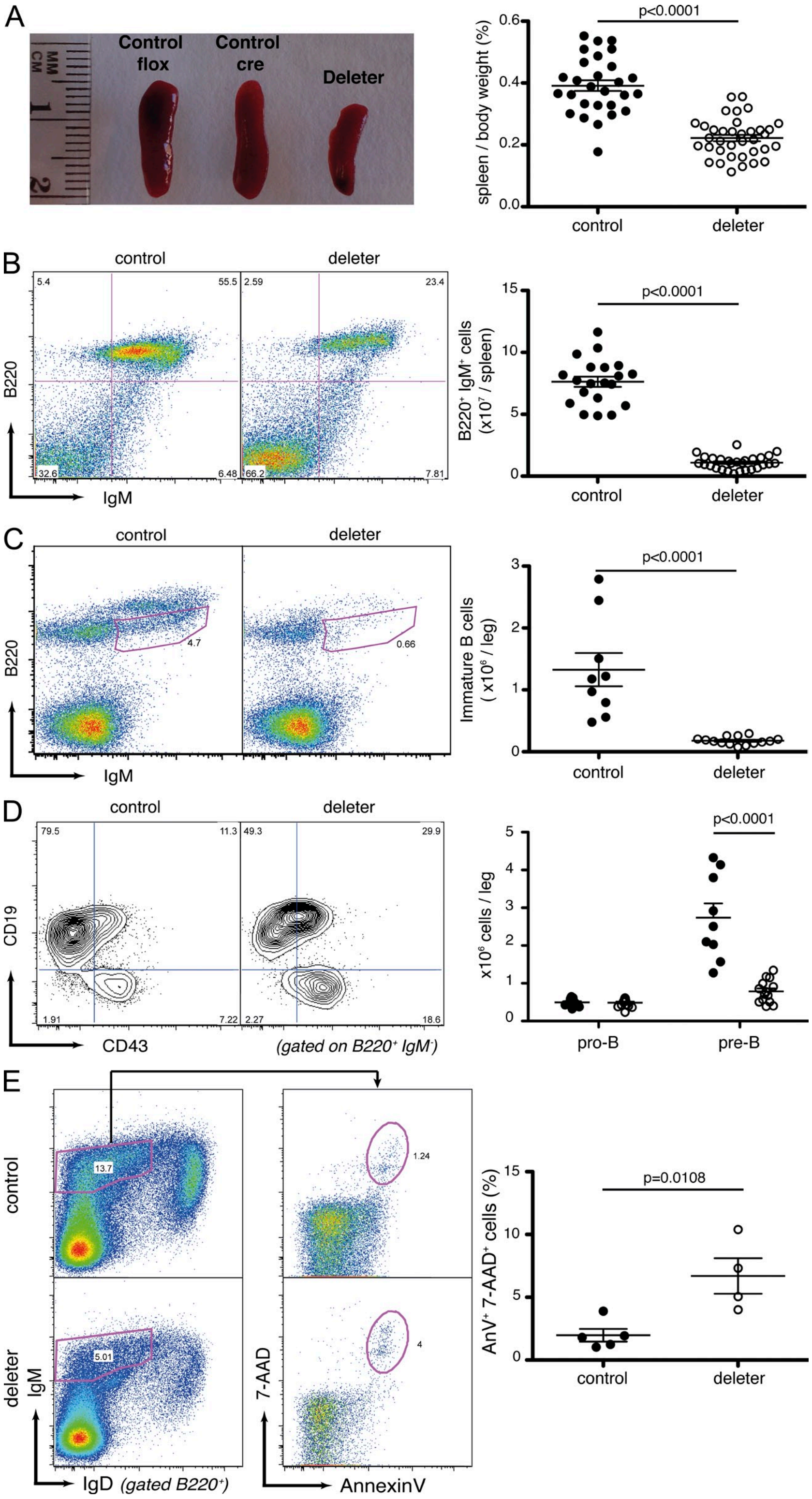

Figure 4. *Mb1*-Cre *Asciz* deletion leads to progressive B cell loss during B cell development. (A) Photograph of representative spleens from *Mb1-Cre^{-}Asciz$^{fl/fl}$*, *Mb1/Mb1-Cre Asciz$^{+/+}$* controls, and an *Mb1/Mb1-Cre Asciz$^{fl/fl}$* deleter (left) and relative spleen weights of controls (n = 28) and deleters (n = 36; right). (B) Representative FACS plots of spleens isolated from an *ASCIZ-Mb* control and a deleter (left) and absolute numbers of splenic B220^{+}IgM^{+} B cells in controls (n = 20) and *Mb1/Mb1-Cre Asciz$^{fl/fl}$* deleters (n = 28; right). The control group contains both *Mb1-Cre^{-}Asciz$^{fl/fl}$* and *Mb1/Mb1-Cre Asciz$^{+/+}$* animals. (C) Representative FACS plots of bone marrows from an *ASCIZ-Mb* control and an *Mb1/Mb1-Cre Asciz$^{fl/fl}$* deleter (left) and absolute cell numbers (right) of B220^{low}IgM^{+} immature B cells (gated as indicated on the FACS plots) in a single hind leg of controls (n = 9) and deleters (n = 14). The control group contains both *Mb1-Cre^{-}Asciz$^{fl/fl}$* and *Mb1/Mb1-Cre Asciz$^{+/+}$* animals. (D) Representative FACS plots of B220^{+}IgM^{-}-gated bone marrow cells from an *ASCIZ-Mb* control and an *Mb1/Mb1-Cre Asciz$^{fl/fl}$* deleter stained for CD43 and CD19 (left). The right panel shows grouped data for pro-B cells (B220^{+}IgM^{-}CD43^{+}CD19^{+}) and pre-B cells (B220^{+}IgM^{-}CD43^{-}CD19^{+}) in controls (n = 9) and deleters (n = 9). The control group contains both *Mb1-Cre^{-}Asciz$^{fl/fl}$* and *Mb1/Mb1-Cre