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

Brenna L. Brady,*‡,†,§ Levi J. Rupp,*‡,§ 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 unarranged or prearranged TCRβ loci. Expression of either a preassembled functional TCRβ gene (Vβ13*N) or the prosurvival BCL2 protein inhibited death and partially rescued proliferative expansion of Dicer-deficient thymocytes. Notably, combined expression of Vβ13*N 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 our 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: 000–000.

Dicer promotes biogenesis of small RNAs that control posttranscriptional gene expression and epigenetic silencing. In fission yeast, Dicer is required for production of short interfering RNAs (siRNAs) that promote epigenetic silencing of repetitive DNA transcription and mating type locus recombination (1). In animals, Dicer is essential for generation of microRNAs (miRNAs) that block translation or induce turnover of target mRNAs (2). Dicer-deficient mice are embryonic lethal (3), whereas mice with tissue-specific deletion of Dicer often exhibit pathological conditions (2). Dicer−/− mouse cells exhibit loss of miRNAs, gene expression changes, altered differentiation, and impaired activity (2), suggesting that Dicer-dependent miRNAs are critical for control of lineage-specific functions. Consistent with this notion, altered expression of individual miRNAs can recapitulate Dicer-deficient phenotypes (4). However, these interpretations are complicated by miRNA-independent Dicer functions and impaired proliferation and survival of Dicer−/− cells. For example, Dicer−/− oocytes and embryonic stem cells exhibit increased transcripts from retrotransposons and other repetitive sequences that correlate with reduced siRNA levels and impaired epigenetic silencing (5, 6). Thus, Dicer−/− phenotypes could arise from loss of miRNA-mediated gene repression, processing of repetitive transcripts, and/or siRNA-dependent epigenetic silencing.

Mouse αβ T and B cell differentiation involves stage-specific regulation of gene expression and genomic recombination or epigenetic silencing of Ag receptor gene segments (7). Transcription and recombination of TCRβ and IgH loci occur in CD4+/CD8− double-negative (DN) thymocytes and bone marrow pro-B cells, respectively. The RAG1/RAG2 (RAG) endonuclease induces double-strand breaks (DSBs) at transcribing TCRβ or IgH V, D, and J gene segments in G1 phase cells, and nonhomologous end-joining proteins repair these DSBs to form V(D)J coding exons upstream of C region exons. Assembly and expression of functional VβDJβJCβ or VμDJμJCμ genes leads to β- or H-selection signals that silence germline Vs and drive proliferative expansion of cells as they differentiate into CD4+/CD8− double-positive (DP) thymocytes or pre-B cells, respectively. Assembly and expression of in-frame VαJoCa or VλJλCa genes in DP or pre-B cells can lead to TCRα/TCRβ or IgH/IgL Ag receptors. Upon their positive selection, Ag receptors signal differentiation into CD4+ or CD8+ single-positive (SP) thymocytes or immature B cells. These cells emigrate from the thymus or bone marrow as CD4+ or CD8+ αβ T cells, or transitional B cells, and can differentiate into effector lineages in peripheral tissues.

