Published in final edited form as:
J Immunol. 2014 October 15; 193(8): 4032–4042. doi:10.4049/jimmunol.1401359.

The microRNA biogenesis machinery modulates lineage commitment during αβ T cell development

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Abstract

Differentiation of $CD4^+$ helper and $CD8^+$ cytotoxic αβ T cells from $CD4^+CD8^+$ thymocytes involves up-regulation of lineage-specifying transcription factors and transcriptional silencing of CD8 or CD4 co-receptors, respectively, in major histocompatibility complex (MHC) II or I restricted thymocytes. Here, we demonstrate that inactivation of the Dicer RNA endonuclease in murine thymocytes impairs initiation of *Cd4* and *Cd8* silencing, leading to development of positively selected, MHCI- and MHCII-restricted mature $CD4^+CD8^+$ thymocytes. Expression of the anti-apoptotic BCL2 protein or inactivation of the p53 pro-apoptotic protein rescues these thymocytes from apoptosis, increasing their frequency and permitting accumulation of $CD4^+CD8^+$ αβ T cells in the periphery. Dicer-deficient MHCI-restricted αβ T cells fail to normally silence *Cd4* and display impaired induction of the CD8-lineage specifying transcription factor Runx3, whereas Dicer-deficient MHCII-restricted αβ T cells show impaired *Cd8* silencing and impaired induction of the CD4-lineage specifying transcription factor Thpok. Finally, we show that the Drosha RNA endonuclease, which functions upstream of Dicer in microRNA biogenesis, also regulates *Cd4* and *Cd8* silencing. Our data demonstrate a previously dismissed function for the microRNA biogenesis machinery in regulating expression of lineage-specifying transcription factors and silencing of *Cd4* and *Cd8* during αβ T cell differentiation.

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Introduction

The generation of distinct cellular lineages from multipotent progenitor cells involves differentiation programs that couple up-regulation of lineage specific genes with silencing of genes expressed in progenitor cells and alternative lineages. The initiation, maintenance, and silencing of gene expression during lineage commitment are regulated by genetic and epigenetic mechanisms. One paradigm for elucidating molecular mechanisms that control gene expression during lineage commitment is the differentiation of $CD4^+$ and $CD8^+$ αβ T cells from $CD4^+CD8^+$ (double-positive or DP) thymocytes that have expressed functional αβ TCRs (1, 2). Assembly and expression of T cell receptor (TCR) β genes drives $CD4^-CD8^-$ (double-negative or DN) thymocytes to differentiate into DP thymocytes (3). This developmental transition initiates rearrangement and expression of TCRα genes, which leads to expression of unique αβ TCRs on immature $CD4^+CD8^+$ thymocytes. Specificities of αβ TCRs are selected through interactions of these antigen receptors with self-peptide/MHC complexes expressed on thymic epithelial cells, a process aided by CD4 and CD8 co-receptors (3, 4). Depending on the affinity of such interactions, thymocytes die by "neglect", are rescued from programmed cell death and further differentiate (positive selection), or are actively deleted (negative selection). Concomitant with positive selection, immature $CD4^+CD8^+$ thymocytes up-regulate lineage-specifying transcription factors and silence *Cd8* or *Cd4* as they differentiate into mature $CD4^+$ or $CD8^+$ (single positive or SP) thymocytes. SP cells exit the thymus and migrate to the spleen, lymph nodes, and other peripheral organs as $CD4^+$ or $CD8^+$ lineage αβ T cells.