Asciz$^{+/+}$* animals. (E) Representative FACS plots of cell death (AnnexinV^{+}7-AAD^{+}) in immature and transitional B cells (B220^{+}IgM^{+}IgD$^{-/low}$) in an *ASCIZ-Mb* control and a deleter (left) and grouped data for *Mb1/Mb1-Cre Asciz$^{+/+}$* controls (n = 5) and *Mb1/Mb1-Cre Asciz$^{fl/fl}$* deleters (n = 4). (A–E) Each data point represents one mouse. All grouped data represent mean ± SEM. Data were obtained in at least two independent experiments. Closed circles indicate controls; open circles indicate deleters.

Young et al., 1994; Cook et al., 2003). We therefore crossed *ASCIZ-Mb* deleters with *SWHEL* mice, which contain a hen egg lysozyme (HEL)–specific prearranged BCR caused by a VDJ-recombined *IgH* locus knockin and an *IgL* transgene that can restore normal B cell development even in RAG KO or SCID mice (Phan et al., 2003). However, neither complementation with the complete HEL-BCR nor its individual HEL-IgH or HEL-IgL components meaningfully increased the numbers of splenic B cells or bone marrow immature B cells in *ASCIZ-Mb* deleters (Fig. 6, C and D). Collectively, the findings that neither provision of a prearranged BCR nor *p53* deletion was able to rescue the B lymphopenia in *ASCIZ-Mb* deleters demonstrate that this defect must be largely unrelated to the DNA damage response functions of ASCIZ.

ASCIZ-deficient lymphopenia is caused by reduced *Dynll1* expression and increased Bim activity

As these data showed that the lymphopenia observed in ASCIZ-deficient mice is most likely DNA damage independent, we

