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Requirement for Dicer in Survival of Proliferating Thymocytes Experiencing DNA Double-Strand Breaks

Brenna L. Brady,*‡§,1 Levi J. Rupp,*‡§,1 and Craig H. Bassing*‡§

The Dicer nuclease generates small RNAs that regulate diverse biological processes through posttranscriptional gene repression and epigenetic silencing of transcription and recombination. Dicer-deficient cells exhibit impaired differentiation, activity, proliferation, and survival. Dicer inactivation in developing mouse lymphocytes impairs their proliferation and survival and alters Ag receptor gene repertoires for largely undefined reasons. To elucidate functions of Dicer in lymphocyte development and Ag receptor locus transcription and recombination, we analyzed mice with conditional Dicer deletion in thymocytes containing unrearranged or preredrarranged TCRβ loci. Expression of either a preassembled functional TCRβ gene (Vβ11NT) or the prosurvival BCL2 protein inhibited death and partially rescued proliferative expansion of Dicer-deficient thymocytes. Notably, combined expression of Vβ11NT and BCL2 completely rescued proliferative expansion of Dicer-deficient thymocytes and revealed that Dicer promotes survival of cells attempting TCRβ recombination. Finally, inclusion of an endogenous preassembled DJβ complex that enhances Vβ recombination increased death and impaired proliferative expansion of Dicer-deficient thymocytes. These data demonstrate a critical role for Dicer in promoting survival of thymocytes experiencing DNA double-strand breaks (DSBs) during TCRβ recombination. Because DSBs are common and ubiquitous in cells, our findings indicate that impaired cellular survival in response to DSBs should be considered when interpreting Dicer-deficient phenotypes. The Journal of Immunology, 2013, 190: 3256–3266.
B cells was partially rescued by Bim deletion, expression of the BCL2 prosurvival protein, or coexpression of preassembled IgH and IgL transgenes that repress Bim, indicating that Dicer-dependent postranscriptional repression of Bim is important for normal B cell development (10). However, because IgH transgenes bypass necessity of IgH recombination for pre-B to pre-B cell development, this finding also suggests additional potential roles of Dicer in control of V(D)J recombination. Bidirectional transcription of V(D)J segments and flanking repetitive sequences has been proposed to generate siRNAs that direct epigenetic silencing (11, 12). Consistent with these models, Dicer+/− B-lineage cells exhibit increased transcription and usage of Dn segments flanked by DNA repeats (10, 13). However, because Dicer+/− B cells exhibit altered VδδJγγ repertoires due to the loss of Dicer-dependent miRNAs that modulate IgH/IgL selection (10, 13), further studies are needed to directly test whether Dicer-dependent siRNAs control IgH recombination. Dicer deletion in DN cells led to loss of miRNAs, impaired DN-to-DP proliferative expansion, and increased apoptosis of thymocytes cycling in vitro, but no effect upon DN-to-DP thymocyte differentiation per se (8, 9), suggesting that Dicer-dependent RNAs regulate thymocyte survival directly and/or during cell division (8). However, because defects in TCRβ recombination or β-selection also impair DN-to-DP proliferative expansion (14–17), additional functions of Dicer may help promote thymocyte development.

We have used mice expressing preassembled functional endogenous TCRβ genes to elucidate V(D)J recombination control mechanisms that were impossible to discover using TCRβ transgenes (18–21). Preassembled functional TCRβ transgenes/gens facilitate study of mechanisms that silence transcription and recombination of germline Vβs on unrearranged TCRβ alleles (19). However, only preassembled TCRβ genes enable study of mechanisms that control transcription and recombination of Vβs located on VDJβ-recombined alleles (21). Our studies with a preassembled Vβ1Dβ1β1.4β1 (Vβ1NT) gene showed that repetitive sequences within Vβ regions correlate with boundaries between chromatin domains that are differentially silenced in response to β-selection signals (20, 21). Bidirectional transcription of Vβs within these domains precedes their epigenetic silencing (20, 21). To elucidate potential roles of Dicer in control of TCRβ germline transcription and recombination, we generated and analyzed mice with Dicer deletion initiating in DN thymocytes that contain unrearranged TCRβ alleles or Vβ1NT alleles.

Materials and Methods

**Mice**

Lck-cre (22), Dicerlox/lox (8), Vβ1NT/NT (21), Vβ14NT/NT (18), Rag1−/− (15), EqBCL2 transgenic (23), and D/J/DJ (24) mice were bred to generate the animals in this study. The background strain of these mice was mixed 129SvEv and C57BL/6. All experimental mice were littermates or age-matched mice between 4 and 6 wk of age. All experiments were conducted in accordance with national guidelines and approved by the Children’s Hospital of Philadelphia Institutional Animal Care and Use Committee.

**Flow cytometry**

Single-cell suspensions were stained with Abs in PBS containing 2% BSA and 1 mM EDTA as described (21). All Abs were purchased from BD Pharmingen: anti-Vβ4 (553364), anti-Vβ5 (553189), anti-Vβ6 (553192), and anti-Vβ8 (553862), anti-Vβ10 (553285), anti-Vβ14 (553258), anti-CD4 (553051), anti-CD8α (553033), anti-TCRβ (553172), anti-B220 (553090), and anti-CD19 (553786), anti-CD11b (553311), anti-CD11c (557401), anti-CD8 (553176), anti-NK1.1 (553165), anti-CD90 (553033), anti-Ter119 (553673), anti-CD25 (552880), and anti-CD117 (553556). Data were acquired on a FACSCalibur (BD Biosciences, San Jose, CA) using CellQuest software (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Analysis of Vβ rearrangements, transcription, and CpG methylation

Analyses of Vβ rearrangements, transcription, and CpG methylation were conducted as described (21).