Differentiation of $CD4^+$ and $CD8^+$ αβ T cells is regulated by αβ TCR-activated signaling pathways that control downstream transcription factors (2, 5). These factors include Runx3, which is required for CD8 lineage effector functions and *Cd4* silencing, and Thpok (encoded by *Zbtb7b*), which drives differentiation of $CD4^+$ SP thymocytes and facilitates *Cd8* silencing (2, 6–10). Runx3 and Thpok are mutually antagonistic as Runx3 represses *Zbtb7b* expression by binding a silencer upstream of the *Zbtb7b* promoter (11, 12), while Thpok represses *Runx3* expression (13–15) and antagonizes Runx-mediated repression of *Cd4*, possibly through binding to the *Cd4* silencer (14, 16). Despite requirement for Runx3 and the *Cd4* silencer in initiation of *Cd4* silencing, neither is required to prevent *Cd4* re-expression in peripheral $CD8^+$ αβ T cells (17, 18), implying that *Cd4* silencing is maintained epigenetically. In contrast to control of *Cd4* expression, lineage-specific *Cd8* transcription appears to be regulated by developmental stage specific *Cd8* enhancers, rather than a *cis*-acting silencer element (19–22). However, Thpok-mediated recruitment of histone deacetylases to *Cd8* enhancers may facilitate *Cd8* silencing in $CD4^+$ cells (10). In addition to Runx3 and Thpok, several transcription factors and chromatin modifying enzymes modulate CD4/CD8 lineage commitment and/or co-receptor expression, yet none of these has been shown to directly regulate initiation of *Cd4* or *Cd8* silencing following positive selection of DP thymocytes (1, 2, 23).

The Dicer and Drosha RNA endonucleases guide cellular differentiation through their ability to control gene expression. Both proteins are required for the biogenesis of microRNAs (miRs), which repress gene expression by binding and destabilizing or blocking translation of mRNAs (24). However, Dicer can also function independently of Drosha to create short-

interfering RNAs (siRNAs), which inhibit gene expression by inducing epigenetic changes that block transcription of target loci (25). While inactivation of Dicer or Drosha initiating in mouse DN thymocytes has been shown to increase apoptosis of immature thymocytes, neither was found to affect CD4 and CD8 lineage commitment or *Cd4* and *Cd8* silencing (26, 27). We demonstrate here that inactivation of Dicer starting in DN thymocytes impairs *Cd4* and *Cd8* silencing, leading to generation of positively selected, MHCI- and MHCII-restricted mature $CD4^+CD8^+$ thymocytes. Expression of the anti-apoptotic BCL2 protein or inactivation of the p53 pro-apoptotic protein rescues these cells from apoptosis, increasing their frequency and permitting accumulation of $CD4^+CD8^+$ αβ T cells in the periphery. We demonstrate that Dicer is required for appropriate initiation of *Cd4* and *Cd8* silencing in thymocytes, but find no evidence for a requirement of Dicer in maintenance of *Cd4* and *Cd8* silencing in peripheral $CD4^+$ or $CD8^+$ αβ T cells. We also show that Dicer-deficient MHCI-restricted αβ T cells exhibit impaired transcriptional silencing of *Cd4* and impaired expression of the *Cd4*-silencing transcription factor Runx3, while Dicer-deficient MHCII-restricted αβ T cells have reduced expression of Thpok, the master regulator of CD4 lineage commitment. We also show that the Drosha RNA endonuclease also regulates *Cd4* and *Cd8* silencing, suggesting a role for miRs in this process during lineage-commitment. Our data demonstrate an unexpected role for the miR biogenesis machinery in promoting appropriate co-receptor silencing and lineage commitment during αβ T cell differentiation.

Materials and Methods

Mice

LckCre (28), *EμBCL2* (29), $Dicer^{flox/flox}$ (26), $Tp53^{flox/flox}$ (30), *Cd4Cre* (28), $Rag1^{-/-}$ (31), $MHCI^{-/-}$ (32), $MHCII^{-/-}$ (33), *OT-I* (34), *OT-II* (35), and $Drosha^{flox/flox}$ (36) mice have been described. Mice were maintained under specific pathogen-free conditions at the Children's Hospital of Philadelphia (CHOP) or the National Institute of Health. Unless otherwise indicated, studies were conducted on littermate or age-matched mice between 4–8 weeks of age. All studies were performed in accordance with regulations and approved by the CHOP or NIAID/NIH Institutional Animal Care and Use Committees.

Flow Cytometry and Gating

Flow cytometry was as described previously (37). Unless otherwise specified, gating was Forward Scatter × Side Scatter → Singlets → Live cells (Invitrogen LIVE/DEAD) → $TCR\beta^{hi}CD24^{lo}$, followed by CD4 and CD8α gating.

Bone marrow chimeras

Single cell suspensions were prepared from tibia and femur bone marrow. $CD4^+$ and $CD8^+$ αβ T cells were removed by magnetic bead depletion (Qiagen). Recipient mice were lethally irradiated (900 rads in two 450 rad doses 4 hours apart) prior to retro-orbital injection. Mice were analyzed 8–10 weeks later.