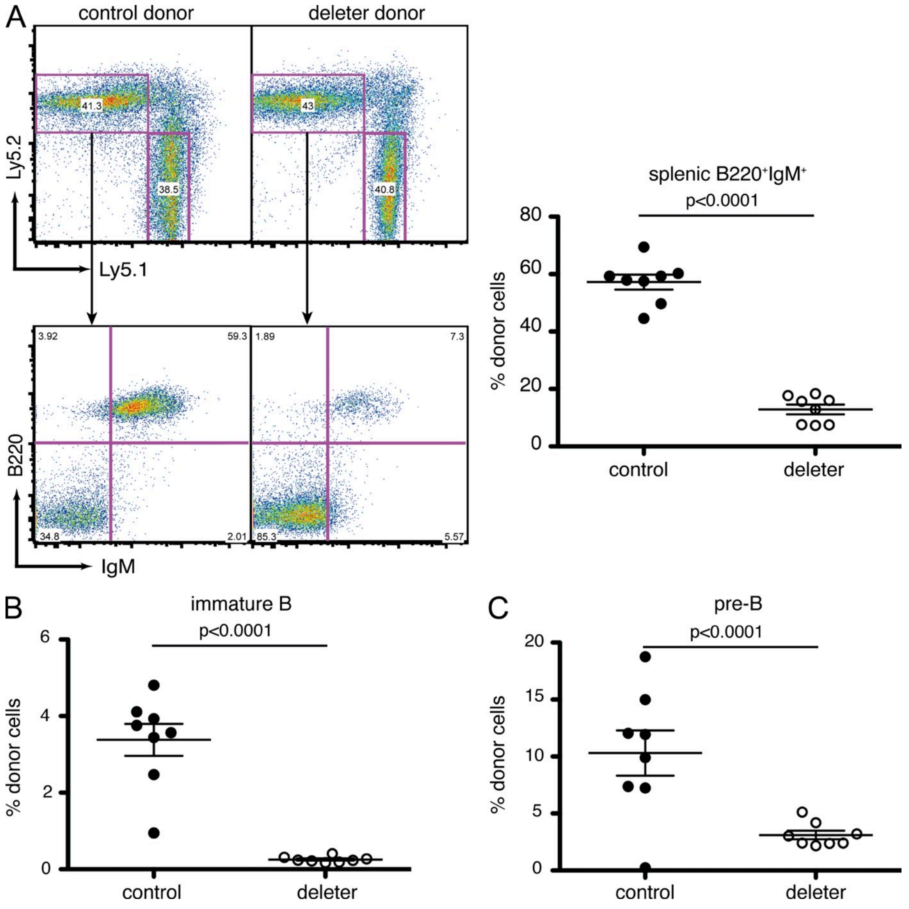

Figure 5. Cell-intrinsic requirement for ASCIZ during B cell development. (A) FACS analysis of spleen cells of lethally irradiated Ly5.1 recipients 8 wk after transplantation with Ly5.2 control or *Mb1/Mb1-Cre Asciz*$^{fl/fl}$ deleter bone marrow and percentage of B220$^+$IgM$^+$ B cells among donor-derived spleen cells (control transplants, *n* = 8; deleter transplants, *n* = 8). Half of the control transplants were performed with bone marrow from a single *Mb1/Mb1-Cre Asciz*$^{+/+}$ donor, the other half with bone marrow from a single *Mb1-Cre*$^-$ *Asciz*$^{fl/fl}$ donor. Two different deleter bone marrows were used for four different recipients each. (B and C) Percentages of donor-derived pre-B and immature B cells in bone marrows from the same recipients shown in A. (A–C) Error bars represent mean ± SEM. Each symbol represents one animal. All data were obtained in one experiment.

explored the possibility that it could be related to the role of ASCIZ as a transcription factor. We recently identified *Dynll1* as the main transcriptional target for ASCIZ in primary mouse fibroblasts (Jurado et al., 2012). Consistently, DYNLL1 protein levels were also dramatically reduced in sorted *ASCIZ-Mb* deleter bone marrow B cell precursors compared with controls (Fig. 7 A). To test whether impaired *Dynll1* expression was a cause of the *ASCIZ-Mb* phenotype, *Asciz* deleter bone marrow was transduced with empty vector or *Dynll1*-containing retroviruses and transplanted together with nontransduced deleter bone marrow carrier cells into lethally irradiated recipients, followed by FACS identification of Ly5.2$^+$ donor-derived cells in recipient spleens 8 wk after transplantation (Fig. 7 B). IRES-GFP tracking of transduced cells revealed a significant enrichment of *Dynll1*-complemented deleter-derived cells in the splenic B220$^+$IgM$^+$ compartment compared with the empty vector control (Fig. 7 B, top), as well as