**Quantification of Dicer deletion**

DN thymocytes were isolated by FACS purification of lineage-negative cells and genomic DNA isolated as described (22). DNA was used as a template for quantitative PCR measurement of Cre-mediated Dicer deletion on an Applied Biosystems ViiA 7 Fast real-time PCR system by quantifying “floxed” Dicer exon 20 sequences and “non-floxed” Dicer exon 19 sequences and calculating the ratio of exon 20 to exon 19 sequences. Primers were: exon 19, forward, 5′-TCATCCTACCGAGCATC3′, reverse, 5′-TCTGAGCTCATGTTTCTGC-3′; exon 20, forward, 5′-AACCTCTGTTGGCTGAGAG-3′, reverse, 5′-TCATGGTTTTTCAAGAGGGGTCT-3′.

**Results**

**Preassembled functional TCRβ genes partially rescue development of Dicer−/− thymocytes**

The Lck-cre transgene and floxed Dicer (Dicerlox) alleles were previously used to create mice with conditional Dicer deletion initiating in DN thymocytes (8, 9). Thus, we generated and analyzed in parallel Dicerlox/lox (Dicer+/+), Lck-cre;Dicerlox/lox (Dicer−/−), Vβ1NT/NT Dicerlox/lox (Vβ1NT/NT), and Lck-cre;Vβ1NT/NT Dicerlox/lox (Vβ1NT/NT;Dicer−/−) mice. Because expression of preassembled functional TCRβ transgenes/genescures the blocks in αβ T lymphocyte development caused by defects in TCRβ recombination (21), we first conducted flow cytometry (FACS) analysis with anti-CD4 and anti-CD8 Abs to quantify the numbers of DN and DP cells in mice of each genotype. Consistent with published observations (8, 9), we found that Dicer−/− mice contained ∼5-fold lower numbers of DN cells and ∼9-fold lower numbers of DP cells as compared with Dicer+/+ mice (Fig. 1A, 1B). However, as compared with Vβ1NT/NT mice, we found that Vβ1NT/NT Dicer−/− mice contained equivalent numbers of DN cells and only ∼3-fold fewer numbers of DP cells (Fig. 1A, 1B). Additionally, the numbers of DN and DP thymocytes in Vβ1NT/NT Dicer−/− mice were higher than in Dicer−/− mice (Fig. 1A, 1B). Notably, we detected similar extents of Dicer deletion in total thymocytes from mice of each genotype (Fig. 1C), indicating that Vβ1NT did not increase DP thymocyte numbers solely by pushing cells through DN-to-DP differentiation prior to Dicer inactivation. Thus, our finding that Vβ1NT expression partially rescues DN-to-DP proliferative expansion of Dicer-deficient thymocytes supports the notion that Dicer is required for normal TCRβ recombination. Importantly, however, note that the inability of Vβ1NT to completely rescue Dicer−/− DP thymocyte cellularity is consistent with the additional proposed role of Dicer in promoting thymocyte survival during cell division (8).

To further investigate the potential requirement of Dicer for normal TCRβ recombination, we next assayed the effects of Vβ1NT expression on the thymocyte development stages in which TCRβ genes are assembled and selected. Assembly and selection of functional TCRβ genes in c-Kit+/CD25+ DN3 cells promotes their concomitant proliferation and differentiation into c-Kit+/CD25+ DN4 and then DP cells (20, 25, 26). We conducted cell counting and FACS analysis of CD4+/CD8− thymocytes with anti-c-Kit and anti-CD25 Abs. Consistent with published findings (8, 9), we observed a ∼5-fold decrease in the numbers of DN3 thymocytes and a ∼90-fold reduction in DN4 cell numbers in Dicer−/− mice relative to Dicer+/+ mice (Fig. 1D, 1E). Mice expressing preassembled TCRβ transgenes/genescures decreased numbers of DN3 thymocytes, yet normal numbers of DN4 cells, owing to enhanced β-selection (18, 21). We detected similar...
numbers of DN3 cells in Vβ1\(^{NT/NT}\) and Vβ1\(^{NT/NT}Dicer^{-/-}\) mice, but ∼8-fold fewer DN4 cells in Vβ1\(^{NT/NT}Dicer^{-/-}\) mice as compared with Vβ1\(^{NT/NT}\) mice (Fig. 1D, 1E). The numbers of DN4 cells in Vβ1\(^{NT/NT}Dicer^{-/-}\) mice were lower than in Dicer\(^{FF}\) mice but higher than in Dicer\(^{-/-}\) mice (Fig. 1D, 1E). These data indicate that Vβ1\(^{NT}\) expression partially rescues DN4 cellularity and the DN3-to-DN4 developmental transition of Dicer-deficient thymocytes, providing further support to our interpretation that Dicer is required for normal TCRβ recombination. We found equivalent deletion of Dicer in DN thymocytes of Dicer\(^{-/-}\) and Vβ1\(^{NT/NT}Dicer^{-/-}\) mice (Fig. 1F), indicating that Vβ1\(^{NT}\) expression does not increase DN4 thymocyte numbers solely by pushing cells through DN3-to-DN4 development prior to Dicer inactivation. However, the inability of Vβ1\(^{NT}\) expression to completely rescue Dicer\(^{-/-}\) DN4 cell numbers and the DN3-to-DN4 developmental transition also is consistent with the additional proposed role of Dicer in promoting thymocyte survival during cell division (8) and a potential requirement of Dicer for T cell development.