Quantitative real-time PCR

RNA isolation and cDNA generation were as described previously (37). Primers: *Cd4* Exon 1F: 5'-GCAGAGTGAAGGAAGGACTGG-3', *Cd4* Intron 1R: 5'-

CAGAACATTCCGGCACATTAGC-3'. Primers for *Zbtb7b* were previously described (38). Primers for *Rorc* and *Foxo1* were purchased from Life Technologies (murine Taqman assays).

Western Blot

Cell pellets were lysed in 1% (vol/vol) SDS buffer under reducing conditions, separated on a 10% SDS-PAGE gel, transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore) and analyzed by immunoblot and chemiluminescence. The anti-Runx antibody was from Epitomics (# 2593-1).

Quantification of miRNA levels

Sorted cells were resuspended in TRIzol (Ambion) and RNA isolated using miRNeasy Mini kit (Qiagen). Reverse transcription was performed using the Taqman microRNA Reverse Transcription Kit (Applied Biosystems) and probe-specific primers. qRT-PCR was performed on a ViiA 7 (Applied Biosystems) instrument using Taqman Universal Master Mix (Applied Biosystems) according to manufacturer's instructions. Matched reverse transcription and qPCR primers for miR-181a, let-7c, and snoRNA202 were from Life Technologies.

Statistical Analyses

Unless otherwise indicated, Student's t-test was utilized for statistical analyses. Error bars are standard error of the mean (SEM).

Results

Suppressing apoptosis of Dicer-deficient thymocytes results in generation of $CD4^+CD8^+$ mature αβ T cells

While previous reports suggested that Dicer does not control lineage commitment of $CD4^+$ and $CD8^+$ αβ T cells (26, 27, 39), we considered the possibility that apoptosis of *Dicer*-deficient thymocytes might mask a role for Dicer in CD4/CD8 lineage commitment. To address this possibility, we analyzed *LckCre:EμBCL2:Dicer*$^{flox/flox}$ (*LBD*), *LckCre:Dicer*$^{flox/flox}$ (*LD*), *EμBCL2:Dicer*$^{flox/flox}$ (*BCL2*), and *Dicer*$^{flox/flox}$ (wild-type or *WT*) mice. We and others have shown that the *LckCre* transgene drives efficient *Dicer* deletion in *LD* and *LBD* DN thymocytes (26, 27, 37). The *EμBCL2* transgene drives expression of the anti-apoptotic BCL2 protein throughout αβ T cell development (29) and inhibits apoptosis of *LD* thymocytes, which results in rescue of thymic DP cellularity as we have previously reported (37). We show here that SP thymocyte numbers are also rescued in *LBD* mice (Supplemental Fig. S1A and S1B), although peripheral αβ T cell numbers are reduced in *LBD* mice relative to controls (Supplemental Fig. S1C and S1D), likely because *Eμ* activity (and therefore transgenic *BCL2* expression) declines as αβ T cells mature (Supplemental Fig. S1E). Notably, BCL2 expression does not alter the extent of Dicer deletion in DN thymocytes of *LBD* mice relative to *LD* mice (37), making it unlikely that phenotypic differences between *LBD* and *LD* mice arise from different efficiencies of Dicer deletion and resultant miR loss. Flow cytometry analyses in *LBD* mice showed large numbers of splenic αβ T cells aberrantly expressing both CD4 and CD8 at varying levels

(hereafter referred to as $CD4^+CD8^+$ cells; Fig. 1A,B), corresponding to ~15% of splenic αβ T cells in 4–8 week old animals. This population was absent from *WT* and *BCL2* mice (Fig. 1A,B). The increased frequency of $CD4^+CD8^+$ αβ T cells was not simply due to loss of $CD4^+$ or $CD8^+$ cells since *LBD* mice exhibited an ~10-fold increase in total number of splenic $CD4^+CD8^+$ cells as compared to control mice (Fig. 1C). We also observed increased frequencies of $CD4^+CD8^+$ αβ T cells in the lymph nodes and blood of *LBD* mice compared to *WT* and *BCL2* mice (Fig. 1D,E). In addition, $CD4^+CD8^+$ αβ T cells were absent in *Dicer*-sufficient, *LckCre:EμBCL2* (*LB* mice (Fig. 1F), indicating that this phenotype of *LBD* mice is not due to combined expression of Cre and BCL2.