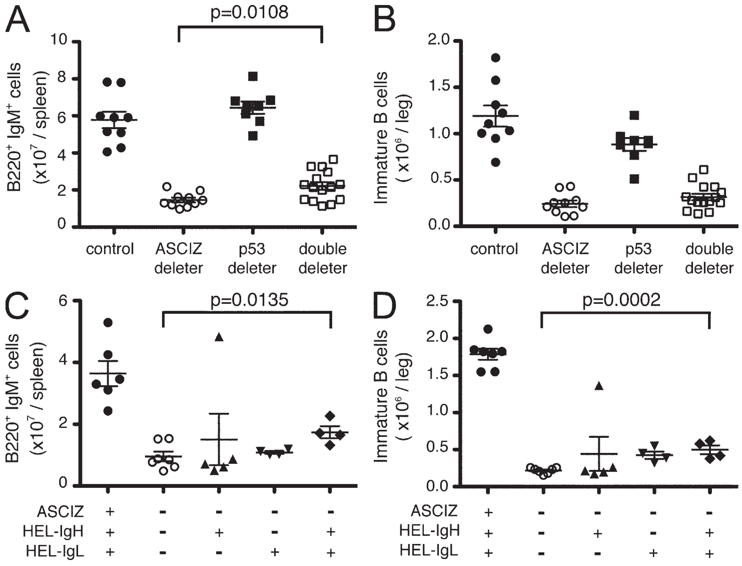

Figure 6. Loss of B cells in the absence of ASCIZ is not rescued by *p53* deletion or complementation with the prearranged SWHEL BCR. (A and B) Absolute splenic B220$^+$IgM$^+$ B cell (A) and bone marrow B220^{low}IgM$^+$ immature B cell numbers (B) in *Mb1/Mb1-Cre Asciz*$^{fl/fl}$ deleters (*n* = 10), *Mb1/Mb1-Cre p53*$^{fl/fl}$ deleters (*n* = 8), *Mb1/Mb1-Cre Asciz*$^{fl/fl}$*p53*$^{fl/fl}$ double deleters (*n* = 15), and controls (*n* = 8). The control group contains four *Mb1-Cre*$^-$*Asciz*$^{fl/fl}$*p53*$^{fl/fl}$ and four *Mb1/Mb1-Cre Asciz*$^{+/+}$*p53*$^{+/+}$ animals. The data were obtained in six independent experiments. (C and D) Absolute splenic B220$^+$IgM$^+$ B cell (C) and bone marrow B220^{low}IgM$^+$ immature B cell numbers (D) in *Mb1/Mb1-Cre Asciz*$^{fl/fl}$ deleters (*n* = 7) and *Mb1/Mb1-Cre Asciz*$^{fl/fl}$ deleters expressing a prearranged SWHEL IgH (HEL-IgH; *n* = 5), SWHEL IgL (HEL-IgL; *n* = 4), or both SWHEL IgH and IgL (*n* = 4), and *Mb1/Mb1-Cre Asciz*$^{+/+}$ controls expressing both SWHEL IgH and IgL (spleen *n* = 6; bone marrow *n* = 7). The data were obtained in three independent experiments. (A–D) Absolute numbers were determined by flow cytometry using gates as shown in Fig. 4. All grouped data represent mean ± SEM. Each symbol represents one animal.

compared with nontransduced deleter-derived cells as an internal control (Fig. 7 B, bottom). Thus, this finding that ectopic DYNLL1 can compensate for loss of ASCIZ demonstrates that ASCIZ regulates B lymphopoiesis as a transcriptional activator of *Dynll1* expression.

As DYNLL1 has been shown to attenuate the proapoptotic activity of the BH3-only protein Bim (Puthalakath et al., 1999) and as Bim is responsible for the elimination of developing B cells (Enders et al., 2003), we hypothesized that the B lymphopenia in *Asciz* deleters may be caused by increased Bim activity. To test this hypothesis, we crossed *ASCIZ-Mb* mice to *Bim* KO mice (Bouillet et al., 1999). Again, *ASCIZ-Mb* deleters had smaller spleens and approximately sevenfold lower splenic B220$^+$IgM$^+$ B cell levels compared with littermate controls (Fig. 8, A and B), and as expected from a previous study (Bouillet et al., 1999), *Bim* KO mice exhibited splenomegaly with highly elevated B cell numbers (Fig. 8, A and B). Remarkably, when combined with the *Bim* KO, the *ASCIZ-Mb* phenotype was completely rescued with highly elevated splenic B cell levels and even splenomegaly similar to *Bim* single mutants ($P < 0.0001$; Fig. 8, A and B). Loss of Bim also increased the numbers of B220^{low}IgM$^+$-gated immature B cells (Fig. 8 C) and B220$^+$IgM$^-$CD43$^-$CD19$^+$ pre-B cells (Fig. 8 D) in the bone marrow of *ASCIZ-Mb* deleters to levels similar to the *Bim* KO alone. These results that the *Bim* KO phenotype is dominant over the *ASCIZ-Mb* deleter phenotype place *Asciz* and *Bim* in a common genetic pathway and, in light of highly reduced DYNLL1 levels, suggest that the *ASCIZ-Mb* phenotype may be caused by excessive Bim activity as the result of impaired *Dynll1* transcription.

DISCUSSION

In this study, we have shown with two independent conditional KO models that ASCIZ is essential for normal B cell development. The resulting B lymphopenia was more severe in the *Mb1*-Cre model, conceivably because in this line *Asciz* is constitutively deleted in every single B cell past the pro-B stage, whereas in the *Mx1*-Cre model large numbers of mature B cells would already have been established in the periphery by the time (4–5 wk of age) of the first poly(I:C) injections. The genetic rescue of this defect by complementation with ectopic DYNLL1 (Fig. 7) but not by deletion of *p53* or complementation with a prearranged BCR (Fig. 6) demonstrates that this phenotype is predominantly caused by loss of ASCIZ's transcriptional function in regulating *Dynll1* expression rather than its role in the DNA damage response. This notion is further supported by the rescue of the *Asciz* deleter phenotype by the absence of Bim, which predominantly functions in DNA damage–independent cell death pathways (Strasser, 2005). It is important to note that the phenotype of *ASCIZ-Mb Bim* double mutants represents to our knowledge the first reported case where reduced peripheral B cell numbers as a result of a B cell developmental defect in the bone marrow are completely rescued all the way to the abnormally elevated B cell levels characteristic of the *Bim* KO alone. For example, in the case of *Mb1-Cre Dicer* deletion, *Bim* KO only partially restored splenic B cell numbers to ∼20% of WT levels (Koralov et al., 2008), and in the case of an *Atp11c* mutation, the *Bim* KO had no beneficial effect at all on peripheral or bone marrow B cell numbers (Siggs et al., 2011). Likewise, even broader inhibition of all intrinsic cell death pathways by transgenic overexpression of the antiapoptotic protein Bcl2 lead to no or only partial improvement of peripheral B cell numbers relative to WT, but much less so relative to the elevated B cell