To directly assess whether Dicer is required for normal TCRβ recombination, we characterized Vβ rearrangement and expression in Vβ1\(^{NT/+}\) Vβ1\(^{NT/NT}\) and wild-type αβ T lineage cells expressing or lacking Dicer. We conducted cell counting and FACs analysis of thymocytes with anti-Vβ and anti-CD8 Abs to quantify the percentages of cells expressing particular Vβs. We compared the percentages, rather than absolute numbers, of thymocytes expressing particular Vβs because mice of Dicer-deficient backgrounds harbor reduced thymic cellularity relative to Dicer-sufficient backgrounds. Most Vβs rearrange at low levels on wild-type alleles in Vβ1\(^{NT/+}\) DN3 thymocytes and are expressed in small fractions of Vβ1\(^{NT/NT}\) DP thymocytes and splenic αβ T lymphocytes (21). Vβ10, which resides just upstream of the Vβ1\(^{NT}\) gene (Fig. 3A), rearranges at a higher frequency than other Vβs on the Vβ1\(^{NT}\) allele, and it is expressed in ∼1% of Vβ1\(^{NT/+}\) and Vβ1\(^{NT/NT}\) αβ T lineage cells (21). On Vβ1\(^{NT}\) alleles, repetitive sequences mark a boundary between

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**FIGURE 1.** A preassembled functional TCRβ gene partially rescues early αβ T cell development. (A) Representative CD4/CD8 FACS data of thymocytes from Dicer\(^{FF}\), Dicer\(^{-/-}\), Vβ1\(^{NT/NT}\), and Vβ1\(^{NT/NT}Dicer^{-/-}\) mice. The average numbers of total thymocytes, the DN, DP, CD4\(^+\) SP, and CD8\(^+\) SP cell gates, and the percentage of thymocyte in each gate are indicated. (B) Graph showing the average numbers of DN and DP thymocytes from mice of the indicated genotypes. Error bars are SE. This experiment was independently performed five times with at least one mouse of each genotype in each experimental replicate. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001. (C) PCR analysis showing equivalent deletion of Dicer in total thymocytes of mice of the indicated genotypes. (D) Representative c-Kit/CD25 FACS data of DN thymocytes from Dicer\(^{FF}\), Dicer\(^{-/-}\), Vβ1\(^{NT/NT}\), and Vβ1\(^{NT/NT}Dicer^{-/-}\) mice. The DN1, DN2, DN3, and DN4 thymocyte quadrants and the percentage of DN cells in each quadrant are indicated. (E) Graph showing the average numbers of DN3 and DN4 cells from mice of the indicated genotypes. Error bars are SE. This experiment was independently performed five times with at least one mouse of each genotype in each experimental replicate. *p < 0.05, **p ≤ 0.01. (F) Quantitative PCR analysis showing equivalent deletion of Dicer in DN thymocytes sort-purified from mice of the indicated genotypes (mice expressing the EµBCL2 transgene are designated B+ in this figure). The dots represent data from individual mice and the bars indicate the average values from mice of each genotype.
Vβ10 segments that recombine and upstream Vβ4/Vβ16 segments that do not recombine (21). Although Dicer deletion had no effect on the percentages of Vβ11NT thymocytes that expressed Vβs other than Vβ10 (Fig. 3B, 3C), we detected ~2-fold decreases in the percentages of Vβ10+ thymocytes upon deletion of Dicer in Vβ11NT+ and Vβ11NT/Dicer−/− mice (Fig. 3B–E). We found no difference in the percentages of Vβ10+ thymocytes between Vβ11NT/DicerF/+ and Vβ11NT/Dicer+/- mice (Fig. 3F, 3G), indicating that Cre expression in Dicer-deficient thymocytes does not affect Vβ10 rearrangement and expression. Notably, we did not notice any significant effect of Dicer deletion on Vβ4, Vβ16, or Vβ14 expression of Vβ11NTs or Vβ11NTNT thymocytes (Fig. 3C, 3E). PCR with specific Vβ and Jβ primers to quantify Vβ rearrangements detected decreases in levels of Vβ rearrangements upon deletion of Dicer in Vβ11NT+ and Vβ11NTNT mice (Fig. 3H). Because Vβ10 recombination on Vβ1NT alleles occurs only in DN3 cells (20), the reduced rearrangement and expression of Vβ10 in Vβ11NT/Dicer−/− and Vβ11NTNT/Dicer−/− thymocytes provides functional evidence that Dicer is inactivated in these DN3 cells. In contrast to mice containing the Vβ1NT allele, we observed no differences in Vβ10 expression between DicerF/F and Dicer−/− mice (Fig. 3I, 3J). These data indicate that Dicer is required for normal recombination of Vβ10 segments on Vβ1NT alleles, but not on wild-type TCRb alleles; they also provide no
evidence that Dicer controls heterochromatin formation over Vβ4 and Vβ16 on Vβ1NT alleles.

Because V(D)J recombination is controlled by modulating accessibility of gene segments to RAG proteins (7), the requirement of Dicer for normal recombination of Vβ10 segments on Vβ1NT alleles could be in promoting Vβ10 accessibility. RAG accessibility is assayed by quantifying steady-state germline transcripts and CpG methylation of gene segments (7). On Vβ1NT alleles, Vβ10 segments are transcribed and exhibit low DNA CpG methylation in both DN and DP thymocytes (20, 21), enabling

![Figure 3](http://www.jimmunol.org/DownloadedFrom/3260-DICER-PROMOTES-LYMPHOCYTE-SURVIVAL.png)
assess the ability of Vβ10 to enter the thymus and the impact of Dicer deletion on thymocyte development.