Dicer promotes B and αβ T cell differentiation through generation of small RNAs that direct miRNA-mediated gene repression and possibly siRNA-mediated control of V(D)J recombination (8–10). Conditional Dicer deletion in pro-B cells caused absence of miRNAs (including one that represses the Bim proapoptotic protein), blocked pro-B to pre-B cell differentiation, and increased pro-B cell apoptosis (10). Development of Dicer−/− pro-
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 posttranscriptional repression of *Bim* is important for normal B cell development (10). However, because IgH transgenes bypass necessity of IgH recombination for pro-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<sup>−/−</sup> B-lineage cells exhibit increased transcription and usage of D<sub>B</sub> segments flanked by DNA repeats (10, 13). However, because Dicer<sup>−/−</sup> B cells exhibit altered V<sub>H</sub>D<sub>H</sub>J<sub>H</sub> 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 TCR<sub>R</sub> 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 TCR<sub>β</sub> genes to elucidate V(D)J recombination control mechanisms that were impossible to discover using TCR<sub>R</sub> transgenes (18–21). Preassembled functional TCR<sub>R</sub> transgenes/gene facilitate study of mechanisms that silence transcription and recombination of germline V<sub>β</sub>S on unarranged TCR<sub>R</sub> alleles (19). However, only preassembled TCR<sub>R</sub> genes enable study of mechanisms that control transcription and recombination of V<sub>β</sub>S located on VDJ<sub>R</sub>-recombined alleles (21). Our studies with a preassembled Vβ1DJβ1.4CB1 (Vβ<sub>1NT</sub>) gene showed that repetitive sequences within V<sub>β</sub> regions correlate with boundaries between chromatin domains that are differentially silenced in response to β-selection signals (20, 21). Bidirectional transcription of V<sub>β</sub>S within these domains precedes their epigenetic silencing (20, 21). To elucidate potential roles of Dicer in control of TCR<sub>R</sub> germline transcription and recombination, we generated and analyzed mice with Dicer deletion initiating in DN thymocytes that contain unrearranged and recombination, we generated and analyzed mice with Dicer deletion in total Rag<sub>2</sub>−/− mice contained equivalent numbers of DN and DP cells, owing to enhanced Dicer<sup>−/−</sup> 5-fold decrease in the numbers of DN3 thymocytes and a ∼90-fold reduction in DN4 thymocytes that repress Bim, indicating that Dicer-deletion in total Rag<sub>2</sub>−/− mice did not increase DP thymocyte numbers solely by pushing cells through DN-to-DP differentiation prior to Dicer inactivation. Thus, our finding that Vβ<sub>1NT</sub> expression partially rescues DN-to-DP proliferative expansion of Dicer-deficient thymocytes supports the notion that Dicer is required for normal TCR<sub>R</sub> recombination. Importantly, however, note that the inability of Vβ<sub>1NT</sub> to completely rescue Dicer<sup>−/−</sup> DP thymocytecellularity 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 TCR<sub>R</sub> recombination, we next assayed the effects of Vβ<sub>1NT</sub> expression on the thymocyte development stages in which TCR<sub>R</sub> genes are assembled and selected. Assembly and selection of functional TCR<sub>R</sub> genes in c-Kit<sup>−/−</sup>/CD25<sup>+</sup> DN3 cells promotes their concomitant proliferation and differentiation into c-Kit<sup>−/−</sup>/CD25<sup>+</sup> DN4 and then DP cells (20, 25, 26). We conducted cell counting and FACS analysis of CD4<sup>+</sup>/CD8<sup>+</sup> 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<sup>−/−</sup> mice relative to Dicer<sup>+/+</sup> mice (Fig. 1D, 1E). Mice expressing preassembled TCR<sub>R</sub> transgenes/gene exhibit 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 Vβ1NT/NT and Vβ1NT/NT Dicer−/− mice, but ~8-fold fewer DN4 cells in Vβ1NT/NT Dicer−/− mice as compared with Vβ1NT/NT mice (Fig. 1D, 1E). The numbers of DN4 cells in Vβ1NT/NT Dicer−/− mice were lower than in Dicer+/FP mice but higher than in Dicer−/− mice (Fig. 1D, 1E). These data indicate that Vβ1NT 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 also observed no differences in thymocyte development between Vβ1NT/Dicerlox/− (Vβ1NT/Dicer−/−) and Lck-cre: Vβ1NT−/− Dicerlox/− (Vβ1NT−/− Dicer−/−) mice (Fig. 2E–H), indicating that Cre expression in Dicer-deficient thymocytes does not impair early αβ T cell development.