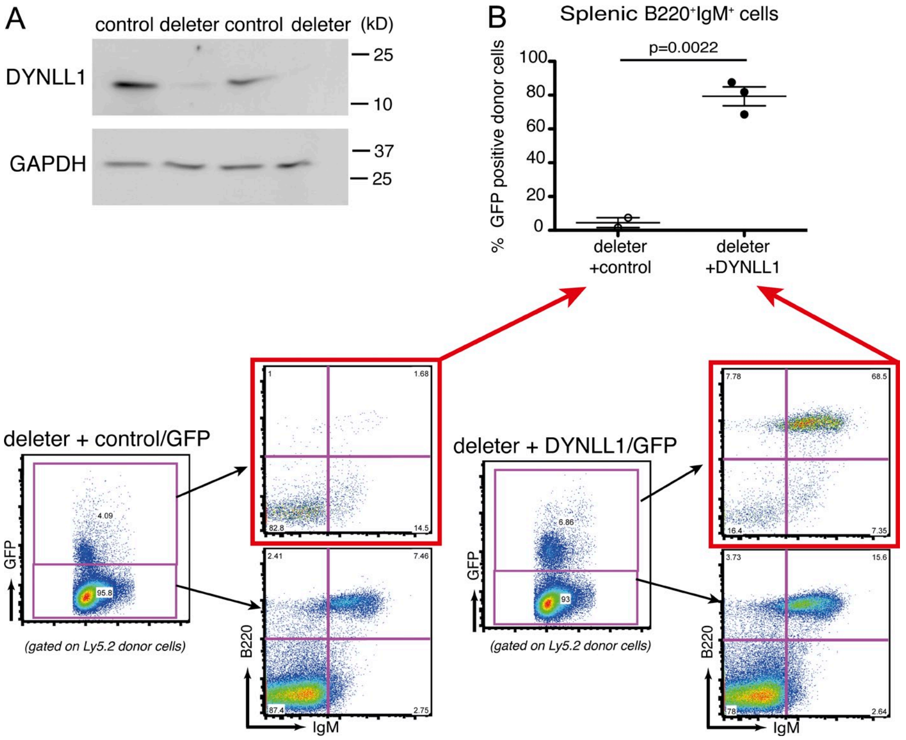

Figure 7. ASCIZ regulates B cell development via DYNLL1. (A) Western blot analysis of DYNLL1 levels in FACS-isolated bone marrow B220$^+$IgM$^-$ cells of two controls and two *Mb1/Mb1-Cre Asciz*$^{fl/fl}$ deleters. GAPDH is used as loading control. Molecular mass standards are indicated on the right. (B) Reconstitution of spleens of lethally irradiated recipients with *Mb1/Mb1-Cre Asciz*$^{fl/fl}$ deleter–derived cells that were retrovirally transduced with MigR1 empty vector (control; $n = 2$) or MigR1-*Dynll1* ($n = 3$). The graph shows the percentage of B220$^+$IgM$^+$ spleen cells among transduced GFP-positive donor-derived cells. FACS plots (below) indicate the gating strategy. Ly5.2-positive donor-derived cells were gated for IRES-GFP expression to detect vector (left)- or *Dynll1* (right)-transduced cells. Error bars represent mean ± SEM. Each symbol represents one animal. All data were obtained in one experiment.