In parallel, we also analyzed Vβ10 transcripts in Vβ1NT/Dicer−/− mice. We found that Dicer deletion had no significant effect on the levels of germline Vβ10 transcripts in Vβ1NT/Dicer−/− thymocytes (Fig. 4E); however, Dicer deletion led to significantly higher total thymocytes, the DN, DP, CD4+ SP, and CD8+ SP cells (Fig. 4F). We also found that Dicer deletion had no effect on Vβ10 CpG methylation in Vβ1NT/Rag1−/− cells (Fig. 4G), but it led to significantly higher Vβ10 CpG methylation in Vβ1NT/Dicer−/− cells (Fig. 4H). The simplest explanation for decreased germline transcription and increased CpG methylation of germline Vβ10 segments in Vβ1NT/Dicer−/−Rag1−/− thymocytes compared with Vβ1NT/Dicer−/− thymocytes is increased apoptosis of Dicer-deficient Vβ1NT/Dicer−/− DN cells attempting TCRβ recombination.

On Vβ1NT alleles, bidirectional transcription of germline Vβ10s in thymocytes precedes their heterochromatin-mediated silencing in mature αβ T cells (21). We observed no difference in the levels of germline Vβ10 transcripts between Vβ1NT/Dicer−/− and Vβ1NT/Dicer−/− splenic αβ T cells (Fig. 4I), consistent with normal silencing. This observation indicates that epigenetic silencing of germline Vβ10s on Vβ1NT alleles does not require Dicer-dependent processing of potential Vβ10 siRNAs.

Dicer is required for survival of thymocytes attempting TCRβ recombination

Expression of BCL2 sustains lymphocyte survival in response to DSBs induced during V(D)J recombination (27). Therefore, as an initial means to assess whether Dicer is required for survival of thymocytes, we examined the ability of Vβ10 to enter the thymus and the impact of Dicer deletion on thymocyte development.

**FIGURE 4.** Dicer is required for survival of DNA cells attempting Vβ10 rearrangements on Vβ1NT alleles. (A) Representative c-KiCD25 FACS data of DNA thymocytes from Vβ1NT/Dicer−/− and Vβ1NT/Dicer−/−Rag1−/− mice. The DNA, DN2, DN3, and DN4 thymocyte quadrants and the percentages of DN cells within each of these quadrants are indicated. (B) Graph showing the average numbers of DN1 and DN4 cells from Vβ1NT/Dicer−/− and Vβ1NT/Dicer−/−Rag1−/− mice. Error bars are SE. This experiment was done three independent times on at least one mouse of each genotype. *p < 0.05, **p < 0.01. (C) Representative CD4/CD8 FACS data of thymocytes from Vβ1NT/Rag1−/− and Vβ1NT/Dicer−/−Rag1−/− mice. The average numbers of total thymocytes, the DN, DP, CD4+ SP, and CD8+ SP cell gates, and the percentages of thymocytes within each of these gates are indicated. (D) Graph showing the average numbers of DN and DP thymocytes from Vβ1NT/Rag1−/− and Vβ1NT/Dicer−/−Rag1−/− mice. Error bars are SE. This experiment was done three independent times on at least one mouse of each genotype. *p < 0.05, **p < 0.01. (E and F) Graphs showing average levels of Vβ1NT gene transcripts or germline transcripts of the other indicated Vβ segments in thymocytes of (E) Vβ1NT/Rag1−/− and Vβ1NT/Dicer−/−Rag1−/− mice or (F) Vβ1NT and Vβ1NT/Dicer−/− mice. Error bars are SE. This experiment was independently performed three times with at least one mouse of each genotype in each experimental replicate. ***p < 0.001. (G and H) Graphs showing average percent Vβ10 DNA CpG methylation at restriction enzyme sites within the intron upstream of Vβ10 (I1 and I2), the Vβ10 promoter (P), or the Vβ10 coding sequence (C) in thymocytes of (G) Vβ1NT/Rag1−/− and Vβ1NT/Dicer−/−Rag1−/− mice or (H) Vβ1NT and Vβ1NT/Dicer−/− mice. Error bars are SE. This experiment was independently performed three times with at least one mouse of each genotype in each experimental replicate. *p < 0.05. (I) Graph showing the average levels of transcripts for rearranged Vβ10 segments, germline (GL) Vβ10 segments, or the Vβ1NT gene in splenic αβ T cells from Vβ1NT and Vβ1NT/Dicer−/− mice. Error bars are SE. No significant differences were detected.
cells attempting TCRβ recombination, we analyzed thymocyte development in Dicer<sup>F/F</sup>, Dicer<sup>b</sup>−/−, Vb<sup>1NT/NT</sup>, and Vb<sup>1NT/NT</sup>Dicer<sup>b</sup>−/− mice lacking or expressing the E<sub>b</sub>BCL2 transgene (the latter are designated B<sub>b</sub> within the figures). We found that E<sub>b</sub>BCL2 expression partially rescued DN3-to-DN4 and DN-to-DP development of Dicer<sup>b</sup>−/− thymocytes (Fig. 5A–D). We also found that combined expression of E<sub>b</sub>BCL2 and Vb<sup>1NT</sup> completely restored DN3-to-DN4 and DN-to-DP development of Dicer<sup>b</sup>−/− thymocytes to levels observed in Dicer<sup>b</sup>+/+ thymocytes (Fig. 5E–H). We found similar deletion of Dicer in DN and total thymocytes of Dicer<sup>b</sup>−/− mice containing E<sub>b</sub>BCL2 and Vb<sup>1NT</sup> (Fig. 1C, 1F), indicating that E<sub>b</sub>BCL2 expression does not increase DN4 and DP thymocyte numbers solely by enabling thymocytes to escape Dicer deletion during the DN3-to-DN4 and DN-to-DP developmental transitions. Although the ability of BCL2 to rescue DN3-to-DN4 and DN-to-DP development of Dicer<sup>b</sup>−/− and Vb<sup>1NT/NT</sup>Dicer<sup>b</sup>−/− thymocytes is consistent with a requirement for Dicer in survival of DN cells attempting TCRβ recombination, this phenotype also could be due to the ability of BCL2 to prevent death of dividing thymocytes.