**Dicer is required for normal TCRβ recombination**

To directly assess whether Dicer is required for normal TCRβ recombination, we characterized Vβ rearrangement and expression in Vβ1NT+/+ Vβ1NT/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-Cβ 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 Vβ1NT/NT DN3 thymocytes and are expressed in small fractions of Vβ1NT+/+ DP thymocytes and splenic αβ T lymphocytes (21). Vβ10, which resides just upstream of the Vβ1NT gene (Fig. 3A), rearranges at a higher frequency than other Vβs on the Vβ1NT allele, and it is expressed in ~1% of Vβ1NT/+/+ and Vβ1NT/NT αβ T-lineage cells (21). On Vβ1NT alleles, repetitive sequences mark a boundary between

**FIGURE 1.** A preassembled functional TCRβ gene partially rescues early αβ T cell development. (A) Representative CD4/CD8 FACS data of thymocytes from Dicer+/FP, Dicer−/−, Vβ1NT/NT, and Vβ1NT/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+/FP, Dicer−/−, Vβ1NT/NT, and Vβ1NT/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 Vβ1NT/ + 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 Vβ1NT/+ and Vβ1NT/− mice (Fig. 3B–E). We found no difference in the percentages of Vβ10+ thymocytes between Vβ1NT/+DicerF/+ and Vβ1NT/+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 Vβ1NT/+ or Vβ1NT/− 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 Vβ1NT/+ and Vβ1NT/− mice (Fig. 3H). Because Vβ10 recombination on Vβ1NT alleles occurs only in DN3 cells (20), the reduced rearrangement and expression of Vβ10 in Vβ1NT/Dicer−/− and Vβ1NT/NT/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 TCRβ alleles; they also provide no

FIGURE 2. Vβ1NT expression partially rescues development of dicer-deficient thymocytes. (A) Representative CD4/CD8 FACS analysis of thymocytes from Vβ1NT/+ and Vβ1NT/−Dicer−/− mice. The average numbers of total thymocytes, the DN, DP, CD4+ SP, and CD8+ SP cell gates, and the percentage of thymocytes in each gate are indicated. (B) Graph showing average numbers of DN and DP thymocytes from Vβ1NT/+ and Vβ1NT/−Dicer−/− mice. Error bars are SE. ***p < 0.001. This experiment was done three independent times on at least one mouse of each genotype. (C) Representative c-Kit/CD25 FACS data of thymocytes from Vβ1NT/+ and Vβ1NT/−Dicer−/− 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 Vβ1NT/+ and Vβ1NT/−Dicer−/− mice. Error bars are SE. This experiment was done three independent times on at least one mouse of each genotype. (E) Representative CD4/CD8 FACS analysis of thymocytes from Vβ1NT/Dicer−/− and Vβ1NT/Dicer−/− (LckCreVβ1NT/Dicer−/−) mice. The DN, DP, CD4+ SP, and CD8+ SP cell gates and the percentages of thymocytes in each gate are indicated. (F) Graph showing average numbers of DN and DP thymocytes from Vβ1NT/Dicer−/− and Vβ1NT/−Dicer−/− mice. Error bars are SE. No significant differences were observed. This experiment was done three independent times on at least one mouse of each genotype. (G) Representative c-Kit/CD25 FACS data of thymocytes from Vβ1NT/Dicer−/− and Vβ1NT/Dicer−/− 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 Vβ1NT/Dicer−/− and Vβ1NT/−Dicer−/− mice. Error bars are SE. No significant differences were observed. This experiment was done three independent times on at least one mouse of each genotype.