Normally, pre-selection DP thymocytes are $CD24^{hi}TCR\beta^{lo}$ cells, become $CD24^{hi}TCR\beta^{hi}$ cells after up-regulation of TCRβ expression during positive selection, and then down-regulate CD24 expression to become $CD24^{lo}TCR\beta^{hi}$ SP mature thymocytes (40); mature peripheral αβ T cells are similarly $CD24^{lo}TCR\beta^{hi}$. We found that $CD4^+CD8^+$ αβ T cells were $CD24^{lo}TCR\beta^{hi}$ (Fig. 1A,B), suggesting that they are mature, post-selection αβ T cells. To further clarify their nature, we performed qPCR for mRNAs that are differentially expressed between thymocytes and mature αβ T cells. Similar to normal splenic $CD4^+$ and $CD8^+$ αβ T cells, splenic $CD4^+CD8^+CD24^{lo}TCR\beta^{hi}$ αβ T cells did not express *Rag1* or *Rorc*, two markers of immature DP thymocytes (Fig. S2A and S2B). Splenic $CD4^+CD8^+CD24^{lo}TCR\beta^{hi}$ αβ T cells also exhibited higher expression of *Foxo1* (Fig. S2C), which is up-regulated in mature αβ T cells. These data indicate that the $CD4^+CD8^+$ αβ T cells that develop in *LBD* mice exhibit multiple features of mature, post-selection αβ T cells and are not simply immature, pre-selection DP thymocytes that exited the thymus. Therefore, our data demonstrate that expression of the pro-survival BCL2 protein throughout development of *Dicer*-deficient αβ T lineage cells permits generation of mature, post-selection peripheral $CD4^+CD8^+$ αβ T cells.

In addition to promoting survival, ectopic BCL2 expression affects other pathways and processes that regulate αβ T lymphocyte differentiation, including NFAT signaling and αβ TCR selection (41, 42). Thus, to rule out the possibility that peripheral $CD4^+CD8^+$ αβ T cells in *LBD* mice arise from effects of BCL2 expression other than promoting survival of *Dicer*-deficient cells, we generated and analyzed *LckCre:p53$^{flox/flox}$:Dicer$^{flox/flox}$* (*LPD*) mice with combined inactivation of Dicer and p53 initiating in DN thymocytes. The p53 protein activates cell cycle checkpoints in response to DNA damage and other cellular stresses, and induces apoptosis when such stresses are too severe (43). Similar to the case for *LBD* mice, we found higher frequencies of mature splenic $CD4^+CD8^+$ αβ T cells in *LPD* mice relative to *WT* mice (Fig. 1G,H). This finding indicates that inactivation of the pro-apoptotic p53 protein in *Dicer*-deficient thymocytes also permits accumulation of splenic $CD4^+CD8^+$ αβ T cells. Consequently, we conclude that inhibiting apoptosis of *Dicer*-deficient αβ T lineage cells unmasks a requirement for Dicer in appropriate CD4/CD8 silencing in mature αβ T cells.

Dicer is required for normal initiation of CD4/CD8 silencing following αβ TCR selection

The splenic $CD4^+CD8^+$ αβ T cells in *LBD* mice could result from impaired initiation of CD4/CD8 silencing upon selection of thymocytes and/or impaired maintenance of