We next evaluated the effect of E<sub>b</sub>BCL2 on the survival of Dicer<sup>b</sup>−/− and Vb<sup>1NT/NT</sup>Dicer<sup>b</sup>−/− thymocytes. Because death of Dicer<sup>b</sup>−/− thymocytes cannot be detected ex vivo by increased annexin V staining or reduced mitochondrial potential (8), we quantified dead thymocytes by FACS analysis using forward and side scatter to distinguish between live and dead cells. We compared the percentages, rather than numbers, of dead thymocytes because mice of Dicer-deficient backgrounds harbor reduced thymic cellularity relative to Dicer-sufficient backgrounds. We detected a ~5-fold increase in the percentage of dead thymocytes in Dicer<sup>b</sup>−/− mice relative to Dicer<sup>F/F</sup> mice (Fig. 5I). We found that E<sub>b</sub>BCL2 expression in Dicer<sup>b</sup>−/− mice lowered the percentage of dead cells to levels observed in Dicer<sup>F/F</sup> and E<sub>b</sub>BCL2 mice (Fig. 5I). We also found that Vb<sup>1NT</sup> expression in Dicer<sup>b</sup>−/− mice lowered the percentage of dead thymocytes to levels slightly above those detected in Dicer<sup>F/F</sup> and Vb<sup>1NT/NT</sup> mice (Fig. 5I), whereas combined E<sub>b</sub>BCL2 and Vb<sup>1NT</sup> expression in Dicer<sup>b</sup>−/− mice lowered the percentage of dead thymocytes to levels found in Dicer<sup>F/F</sup>, Vb<sup>1NT/NT</sup>, and E<sub>b</sub>BCL2 mice (Fig. 5I). Because expression of preassembled TCRβ transgenes/genes inhibits RAG activity in DN cells and bypasses necessity of TCRβ recombination for thymocyte development, the ability of Vb<sup>1NT</sup> to decrease the percentage of dead Dicer<sup>b</sup>−/− thymocytes supports the idea that Dicer is required for survival of cells attempting TCRβ recombination. Importantly, however, note that because expression of both Vb<sup>1NT</sup> and E<sub>b</sub>BCL2 further decreases the percentage of dead Dicer<sup>b</sup>−/− thymocytes, our findings are consistent with an additional role of Dicer in promoting thymocyte survival during cell division (8). The notion that Dicer promotes survival of proliferating thymocytes is also supported by our data that Dicer deletion in Vb<sup>1NT/NT</sup>Rag1<sup>+/−</sup> mice leads to reduced numbers of DN4 and DP cells (Fig. 4A–D).

To evaluate whether Dicer is required for survival of DN cells attempting TCRβ recombination, we analyzed Vβ10 expression and rearrangement in thymocytes of Vb<sup>1NT/NT</sup> and Vb<sup>1NT/NT</sup>Dicer<sup>b</sup>−/− mice lacking or expressing the E<sub>b</sub>BCL2 transgene. We detected an ~12-fold increase in the percentages of Vβ10<sup>+</sup> thymocytes in Vb<sup>1NT/NT</sup>Dicer<sup>b</sup>−/− mice expressing E<sub>b</sub>BCL2 as compared with Vb<sup>1NT/NT</sup>Dicer<sup>b</sup>−/− mice lacking E<sub>b</sub>BCL2 (Fig. 5J). The elevated percentages of Vβ10<sup>+</sup> thymocytes in Vb<sup>1NT/NT</sup>Dicer<sup>b</sup>−/− mice expressing E<sub>b</sub>BCL2 were comparable to those in Vb<sup>1NT/NT</sup> mice expressing E<sub>b</sub>BCL2 (Fig. 5J). The increased percentages of Vβ10<sup>+</sup> cells in Vb<sup>1NT/NT</sup>Dicer<sup>b</sup>−/− and Vb<sup>1NT/NT</sup>Dicer<sup>b</sup>−/− mice expressing E<sub>b</sub>BCL2 corresponded with higher levels of Vβ10 recombination (Fig. 5K). The ability of BCL2 to rescue Vβ10<sup>+</sup> thymocytes in Vb<sup>1NT/NT</sup>Dicer<sup>b</sup>−/− mice is consistent with the notion that Dicer is required for survival of cells attempting TCRβ recombination. Because BCL2 affects αβ TCR selection (28), and αβ TCR selection can alter Vβ repertoire and levels of Vβ rearrangements between nonselected TCRβ<sup>ab</sup> DP thymocytes and selected TCRβ<sup>ab</sup> SP thymocytes (29), these phenotypes also could be caused by differences in selection of Vβ10<sup>+</sup> cells among mice of these genotypes. Although E<sub>b</sub>BCL2 expression caused an ~2-fold increase in the ratio of TCRβ<sup>hig</sup>V<sub>b</sub>10<sup>+</sup> to TCRβ<sup>ab</sup>V<sub>b</sub>10<sup>+</sup> thymocytes in Vb<sup>1NT/NT</sup>Dicer<sup>b</sup>−/− mice (Fig. 5L), this change was lower than the ~4-fold increase in the percentage of Vβ<sup>10</sup>− thymocytes observed between these mice. Additionally, the ratio of TCRβ<sup>hig</sup>V<sub>b</sub>10<sup>+</sup> to TCRβ<sup>ab</sup>V<sub>b</sub>10<sup>+</sup> thymocyte numbers in Vb<sup>1NT/NT</sup>Dicer<sup>b</sup>−/− mice was not altered by E<sub>b</sub>BCL2 expression (Fig. 5L). Collectively, these data indicate that the increased frequency of Vβ10<sup>+</sup> cells in Vb<sup>1NT/NT</sup>Dicer<sup>b</sup>−/− and Vb<sup>1NT/NT</sup>Dicer<sup>b</sup>−/− mice expressing E<sub>b</sub>BCL2 compared with those lacking E<sub>b</sub>BCL2 resulted from both greater survival of DN cells attempting Vβ10<sup>+</sup> rearrangements and increased selection of Vβ10<sup>+</sup> DP thymocytes.