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.** Dicer is required for expression and accessibility of Vβ10 segments on Vβ1NT alleles. (A) Schematic representation of the Vβ1NT TCRβ locus showing the relative locations of the preassembled Vβ1NT coding join and the germline Vβ and DJβ2-Jβ2 segments. (B) Representative TCRβ/Vβ10 FACS data from Vβ1NT/+ and Vβ1NT/Dicer−/− mice. The Vβ10+ cell gate and the percentage of thymocytes in this gate are indicated. (C) Graph showing the average percentages of TCRβhigh thymocytes expressing the indicated Vβ segments in 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 < 0.01. (D) Representative TCRβ/Vβ10 FACS data from Vβ1NT/+ and Vβ1NT/Dicer−/− mice. The Vβ10+ cell gate and the percentage of thymocytes within these gates are indicated. (E) Graph showing the average percentages of TCRβhigh thymocytes expressing the indicated Vβ segments in 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. (F) Representative TCRβ/Vβ10 FACS data from Vβ1NT/DicerF/+ and Vβ1NT/Dicer−/− mice. The Vβ10+ cell gate and the percentage of thymocytes in this gate are indicated. (G) Graph showing average percentage of TCRβhigh thymocytes from Vβ1NT/DicerF/+ and Vβ1NT/Dicer−/− mice. Error bars are SE. No significant differences were observed. This experiment was done three independent times on at least one mouse of each genotype. (H) Representative PCR analysis of rearrangements involving the indicated Vβ segments to DJβ1.1/DJβ1.2 or DJβ2.1/DJβ2.2 complexes in thymocytes of Vβ1NT/+ and Vβ1NT/Dicer−/− mice. A Cβ PCR control for genomic DNA content is also shown. This experiment was done three independent times on one mouse of each genotype. (I) Representative TCRβ/Vβ10 FACS data from DicerF/F and Dicer−/− mice. The Vβ10+ cell gate and the percentage of thymocytes in this gate are indicated. (J) Graph showing average percentages of TCRβhigh thymocytes expressing the indicated Vβ segments in DicerF/F and Dicer−/− mice. Error bars are SE. No significant differences were detected. This experiment was done three independent times on at least one mouse of each genotype.
assessment of Vβ10 accessibility through analysis of total thymocytes isolated from mice on a Vβ10NT background (21). To assess whether Dicer promotes accessibility of Vβ10 segments on Vβ10NT alleles, we analyzed Vβ10NT/Rag1−/− and Vβ10NT/Dicer−/− thymocytes (Fig. 4A–D) to avoid potential confounding effects from recombination of transcribing Vβ10 segments. In parallel, we also analyzed Vβ10NT and Vβ10NT/Dicer−/− thymocytes to evaluate whether cells with accessible Vβ10 segments are lost while attempting recombination. We found that Dicer deletion had no significant effect on relative levels of germline Vβ10 transcripts in Vβ10NT/Rag1−/− thymocytes (Fig. 4E); however, Dicer deletion led to ~4-fold lower relative levels of germline Vβ10 transcripts in Vβ10NT cells (Fig. 4F). We also found that Dicer deletion had no effect on Vβ10 CpG methylation in Vβ10NT/Rag1−/− cells (Fig. 4G), but it led to significantly higher Vβ10 CpG methylation in Vβ10NT cells (Fig. 4H). The simplest explanation for decreased germline transcription and increased CpG methylation of germline Vβ10 segments in Vβ10NT/Dicer−/−/Rag1−/− thymocytes compared with Vβ10NT/Dicer−/− thymocytes is increased apoptosis of Dicer-deficient Vβ10NT DN cells attempting TCRβ recombination.