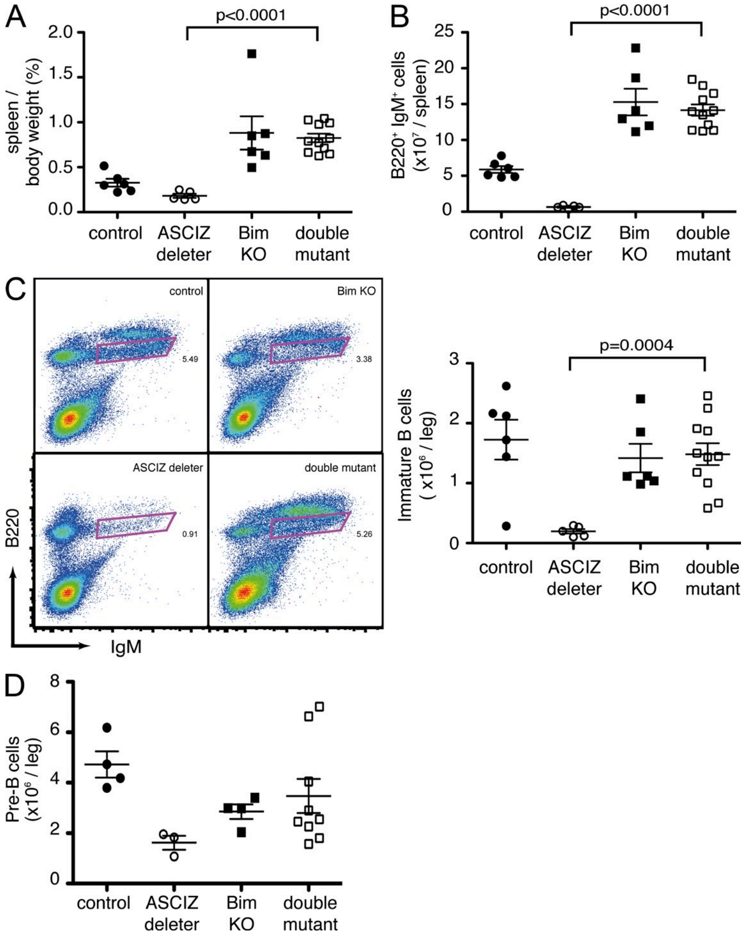

Figure 8. Impaired B cell development in the absence of ASCIZ is rescued by deletion of *Bim*. (A and B) Relative spleen weights (A) and total numbers of splenic B220$^+$IgM$^+$ B cells (B) in *Mb1/Mb1-Cre Asciz*$^{fl/fl}$ deleters (n = 5), *Bim* KO (n = 6), double mutants (n = 11), and controls (n = 6). All controls and all *Bim* KO mice are *Mb1/Mb1-Cre Asciz*$^{+/+}$. Data were obtained in five independent experiments. (C) Bone marrow phenotyping (left) and total cell numbers (right) for B220^{low}IgM$^+$-gated immature B cells in one hind limb of *Mb1/Mb1-Cre Asciz*$^{fl/fl}$ deleters (n = 5), *Bim* KO mice (n = 6), double mutants (n = 11), and controls (n = 6). The animals are the same as in A. (D) Pre-B cell numbers (B220$^+$IgM$^-$CD43$^-$CD19$^+$) in *Mb1/Mb1-Cre Asciz*$^{fl/fl}$ deleters (n = 3), *Bim* KO mice (n = 4), double mutants (n = 9), and controls (n = 4). All controls and all *Bim* KO mice are *Mb1/Mb1-Cre Asciz*$^{+/+}$. The data were obtained in three independent experiments. (A–D) Each symbol represents one mouse. Error bars represent mean ± SEM.

levels in the transgene control, in the case of *Dicer* (Koralov et al., 2008), *Atp11c* (Siggs et al., 2011; Yabas et al., 2011), or *Stat5* (Malin et al., 2010) mutations. These considerations indicate that absence of *Bim* rescues the *Asciz* deleter phenotype not just by generally elevating B cell numbers, but in a highly specific manner suggestive of functions in a common genetic pathway.

As cell death during B cell development, which is known to be at least partially regulated by Bim (Enders et al., 2003), is elevated in *Asciz* deleters (Fig. 4 E) and as Bim's proapoptotic function has been reported to be held in check by DYNLL1 (Puthalakath et al., 1999), the simplest explanation for our findings is a model in which ASCIZ positively regulates DYNLL1 expression, which may in turn prevent excessive Bim-dependent apoptosis during B cell development. However, given that DYNLL1 has been proposed to regulate >100 diverse proteins (Rapali et al., 2011), it is remarkable that deletion of Bim alone was sufficient to rescue the *ASCIZ-Mb* deleter phenotype. In this sense, it is formally possible that other defects that indirectly lead to increased Bim-dependent apoptosis could contribute to the phenotype described here in addition to the direct effects lower DYNLL1 levels may have on Bim activity. For example, DYNLL1 was originally identified as a component of the dynein motor complex (King and Patel-King, 1995) that has recently been shown to be involved in the regulation of BCR clustering (Schnyder et al., 2011), a feature which could conceivably affect Bim activation by BCR signaling pathways. Nevertheless, the restoration of normal B cell development in *ASCIZ-Mb* deleters by ectopic *Dynll1* expression (Fig. 7), to our knowledge, represents the first in vivo evidence in any vertebrate species that reduced DYNLL1 levels are a direct cause of a severe developmental defect.

A unique feature of ASCIZ as a regulator of *Dynll1* expression is that this activity is inhibited by high DYNLL1 levels as a result of >10 DYNLL1-binding sites in its transcription activation domain (Jurado et al., 2012), which allows for an efficient feedback mechanism to maintain free DYNLL1 at a homeostatic level. This feedback mechanism may serve to set a default threshold of apoptosis in developing B cells by providing sufficient DYNLL1 to prevent Bim from unnecessarily killing normal cells, yet also ensures that DYNLL1 levels are not too high to preclude legitimate Bim-dependent elimination of dysfunctional or autoreactive B cells. In this way, ASCIZ-dependent regulation of DYNLL1 levels could tone the strength of death signals in the B cell lineage in a manner analogous to the recently described role of schnurri-2 during T cell development (Staton et al., 2011).