We sought another means to show that Dicer is required for survival of cells attempting TCRβ recombination. We previously showed that TCRβ alleles containing a preassembled DJβ1 complex (DJ alleles) exhibit increased Vβ rearrangement frequencies relative to wild-type alleles (24). Despite the ability of preassembled functional TCRβ genes to activate β-selection signals that inhibit RAG activity and Vβ recombination, ~5% of Vβ<sup>10</sup>−/− DN3 cells assemble VDJβ1 joins on wild-type alleles (21). Thus, we reasoned that comparison of DN3-to-DN4 and DN-to-DP thymocyte development between Vb<sup>1NT</sup> and Vb<sup>1NT/NT</sup>Dicer<sup>b</sup>−/− mice on wild-type and Dicer-deficient backgrounds would enable us to determine whether Dicer is required for survival of cells attempting TCRβ recombination. To validate this approach, we first needed to show that Vβ rearrangement frequencies are increased on DJ alleles in Vb<sup>1NT/NT</sup> mice relative to on wild-type alleles in Vb<sup>1NT</sup> mice. We observed greater percentages of TCRβ<sup>hig</sup> thymocytes expressing each Vβ assayed in Vb<sup>1NT/NT</sup> mice relative to Vb<sup>1NT</sup> mice (Fig. 6A, 6B). We also detected higher levels of Vβ-to-DJβ1 rearrangements involving DJβ1.1 or DJβ1.2 complexes of all Vβs assayed in Vb<sup>1NT/NT</sup> cells as compared with in Vb<sup>1NT</sup> cells (Fig. 6C). Because β-selection does not alter Vβ repertoire during DN3-to-DN4 and DN-to-DP development (30), these data confirm that Vβ recombination frequencies are higher on DJ alleles in Vb<sup>1NT/NT</sup> mice as compared with wild-type alleles in Vb<sup>1NT</sup> mice.

To directly evaluate whether Dicer is required for survival of DN thymocytes attempting TCRβ recombination, we analyzed the effects of Dicer deletion upon Vβ repertoire and DN3-to-DN4 and DN-to-DP thymocyte development in Vb<sup>1NT/NT</sup> and Vb<sup>1NT</sup> mice. We observed equivalent percentages of thymocytes expressing each Vβ assayed in Vb<sup>1NT/NT</sup> and Vb<sup>1NT</sup>Dicer<sup>b</sup>−/− mice (Fig. 6A, 6B). We also detected higher levels of Vβ-to-DJβ1 rearrangements involving DJβ1.1 or DJβ1.2 complexes of all Vβs assayed in Vb<sup>1NT/NT</sup> cells as compared with in Vb<sup>1NT</sup> cells (Fig. 6C). Because β-selection does not alter Vβ repertoire during DN3-to-DN4 and DN-to-DP development (30), these data confirm that Vβ recombination frequencies are higher on DJ alleles in Vb<sup>1NT/NT</sup> mice as compared with wild-type alleles in Vb<sup>1NT</sup> mice.
caused ∼2-fold decreases in the numbers of DN3 and DN4 thymocytes (Fig. 6G, 6H), whereas deletion of Dicer in Vβ1<sup>NT/DJ</sup> mice had no significant effect (albeit a ∼2-fold reduction) on DN3 thymocyte numbers, yet caused a ∼10-fold decrease in DN4 cell numbers (Fig. 6H). These data indicate that Dicer deletion caused a more pronounced loss of DN4 cells in Vβ1<sup>NT/DJ</sup> mice as com-