On Vβ10NT 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 Vβ10NT and Vβ10NT/Dicer−/− splenic αβ T cells (Fig. 4I), consistent with normal silencing. This observation indicates that epigenetic silencing of germline Vβ10s on Vβ10NT 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

**FIGURE 4.** Dicer is required for survival of DN cells attempting Vβ10 rearrangements on Vβ10NT alleles. (A) Representative c-Ki67/CD25 FACS data of DN thymocytes from Vβ10NT/Rag1−/− and Vβ10NT/Dicer−/−/Rag1−/− mice. The DN1, 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 DN3 and DN4 cells from Vβ10NT/Rag1−/− and Vβ10NT/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 Vβ10NT/Rag1−/− and Vβ10NT/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 Vβ10NT/Rag1−/− and Vβ10NT/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 and Vβ10 alleles. (G) Graph showing average levels of Vβ10 NT gene transcripts or germline transcripts of the other indicated Vβ segments in thymocytes of (E) Vβ10NT/Rag1−/− and Vβ10NT/Dicer−/−/Rag1−/− mice or (F) Vβ10NT and Vβ10NT/Dicer−/−/Rag1−/− 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) Vβ10NT/Rag1−/− and Vβ10NT/Dicer−/−/Rag1−/− mice or (H) Vβ10NT and Vβ10NT/Dicer−/−/Rag1−/− 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 Vβ10NT gene in splenic αβ T cells from Vβ10NT and Vβ10NT/Dicer−/− mice. Error bars are SE. No significant differences were detected.
cells attempting TCRβ recombination, we analyzed thymocyte development in Dicer+/+, Dicer++, Vβ11NT/NT, and Vβ11NT/NT Dicer−/− mice lacking or expressing the EμBCL2 transgene (the latter are designated B+ within the figures). We found that EμBCL2 expression partially rescued DN3-to-DN4 and DN-to-DP development of Dicer−/− thymocytes (Fig. 5A–D). We also found that combined expression of EμBCL2 and Vβ1NT completely restored DN3-to-DN4 and DN-to-DP development of Dicer−/− thymocytes to levels observed in Dicer+/+ thymocytes (Fig. 5E–H). We found similar deletion of Dicer in DN and total thymocytes of Dicer−/− mice and Dicer−/− mice containing EμBCL2 and/or Vβ1NT (Fig. 1C, 1F), indicating that EμBCL2 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−/− mice 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 examined the effect of EμBCL2 on the survival of Dicer−/− and Vβ11NT/NT Dicer−/− thymocytes. Because death of Dicer−/− thymocytes cannot be detected ex vivo by increased annexin V staining or reduced mitochondrial potential (8), we reasoned that comparison of DN3-to-DN4 and DN-to-DP thymocyte development in Vβ11NT/Dicer−/− and Vβ11NT/NT/Dicer−/− thymocytes is consistent with a requirement for Dicer in survival of DN cells attempting TCRβ recombination. We observed greater percentages of TCRβ−thymocytes expressing each Vβ assayed in Vβ11NT/Dicer−/− mice relative to wild-type alleles in Vβ11NT/Dicer−/− mice (Fig. 6A, 6B). We also detected higher levels of Vβ10 DP thymocytes. Because death of Dicer−/− thymocytes supports the idea that Dicer is required for survival of cells attempting TCRβ recombination. Importantly, however, note that because expression of both Vβ11NT and EμBCL2 further decreases the percentage of dead Dicer−/− 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 Vβ11NT/Rag1−/− 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 the effects of Dicer deletion upon Vβ repertoire and DN3-to-DN4 and DN-to-DP thymocyte development in Vβ11NT/Dicer−/− and Vβ11NT/NT mice. We observed equivalent percentages of thymocytes expressing each Vβ assayed in Vβ11NT/Dicer−/− and Vβ11NT/NT/Dicer−/− mice (Fig. 6A, 6B). We also detected similar levels of Vβ10 DJ rearrangements involving DJβ1.1 or DJβ1.2 complexes of all Vβs assayed in Vβ11NT cells as compared with those observed in Dicer−/− mice (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 Vβ11NT/Dicer−/− mice as compared with wild-type alleles in Vβ11NT/NT 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 Vβ11NT/Dicer−/− and Vβ11NT/NT mice. We observed equivalent percentages of thymocytes expressing each Vβ assayed in Vβ11NT/Dicer−/− and Vβ11NT/NT/Dicer−/− mice (Fig. 6A, 6B). We also detected similar levels of Vβ10 DJ rearrangements involving DJβ1.1 or DJβ1.2 complexes in Vβ11NT/Dicer−/− and Vβ11NT/NT mice (Fig. 6C). Additionally, we found that Vβ recombination frequencies in Dicer−/− mice were comparable to wild-type alleles in Vβ11NT/NT mice (Fig. 6D–F). We also found that deletion of Dicer in Vβ11NT mice
caused ∼2-fold decreases in the numbers of DN3 and DN4 thy-
mocytes (Fig. 6G, 6H), whereas deletion of Dicer in Vβ1NT/DJ
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 Vβ1NT/DJ 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+ and B+Dicer−/− mice (mice expressing the EμBCL2 transgene are designated B+ in this figure). The average numbers of total thymocytes, the DN, DP, CD4+ SP, and CD8+ 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+ and B+Dicer−/− 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+Vb1NT/NT and B+Vb1NT/NTDicer−/− mice. The average numbers of total thymocytes, the DN, DP, CD4+ SP, and CD8+ 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+Vb1NT/NT and B+Vb1NT/NTDicer−/− 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 ≤ 0.001. (J) Graph showing average percentages of Vβ10+ TCRβhi and Vβ10+ TCRβint thymocytes from mice of the indicated genotypes. Error bars are SE. **p ≤ 0.01. (K) 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. This experiment was done three independent times on one mouse of each genotype. (L) Graph showing the average ratios of the percentages of Vβ10+ TCRβhi and Vβ10+ TCRβint 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 Vβ11NT/+/ mice. Because the assembly of functional VDJCβ genes is required for DN3-to-DN4 development and the only phenotypic difference between Vβ11NT/DJ and Vβ11NT/Dj 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 Vβ11NT/DJ and Vβ11NT/Dj mice. The Vβ14+ and Vβ6+ cell gates are indicated. (B) Graph showing average percentages of TCRβhigh thymocytes expressing the indicated Vβ segments in Vβ11NT/, Vβ11NT/Dj, and Vβ11NT/Dj 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 Vβ11NT/DJ and Vβ11NT/Dj 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 Vβ11NT/Dj 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.
Discusion