While the work reported here was in progress, another group reported that a similarly targeted conditional *Asciz* deletion using *CD19*-Cre leads to increased rates of peripheral B cell lymphoma but only very modest B cell developmental defects with normal splenic B cell numbers (Loizou et al., 2011). The simplest explanation for the differing phenotypes is that *CD19*-Cre is known to be much less efficient at recombining loxP-flanked genes than *Mb1*-Cre during early B cell developmental stages (Rickert et al., 1997; Hobeika et al., 2006; Kwon et al., 2008), which would have attenuated the severity of the pre-B and immature B cell defects and obscured the splenic defect. Conversely, the absence of B cell lymphomas in both our *Mb1*-Cre and *Mx1*-Cre *Asciz* deleters up to 2 yr of age (Fig. 1, D and E) could simply be caused by much lower peripheral B cell numbers or alternatively could potentially be affected by a different genetic background, which was not specified in the other study. Thus, although it is well possible that DNA base damage–related functions of ASCIZ affect the onset of germinal center–derived B cell lymphomas, our work has uncovered a major unexpected function of ASCIZ as a novel regulator of DNA damage–independent cell death signaling pathways during early B cell development in the bone marrow.

A notable result from the *ASCIZ-Mx* deleters was that despite efficient deletion of ASCIZ in the T cell lineage (Figs. 1 B and 3 A), there were no noticeable differences in circulating T cell levels or thymic T cell developmental stages (Fig. 3, B and C), which is consistent with findings that basal Bim activity in T cells is regulated via DYNLL1-independent mechanisms (Zhu et al., 2004). Thus, in contrast to V(D)J recombination defects that lead to a SCID phenotype affecting both lymphocyte lineages (Mombaerts et al., 1992; Shinkai et al., 1992; Blunt et al., 1995), the phenotype of *ASCIZ-Mx* deleters more closely resembles CVID (common variable immunodeficiencies), a heterogeneous group of hereditary B cell dysfunctions in which most of the underlying mutations remain to be identified. This raises the possibility that mutations in *Asciz*, and by extension mutations in *Dynll1*, could be involved in a subset of human CVID cases of unexplained etiology.

MATERIALS AND METHODS

Mice. All experimental procedures were approved by the St. Vincent's Hospital Melbourne Animal Ethics Committee. Mice were maintained on a C57BL/6 background and housed in specific pathogen–free microisolators to minimize pathogen exposure at the St. Vincent's Hospital Melbourne Bioresources Centre.

The *Asciz* gene was targeted in C57BL/6 embryonic stem cells by integrating loxP sites on both sides of exon D (Fig. 1 A) as described previously (Jurado et al., 2010), followed by removal of the FRT-flanked PGK-neo cassette by crossing with *ACTB-FLPe* transgenic mice on a C57BL/6 background as a contracted service by Ozgene Pty Ltd. After removal of the PGK-neo cassette, the floxed *Asciz* allele was backcrossed twice to WT C57BL/6 mice to remove the *FLPe* transgene and for embryo transfer into our mouse facility.

Mx1-cre (Kühn et al., 1995), *Mb1*-Cre (Hobeika et al., 2006; provided by M. Reth, Max Planck Institute of Immunobiology, Freiburg, Germany), *p53* floxed (Jonkers et al., 2001), *SWHEL* (Phan et al., 2003; provided by R. Brink, Garvan Institute, Darlinghurst, New South Wales, Australia), and *Bim* KO (Bouillet et al., 1999) mice have been described previously. All of these lines had previously been backcrossed to C57BL/6 for at least 10 generations. As *Mb1*-Cre is a knockin and homozygotes lack B cells, only *Mb1/Mb1*-Cre heterozygotes were used for phenotypic analyses. *SWHEL* mice were heterozygous for the *HEL-IgH* knockin and transgenic for *HEL-IgL*.

Unless otherwise specified, mice were used at 6–14 wk of age. The *ASCIZ-Mx* control group contained *Mx1-Cre*$^{+}$*Asciz*$^{fl/+}$ and *Mx1-Cre*$^{-}$*Asciz*$^{fl/fl}$ mice. Control groups for other experiments contained *Mb1/Mb1*-Cre–only mice and in some cases also *Asciz*$^{fl/fl}$ or *p53*$^{fl/fl}$ mice without Cre expression as indicated in the figure legends. Genotyping of mice was performed by PCR using the primer sets shown in Table S1.

Mx-Cre induced ASCIZ deletion. 4–5-wk-old *ASCIZ-Mx* controls and deleters were injected every other day with 25 mg/kg poly(I:C) (Poly(I:C)-LMW, tlrl-picw; InvivoGen) to induce Cre expression. Mice were bled at 4, 8, 12, and 16 wk and 6, 9, and 12 mo after injection for blood cell analyses and observed for long-term survival for up to 24 mo of age. Additional mice were sacrificed at 4 and 16 wk after poly(I:C) injection for bone marrow and spleen cell analyses.