FIGURE 5. EμBCL2 expression prevents the death of Dicer-deficient thymocytes attempting TCRβ recombination. (A) Representative CD4/CD8 FACS analysis of thymocytes from B<sup>+</sup> and B<sup>+</sup>Dicer<sup>−/−</sup> mice (mice expressing the EμBCL2 transgene are designated B<sup>+</sup> in this figure). The average numbers of total thymocytes, the DN, DP, CD4<sup>+</sup> SP, and CD8<sup>+</sup> SP cell gates, and the percentage of thymocytes in each gate are indicated. (B) Graph showing average numbers of DN and DP thymocytes from mice of the indicated genotypes. Error bars are SE. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001. (C) Representative c-Kit/CD25 FACS data of thymocytes from B<sup>+</sup> and B<sup>+</sup>Dicer<sup>−/−</sup> mice. The DN1, DN2, DN3, and DN4 quadrants and the percentage of DN cells in each quadrant are indicated. (D) Graph showing average numbers of DN3 and DN4 thymocytes from mice of the indicated genotypes. Error bars are SE. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001. (E) Representative CD4/CD8 FACS analysis of thymocytes from B<sup>+</sup>Vβ1<sup>NT/DJ</sup> and B<sup>+</sup>Vβ1<sup>NT/DJ</sup>Dicer<sup>−/−</sup> mice. The average numbers of total thymocytes, the DN, DP, CD4<sup>+</sup> SP, and CD8<sup>+</sup> SP cell gates, and the percentage of thymocytes in each gate are indicated. (F) Graph showing average numbers of DN and DP thymocytes from mice of the indicated genotypes. Error bars are SE. *p < 0.05, **p ≤ 0.01, ***p < 0.001. (G) Representative c-Kit/CD25 FACS data of thymocytes from B<sup>+</sup>Vβ1<sup>NT/DJ</sup> and B<sup>+</sup>Vβ1<sup>NT/DJ</sup>Dicer<sup>−/−</sup> mice. The DN1, DN2, DN3, and DN4 quadrants and the percentage of DN cells in each quadrant are indicated. (H) Graph showing average numbers of DN3 and DN4 thymocytes from mice of the indicated genotypes. Error bars are SE. *p < 0.05, **p ≤ 0.01. (I) Graph showing the average percentage of dead thymocytes in mice of the indicated genotypes. Error bars are SE. **p < 0.01, ***p ≤ 0.001. (J) Graph showing the ratio of percentages of Vβ10<sup>+</sup>TCRB<sup>hi</sup> and Vβ10<sup>+</sup>TCRB<sup>int</sup> thymocytes from mice of the indicated genotypes. Error bars are SE. Each experiment in this figure was independently performed three times with at least one mouse of each genotype in each replicate. **p ≤ 0.01.
pared with Vβ1NT/+ mice. Because the assembly of functional VDJβ genes is required for DN3-to-DN4 development and the only phenotypic difference between Vβ1NT/DJ and Vβ1NT/+ mice is the frequency of Vβ recombination, these data provide unequivocal evidence that Dicer is required for survival of DN thymocytes attempting TCRβ recombination.

FIGURE 6. Dicer is required for survival of DN thymocytes attempting Vβ recombination. (A) Representative TCRβ/Vβ14 and TCRβ/Vβ6 FACS data from Vβ1NT/DJ and Vβ1NT/DJ/Dicer−/− mice. The Vβ14+ and Vβ6+ cell gates are indicated. (B) Graph showing average percentages of TCRβhigh thymocytes expressing the indicated Vβ segments in Vβ1NT/+, Vβ1NT/Dicer−/−, Vβ1NT/DJ, and Vβ1NT/DJRc−/− mice. Error bars are SE. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001. (C) Representative PCR analysis of rearrangements involving the indicated Vβ segments to DJβ1.1/DJβ1.2 or DJβ2.1/DJβ2.2 complexes in thymocytes of mice of the indicated genotypes. A Cβ PCR control for genomic DNA content is also shown. (D) Graph showing the average percentage of dead thymocytes in mice of the indicated genotypes. Error bars are SE. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001. (E) Representative CD4/CD8 FACS data of thymocytes from Vβ1NT/DJ and Vβ1NT/DJ/Dicer−/− mice. The average numbers of total thymocytes and the DN, DP, CD4+ SP, and CD8+ SP cell gates are indicated. (F) Graph showing the average numbers of DN and DP thymocytes from mice of the indicated genotypes. Error bars are SE. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001. (G) Representative c-Kit/CD25 FACS data of DN thymocytes from Vβ1NT/DJ and Vβ1NT/DJ/Dicer−/− mice. The DN1, DN2, DN3, and DN4 thymocyte quadrants and the percentages of DN cells within each of these quadrants are indicated. (H) Graph showing the average numbers of DN3 and DN4 cells from mice of the indicated genotypes. Error bars are SE. Each experiment in this figure was independently performed three times with at least one mouse of each genotype in each experimental replicate. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001.
Discussion

We have shown that Dicer is required for normal survival of DN thymocytes attempting Vβ recombination. TCRβ genes are assembled in G0/G1 phase cells through an ordered process involving D.Jb to D.Jβ and then Vβ to D.Jβ recombination (7). Expression of functional TCRβ genes drives DN3 cells into S phase and through many cell cycles as they differentiate into DN4 cells (20, 31). Vβ to D.Jβ recombination is repressed through TCRβ-mediated feedback inhibition signals (7). However, functional TCRβ genes can be detected on both alleles in 1–10% of αβ T cells (7), indicating that DN3 cells can attempt Vβ recombination while experiencing proliferation signals from assembled VβDJβCβ genes. The increased death and decreased proliferative expansion of Vβ1NT/Dicerβ−/− thymocytes compared with Vβ1NT+/+Dicer−/− thymocytes demonstrates that Dicer is required for survival of DN3 cells that attempt Vβ recombination while experiencing proliferation signals from TCRβ genes assembled first on other alleles. Despite TCRβ-mediated feedback inhibition, ~6% of Vβ1NT/+ and Vβ1NT/NT splenic αβ T cells contain Vβ-to-D.Jβ2 rearrangements that replace Vβ1NT genes (21), revealing that a significant percentage of DN3 thymocytes that have assembled functional VβDJβCβ1 genes can attempt replacement Vβ-to-D.Jβ2 rearrangements. The increased death, decreased proliferative expansion, reduced levels of Vβ10+/D.Jβ2 rearrangements, and decreased frequency of Vβ10+ thymocytes in Vβ1NT/NT/Dicer−/− mice relative to Vβ1NTNT mice demonstrate that Dicer also is required for survival of DN3 cells that attempt Vβ-to-D.Jβ2 recombination while experiencing proliferation signals from VβDJβCβ1 genes assembled on the same allele.