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 Dß-to-Jß and then Vß-to-DJß 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). Vß-to-DJß recombination is repressed through TCRß-mediated feedback inhibition signals (7). However, functional TCRß genes can be detected on both alleles in 1–10% of αß T cells (7), indicating that DN3 cells can attempt Vß recombination while experiencing proliferation signals from assembled VßDJßCß genes. The increased death and decreased proliferative expansion of VßßNTNDicer−/− thymocytes compared with VßßNT/Dicer−/− thymocytes demonstrates that Dicer is required for survival of DN3 cells that attempt Vß recombination while experiencing proliferation signals from TCRß genes assembled first on other alleles. Despite TCRß-mediated feedback inhibition, ~>6% of VßßNT and VßßNTNT splenic αß T cells contain Vß-to-DJß2 rearrangements that replace VßßNT genes (21), revealing that a significant percentage of DN3 thymocytes that have assembled functional VßDJßCß genes can attempt replacement Vß-to-DJß2 rearrangements. The increased death, decreased proliferative expansion, reduced levels of Vßß10-to-DJß2 rearrangements, and decreased frequency of Vßß10+ thymocytes in VßßNTNT/Dicer−/− mice relative to VßßNTNT mice demonstrate that Dicer also is required for survival of DN3 cells that attempt Vß-to-DJß2 recombination while experiencing proliferation signals from VßDJßCß genes assembled on the same allele.

Although preassembled functional TCRß genes inhibit Vß recombination, their suppression of other VDJ recombination events likely contributes to their ability to partially rescue the development of Dicer-deficient thymocytes. In DN cells, Dß-to-Jß rearrangements occur on both alleles and are not subject to feedback inhibition (7). Despite this lack of regulation, Dß-to-Jß recombination is decreased on wild-type alleles in αß T cells of VßßNT/Dicer−/− mice (18, 21), likely because expression of preassembled TCRß genes/transgenes accelerates DN3-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 Dß-to-Jß recombination while experiencing TCRß proliferation signals. In DN cells, the RAG proteins also promote TCRß, TCRδ, and IgH recombination and induce “off-target” DSBS at other genetic loci (7). Because preassembled TCRß genes/transgenes downregulate RAG activity in DN cells (7), the decreased death and increased proliferative expansion of VßßNTNTDicer−/− thymocytes as compared with Dicer−/− thymocytes is consistent with a requirement for Dicer in survival of DN3 cells that induce RAG DSBS outside of TCRß 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 DS BS responses during VDJ 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 VDJ 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 VDJ recombination.

Our data also reveal a requirement for Dicer in promoting survival of proliferating thymocytes. The ability of VßßNT, 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 VßßNT 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, Brca2, or Blm proteins that repair DNA replication-associated DSBS exhibit impaired proliferative expansion and increased apoptosis of thymocytes (43–45). Expression of the EßBCL2 transgene, but not preassembled TCRßTCRα 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 αß 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 αß 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 αß 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 αß 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 αß T lymphocyte lineages and thus not disrupt adaptive immune system homeostasis. In contrast, Dicer deletion specifically in αß 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.