CD4/CD8 silencing following thymic egress of mature post-selection thymocytes. To determine whether initiation of CD4/CD8 silencing is impaired in *LBD* mice, we analyzed CD4 and CD8 expression on pre- and post-selection *LBD* and control thymocytes. We found that 18% of $CD24^{lo}TCR\beta^{hi}$ mature thymocytes in *LBD* mice aberrantly expressed both CD4 and CD8, whereas *WT* and *BCL2* mice contained essentially no $CD4^+CD8^+$ mature thymocytes (Fig. 2A,B). The increased frequency of $CD4^+CD8^+$ cells was not simply due to loss of $CD4^+$ or $CD8^+$ cells since *LBD* mice exhibited an ~5-fold increase in the total number of thymic $CD4^+CD8^+$ cells relative to controls (Fig. 2C). We found similar increased frequencies of $CD4^+CD8^+CD24^{lo}TCR\beta^{hi}$ thymocytes in *LPD* mice relative to *WT* mice (Fig. 2D,E). In addition, we found that 5% of $CD24^{lo}TCR\beta^{hi}$ mature thymocytes in *LD* mice express both CD4 and CD8. While this frequency is above the levels observed in mature thymocytes of *WT* and *BCL2* mice (Fig. 2A,B), the limited numbers of these thymocytes (<1% of total thymocytes in *LD* mice) could explain why they were not observed in previous analyses of *LD* mice (26, 27). To evaluate whether incomplete deletion and inactivation of Dicer could account for impaired initiation of CD4/CD8 silencing in only a subset of *LBD* and *LD* thymocytes, we quantified the expression of two miRs that are highly expressed in thymocytes (miR-181a and let-7c) (44). Post-selection ($CD24^{lo}TCR\beta^{hi}$) thymocytes from *LD* and *LBD* mice exhibited >80% reduction in miR-181a and let-7c levels (Fig. S3A, B), indicating comparable and substantial inactivation of Dicer in post-selection thymocytes of both *LD* and *LBD* mice. Furthermore, the levels of each miR were reduced similarly among post-selection $CD4^+CD8^+$, or $CD4^+CD8^+$ thymocytes from *LBD* and *LD* mice (Fig. S3A, B), indicating that Dicer is required for normal initiation of CD4/CD8 silencing in only a subset of post-selection thymocytes. Regardless, our data show that $CD4^+CD8^+$ mature thymocytes arise after positive selection of *Dicer*-deficient thymocytes, and that expression of BCL2 or inactivation of p53 is not required for development of these cells. Therefore, we conclude that Dicer is required for appropriate initiation of *Cd4* and/or *Cd8* silencing during intrathymic αβ T cell differentiation.

Cd4 and *Cd8* silencing is maintained in *Dicer*-deficient αβ T cells

A requirement for Dicer in initiation of *Cd4* and/or *Cd8* silencing does not preclude a role for Dicer in maintenance of *Cd4* and *Cd8* silencing in mature αβ T cells. To determine whether Dicer is also required for appropriate maintenance of *Cd4* and *Cd8* silencing, we employed a genetic approach. The *Cd4Cre* transgene drives expression of Cre recombinase and deletion of $Dicer^{flox}$ alleles initiating in DP thymocytes (27, 39). However, published reports have shown that *Cd4Cre*-mediated *Dicer* deletion does not lead to appreciable loss of Dicer-dependent miRs until after initiation of *Cd4* and *Cd8* silencing and CD4/CD8 lineage commitment (27, 39, 45). In contrast, peripheral αβ T cells of $Cd4Cre{:}Dicer^{flox/flox}$ mice exhibit near complete deletion of $Dicer^{flox}$ alleles, low expression of miRs, and phenotypes indicative of Dicer inactivation (27, 39, 45). Based on these observations, we reasoned that *Cd4Cre*-mediated deletion of *Dicer* starting in $CD4^+CD8^+$ thymocytes would allow initiation of *Cd4* and *Cd8* silencing before substantial loss of Dicer-dependent miRs, and thereby permit evaluation of whether Dicer has a role in maintenance of co-receptor silencing in $CD4^+$ and $CD8^+$ αβ T cells. Thus, we generated and analyzed $Cd4Cre{:}E\mu BCL2{:}Dicer^{flox/flox}$ (*CBD*) mice. In striking contrast to *LBD* mice, *CBD* mice had neither mature $CD4^+CD8^+$ thymocytes (Fig. 3A,B) nor $CD4^+CD8^+$ splenic αβ T cells (Fig.

3C,D). We conclude that Dicer and miRs are not required for the maintenance of *Cd4* and *Cd8* silencing in mature splenic αβ T cells.