Although preassembled functional TCRβ genes inhibit Vβ recombination, their suppression of other V(D)J recombination events likely contributes to their ability to partially rescue the development of Dicer-deficient thymocytes. In DN cells, D.Jb to D.Jβ rearrangements occur on both alleles and are not subject to feedback inhibition (7). Despite this lack of regulation, D.Jb to D.Jβ recombination is decreased on wild-type alleles in αβ T cells of Vβ1NTNT mice (18, 21), likely because expression of preassembled TCRβ genes/transgenes accelerates D3N-to-DN4 thymocyte development (18, 32). The decreased death and proliferative expansion of Dicer−/− thymocytes relative to Dicer+/− thymocytes may reflect that Dicer is required for survival of DN3 cells that attempt D.Jb to D.Jβ recombination while experiencing TCRβ proliferation signals. In DN cells, the RAG proteins also promote TCRγ, TCRδ, and IγH recombination and induce “off-target” DSBS at other genetic loci (7). Because preassembled TCRβ genes/transgenes downregulate RAG activity in DN cells (7), the decreased death and increased proliferative expansion of Vβ1NT/NT Dicer−/− thymocytes as compared with Dicer−/− thymocytes is consistent with a requirement for Dicer in survival of DN3 cells that induce RAG DSBS outside of TCRβ loci while experiencing proliferation signals. Normal DP-to-Sp thymocyte development in Dicer−/− mice indicates that Dicer is not required for survival in response to RAG DSBS induced in DP thymocytes that do not experience proliferation signals. We propose that this would be the case in DN3 cells not undergoing β-selection.

Dicer-generated miRNAs are required for normal survival of mammalian cells in response to DSBS (33). We showed that a 2-fold reduction in expression levels of histone H2AX leads to impaired DSBS responses during V(D)J recombination (34). Because loss of Dicer-dependent miRNAs can lead to 2-fold changes in the expression of hundreds of proteins, including factors that function in the same pathways (35, 36), impaired survival of Dicer−/− DN cells that induce RAG DSBS could be due to altered constitutive expression of DNA damage response proteins. Upon induction of DSBs in nonlymphoid cells, the ATM, p53, and/or p38MAPK proteins signal to increase generation of mature miRNAs that promote cellular survival (37, 38). Because RAG DSBS signal through ATM, p53, and p38MAPK to eliminate DN cells that attempt to proliferate with unrepaired TCR loci (39, 40), impaired survival of Dicer−/− thymocytes could be due to their inability to upregulate expression of prosurvival miRNAs in response to RAG DSBS. Recent studies have revealed a requirement for Dicer in the ability of mammalian cells to respond to and repair DSBS by processing dsRNAs formed upon transcription of broken DNA ends (41, 42). Perhaps transcription of RAG-generated hairpin coding ends generates dsRNAs that Dicer processes to promote V(D)J recombination and inhibit apoptosis of recombining cells. Comparison of miRNA, mRNA, and protein expression in wild-type, Dicer−/−, and Rag1−/− DN3 cells and follow-up functional studies will be required to elucidate the precise mechanisms by which Dicer sustains survival of thymocytes during V(D)J recombination.

Our data also reveal a requirement for Dicer in promoting survival of proliferating thymocytes. The ability of Vβ1NT, alone or in combination with Rag1 deficiency, to only partially rescue DN3-to-DN4 and DN-to-DP development in Dicer−/− mice is consistent with the postulated requirement for Dicer in survival of dividing thymocytes (8). Our data that combined expression of Vβ1NT and Bcl2 completely rescued these developmental transitions in Dicer−/− mice provide strong evidence that Dicer is required for the survival of proliferating thymocytes. DNA replication-associated DSBS are common and unavoidable in each S phase. Similar to mice with conditional Dicer deletion in DN cells, mice with thymocyte-specific inactivation of the Brcal, Brcal2, or Blm proteins that repair DNA replication-associated DSBS exhibit impaired proliferative expansion and increased apoptosis of thymocytes (43–45). Expression of the EμBcl2 transgene, but not preassembled TCRβ/TCRα transgenes, rescued these phenotypes (43–45), revealing that the ability of thymocytes to survive in response to DNA replication-associated DSBS is required for normal DN-to-DP proliferative expansion. Therefore, we conclude that Dicer also promotes αβ T cell differentiation by controlling cellular survival and death decisions in response to DNA replication-associated DSBS.

Our findings suggest that impaired proliferation and survival of cells in response to replication-associated DSBS could contribute to Dicer-deficient phenotypes. Conditional Dicer deletion in DN cells leads to lower numbers of developing and mature αβ T cells, but not γδ T cells that develop with less cellular expansion, nor any obvious pathological conditions (8, 9). However, Dicer deletion in DP cells after thymocyte proliferative expansion has minimal impact on thymocyte numbers but leads to lower numbers of peripheral αβ T cells that exhibit impaired survival during proliferation, and it causes lethal inflammatory disease late in life (9, 46–49). Moreover, Dicer deletion in mature αβ T cells as they differentiate into regulatory T cells has no effect on the development or numbers of these immunosuppressive cells, but it ablates regulatory T cell function and causes lethal inflammatory disease by 2 mo of age (49, 50). Considering the requirement for Dicer in survival of proliferating cells, Dicer deletion in DN thymocytes would cause death of cells in all mature αβ T lymphocyte lineages and thus not disrupt adaptive immune system homeostasis. In contrast, Dicer deletion specifically in αβ regulatory T cells preceding Ag-dependent proliferation would impair their survival and necessary immunoregulatory functions. Because DSBS are induced by replication, transcription, and byproducts of metabolism and are thus ubiquitous, our findings indicate that impaired DNA damage responses should be considered when interpreting Dicer-deficient phenotypes.
Disclosures

The authors have no financial conflicts of interest.

References