Bone marrow transplantation and retroviral DYNLL1 complementation. For retroviral complementation experiments, the human *Dynll1* cDNA (Puthalakath et al., 1999) was cloned into the MigR1 vector (Pear et al., 1993), and virus was produced using the Phoenix packaging cell line. Donor cells were isolated from Ly5.2 *ASCIZ-Mb* deleters and controls (*Asciz*$^{fl/fl}$ without *Cre* expression or *Mb1/Mb1*-Cre–only mice). Hematopoietic stem cell–enriched fractions (Lin^{-}ckit^{+}Sca^{+}) were FACS isolated from whole bone marrow of *ASCIZ-Mb* deleters as described previously (Walkley et al., 2005) and cultured overnight in StemPro-34 medium (Invitrogen) with 10 ng/ml mSCF, 20 ng/ml mTPO, 20 ng/ml mIGF2, and 10 ng/ml hFGF1 (Zhang and Lodish, 2004) before two rounds of infection with MIGR1 control or DYNLL1 retroviruses. Equal numbers (4,000–5,000) of cells were then transplanted by tail vein injection into lethally irradiated Ly5.1 recipient mice together with 200,000 nontransduced bone marrow cells of the same donor genotype. Mice were sacrificed 8 wk after transplant for bone marrow and spleen cell analyses. Ly5.2-stained donor-derived cells were further gated for *IRES-GFP* expression to distinguish between carrier cells and retrovirally transduced cells for the analyses shown in Figs. 5 and 7.

Cytometry and FACS analyses. Spleens were weighed and passed through a 70-µm cell strainer to allow preparation of a homogeneous cell suspension. Cells were collected in PBS 2% FBS. Bone marrow cells were isolated from hind limbs. Typically, one leg (femur plus tibia) was cleaned of soft tissues, and bones were flushed with PBS 2% FBS with a syringe connected to a 23G needle. Blood, spleen, and bone marrow cellularities were determined using an automated KX-21N cell counter (Sysmex). Cells were stained with appropriate antibodies in PBS 2% FBS on ice. AnnexinV staining was performed in its own buffer (eBioscience) at room temperature for 15 min according to the manufacturer's recommendation. Stained cells were analyzed on an LSRFortessa (BD). Compensation for multicolor stains and data acquisition were performed using the FACSDiva software (BD), and data were analyzed using FlowJo (Tree Star).

Antibodies used were as follows: B220-APC (eBioscience), B220-FITC (BD), IgM-FITC (eBioscience), IgM-eFluor650 (eBioscience), IgM-biotin (eBioscience), IgD-eFluor450 (eBioscience), IgD–Alexa Fluor 647 (eBioscience), CD19-FITC (eBioscience), CD19-PerCP-Cy5.5 (eBioscience), CD43-PE (BD), CD4-PE (BD), CD4-eFluor450 (eBioscience), CD8a-FITC (eBioscience), CD8a-eFluor450 (eBioscience), CD44-APC (eBioscience), CD25-PE (eBioscience), Ly5.1-PECy7 (eBioscience), Ly5.2-biotin (eBioscience), streptavidin-Qdot605 (Invitrogen), and AnnexinV-eFluor450 detection kit (eBioscience). Bone marrow B cell populations were defined according to the following surface marker profiles: pro-B cells, B220^{+}IgM^{-}CD43^{+}CD19^{+}; pre-B cells, B220^{+}IgM^{-}CD43^{-}CD19^{+}; and immature B cells, B220^{low}IgM^{+}.

Blot analyses. For Southern blots, DNA was isolated from tissues or cell suspensions by overnight digestion with 0.5 mg/ml proteinase K in 1% SDS at 55°C followed by isopropanol precipitation and ScaI endonuclease treatment. Membranes were incubated overnight with a radioactive *Asciz* probe and exposed to phosphorimager screens as described previously (Jurado et al., 2010, 2012). For Western blots, splenic and bone marrow B cells were isolated from total cell suspensions using B220 (CD45R)-MACS beads (Miltenyi Biotec) and an auto-MACS Pro Separator (Miltenyi Biotec) according to the manufacturer's instructions. Bone marrow B cell precursors were isolated using a FACSAria (BD) at the St. Vincent's Institute FACS facility. Cells were lysed in SDS gel loading buffer before electrophoresis and transfer to polyvinyldifluoride membranes. Antibodies used were as follows: actin (EMD Millipore/Merck), ASCIZ (McNees et al., 2005; available from EMD Millipore/Merck), CTNNB1 (Sigma-Aldrich), DYNLL1/2 (Santa Cruz Biotechnology, Inc.), and GAPDH-HRP (Cell Signaling Technology).

Statistical analyses. P-values were calculated by two-tailed unpaired Student's *t* test. Grouped data represent the mean ± SEM. Numbers of mice per analysis are indicated in the figure legends.

Online supplemental material. Table S1 contains oligonucleotide primers for PCR genotyping. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20120785/DC1.

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Author contributions: S. Jurado designed, performed, and analyzed experiments and wrote the paper; K. Gleeson and K. O'Donnell performed experiments and provided assistance; D.J. Izon, C.R. Walkley, and D.M. Tarlinton designed, discussed, and analyzed experiments; A. Strasser discussed experiments and provided reagents; and J. Heierhorst designed, discussed, and analyzed experiments and wrote the paper.

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