Dicer controls initiation of both *Cd4* and *Cd8* silencing

The $CD4^+CD8^+$ αβ T cells in *LBD* mice could comprise MHCI-restricted cells with impaired *Cd4* silencing, MHCII-restricted cells with impaired *Cd8* silencing, or both. To address this issue we restricted the ability of thymocytes to develop on MHCI or MHCII by transferring bone marrow cells from *LBD* or *BCL2* mice into irradiated $MHCI^{-/-}$ or $MHCII^{-/-}$ recipient mice.

To determine whether Dicer is required for appropriate initiation of *Cd8* silencing during development of MHCII-restricted $CD4^+$ αβ T cells, we analyzed irradiated $MHCI^{-/-}$ mice (32) reconstituted with *LBD* or *BCL2* bone marrow cells. It has been shown that BCL2 expression in $MHCI^{-/-}$ mice allows development of small numbers of splenic $CD8^+$ T cells (46). We found the same in $MHCI^{-/-}$ mice reconstituted with *BCL2* or *LBD* bone marrow cells (Fig. 4C). However, we also observed that ~15% of $CD24^{lo}TCR\beta^{hi}$ mature thymocytes aberrantly expressed both CD4 and CD8 in $MHCI^{-/-}$ mice reconstituted with *LBD* bone marrow, while only 2% of $CD24^{lo}TCR\beta^{hi}$ mature thymocytes were $CD4^+CD8^+$ in $MHCI^{-/-}$ mice reconstituted from *BCL2* cells (Fig. 4A,B). We also found that ~15% of splenic αβ T cells were $CD4^+CD8^+$ in $MHCI^{-/-}$ mice reconstituted with *LBD* bone marrow cells, while only 0.5% of splenic αβ T cells expressed both CD4 and CD8 in $MHCI^{-/-}$ mice reconstituted with *BCL2* bone marrow (Fig. 4C,D). While we cannot rule out that the $CD4^+CD8^+$ αβ T cells in $MHCI^{-/-}$ mice reconstituted with *LBD* cells developed from $CD8^+$ cells that failed to silence *Cd4*, the substantial population of $CD4^+CD8^+$ αβ T cells after transfer of *LBD* versus *BCL2* cells is more consistent with Dicer inactivation leading to impaired initiation of *Cd8* silencing during intrathymic differentiation of MHCII-restricted αβ T cells.

To determine whether Dicer is required for appropriate initiation of *Cd4* silencing during development of MHCI-restricted $CD8^+$ αβ T cells, we analyzed irradiated $MHCII^{-/-}$ mice (33) reconstituted with bone marrow from *LBD* or *BCL2* mice. As previously shown (33, 46), a small population of $CD4^+$ αβ T cells does develop in $MHCII^{-/-}$ mice, although the vast majority of cells are $CD8^+$ (Fig. 4E). We found that ~16% of $CD24^{lo}TCR\beta^{hi}$ mature thymocytes expressed both CD4 and CD8 in $MHCII^{-/-}$ mice reconstituted with *LBD* bone marrow, but only ~4% of these cells were $CD4^+CD8^+$ in $MHCII^{-/-}$ mice reconstituted with *BCL2* cells (Fig. 4E,F). We also found that ~10% of splenic αβ T cells were $CD4^+CD8^+$ in $MHCII^{-/-}$ mice reconstituted from *LBD* bone marrow, but only ~2.5% of splenic αβ T cells expressed both CD4 and CD8 in $MHCII^{-/-}$ mice reconstituted from *BCL2* cells (Fig. 4G,H). Although we cannot rule out that the $CD4^+CD8^+$ αβ T cells in $MHCII^{-/-}$ mice reconstituted with *LBD* cells developed from $CD4^+$ cells that failed to silence *Cd8*, the substantial population of $CD4^+CD8^+$ αβ T cells after transfer of *LBD* versus *BCL2* cells is more consistent with Dicer inactivation causing impaired initiation of *Cd4* silencing during intrathymic differentiation of MHCI-restricted αβ T cells.

Based on our analyses of *MHCI*$^{-/-}$ and *MHCII*$^{-/-}$ mice reconstituted with *LBD* or *BCL2* cells, we conclude that Dicer expression in immature DP thymocytes is needed for appropriate initiation of *Cd4* and *Cd8* silencing in MHCI and MHCII-restricted cells, respectively.

Dicer regulates *Cd4* and *Cd8* silencing and expression of *Runx3* and *Thpok* in positively-selected αβ T cells

To gain further support for our conclusion that Dicer is required for appropriate initiation of both *Cd4* and *Cd8* silencing, we generated *LBD* mice that express the MHCI-restricted OT-I αβ TCR transgene, which normally promotes positive selection of only CD8$^+$ T cells (34), or the MHCII-restricted OT-II αβ TCR transgene, which normally promotes positive selection of only CD4$^+$ T cells (35). We generated these mice on a *Rag1*$^{-/-}$ background (*LBD R1 OT-I* and *LBD RI OT-II* mice) to prevent TCRβ and TCRα gene rearrangements that could subvert the ability of these αβ TCR transgenes to restrict MHC specificity. We found that positively-selected *LBD R1 OT-I* mature thymocytes (Figs. 5A, B) and αβ T cells (Figs. 5C, D) exhibited impaired *Cd4* silencing, with ~35–45% of cells aberrantly expressing both CD4 and CD8, indicating that Dicer is required for appropriate initiation of *Cd4* silencing in cells expressing an MHCI-restricted αβ TCR transgene. Similarly, we found that positively-selected *LBD R1 OT-II* mature thymocytes (Figs. 5E, F) and αβ T cells (Figs. 5G, H) exhibited impaired *Cd8* silencing, with ~5% of mature thymocytes and ~25% of mature splenic αβ T cells expressing both CD4 and CD8, indicating that Dicer is also required for normal *Cd8* silencing in cells expressing an MHCII-restricted αβ TCR transgene. Collectively, these data demonstrate that Dicer ensures appropriate silencing of both *Cd4* and *Cd8* in positively-selected αβ T cells.

The expression of both CD4 and CD8 on Dicer-deficient αβ T cells could result from impaired transcriptional or translational silencing of *Cd4* and *Cd8*. To evaluate the role of Dicer in control of co-receptor transcriptional silencing, we conducted qRT-PCR analyses of primary (un-spliced) *Cd4* transcripts in mature αβ T cells sorted from spleens of *LBD R1 OT-I* and control mice. We detected similarly high levels of primary *Cd4* transcripts in *LBD R1 OT-I* CD4$^+$CD8$^+$ cells and *WT* CD4$^+$ cells (Fig. 5I). In contrast, we were unable to detect primary *Cd4* transcripts in CD8$^+$ cells of *WTR1 OT-1*, or *LBD R1 OT-I* mice (Fig. 5I). These results demonstrate that Dicer is required for appropriate transcriptional silencing of *Cd4* in MHCI-restricted αβ T cells.

Following positive selection, *Runx3* expression is up-regulated in MHCI-restricted thymocytes and drives appropriate initiation of *Cd4* transcriptional silencing in CD8 lineage cells (6, 7). To determine whether Dicer controls expression of *Runx3* in positively selected, MHCI-restricted αβ T cells, we conducted Western blot analyses of Runx3 protein in mature αβ T cells sorted from spleens of *LBD R1 OT-I* and control mice. We detected a decreased level of Runx3 protein in CD4$^+$CD8$^+$ cells of *LBD R1 OT-I* mice as compared to CD8$^+$ cells of *WT* and *LBD R1 OT-I* mice (Fig. 5J), indicating that Dicer is required for appropriate expression of Runx3 in positively-selected, MHCI-restricted αβ T cells.

Analogous to Runx3 up-regulation in MHCI-restricted cells, positive selection of MHCII-restricted cells induces Thpok expression, which drives CD4 lineage commitment and

facilitates *Cd8* silencing (8–10). Given that Runx3 expression was impaired in Dicer-deficient MHCI-restricted αβ T cells, we hypothesized that Dicer might similarly control expression of Thpok in MHCII-restricted cells. To test this hypothesis, we performed qRT-PCR analyses for *Zbtb7b* mRNA (since we were unable to isolate enough cells for Western blot with available Thpok antibodies) in sorted cells from *LBD R1 OT-II* and control mice. We found a lower level of *Zbtb7b* mRNA in $CD4^+CD8^+$ cells from *LBD R1 OT-II* mice relative to $CD4^+$ cells from control *R1 OT-II* mice (Fig. 5K), revealing that Dicer is also required for normal expression of *Zbtb7b* in positively-selected, MHCII-restricted αβ T cells. Collectively, these data demonstrate that Dicer promotes appropriate expression of "master" transcriptional regulators of the CD4 and CD8 αβ T cell lineages in MHC II- or I-restricted cells, respectively, following positive selection.

Drosha is also required for normal *Cd4* and *Cd8* silencing during αβ T cell development

Dicer could regulate *Cd4* and *Cd8* silencing through generation of siRNAs that directly halt transcription of these loci and/or via biogenesis of miRs that indirectly regulate expression of Runx3, Thpok, or other factors that control CD4 and CD8 expression. To determine if Dicer-dependent siRNAs and/or miRs regulate initiation of *Cd4* and *Cd8* silencing, we generated and analyzed *LckCre:EμBCL2:Drosha*$^{flox/flox}$ (*LBDr*) mice because the Drosha RNA endonuclease is required for production of miRs, but not siRNAs (47). We observed that ~ 40% of $CD24^{lo}TCR\beta^{hi}$ mature thymocytes in *LBDr* mice were $CD4^+CD8^+$ (Fig. 6A,B), revealing that Drosha is required for appropriate initiation of *Cd4* and/or *Cd8* silencing. We also found that ~20% of mature splenic αβ T cells in *LBDr* mice were $CD4^+CD8^+$ (Fig. 6C,D), indicating that ectopic expression of *BCL2* throughout development of *Drosha*-deficient αβ T cells permits the accumulation of mature post-selection splenic $CD4^+CD8^+$ αβ T cells. Since these data demonstrate that both Drosha and Dicer are required for regulation of CD4 and CD8 expression in mature αβ T cells, we conclude that miRs) likely control the appropriate initiation of *Cd4* and *Cd8* silencing during αβ T cell differentiation.

Discussion

We have demonstrated that expression of the Dicer and Drosha proteins in thymocytes is required for appropriate initiation of *Cd4* and *Cd8* silencing during intrathymic differentiation of $CD8^+$ and $CD4^+$ αβ T cells, respectively. The positive selection of $CD24^{hi}TCR\beta^{lo}CD4^+CD8^+$ immature thymocytes activates intracellular signals that up-regulate TCRβ expression and down-regulate CD24 expression as these cells differentiate into lineage-committed $CD24^{lo}TCR\beta^{hi}CD4^+$ or $CD24^{lo}TCR\beta^{hi}CD8^+$ mature thymocytes that exit the thymus as mature $CD4^+$ or $CD8^+$ αβ T cells (40). Our detection of $CD24^{lo}TCR\beta^{hi}CD4^+CD8^+$ mature thymocytes in mice with Dicer or Drosha inactivation starting in DN thymocytes demonstrates that a Dicer- and Drosha-dependent mechanism(s) is required for the appropriate initiation of *Cd4* and *Cd8* silencing. The increased presence of these cells and the generation of peripheral $CD4^+CD8^+$ αβ T cells following BCL2 expression (or p53 inactivation) indicates that apoptosis of *Dicer*- and *Drosha*-deficient αβ T cells after thymic emigration obscures the critical role of Dicer and Drosha in *Cd4* and *Cd8* silencing. For this reason, previous analyses of splenic αβ T cells in mice with Dicer or

Drosha inactivation starting in DN thymocytes failed to discover that Dicer and Drosha control *Cd4* and *Cd8* silencing (26, 27). We previously showed that ectopic BCL2 expression in *Dicer*-deficient thymocytes similarly unmasks a requirement for Dicer in promoting survival of DN thymocytes that attempt TCRβ gene rearrangements (37). Thus, our observations indicate that suppressing apoptosis should be standard practice when analyzing and interpreting phenotypes of *Dicer*- or *Drosha*-deficient cells.

Our discovery that *Dicer*- and *Drosha*-deficient αβ T cells exhibit similar defects in CD4 and CD8 expression provides strong support for our conclusion that miRs regulate *Cd4* and *Cd8* silencing during CD4/CD8 lineage commitment. In this context, while our data do not exclude another mechanism, miR biogenesis is the only known process for which both Drosha and Dicer are required. How might miRs control the initiation of *Cd4* and *Cd8* silencing? TCR-activated signaling pathways regulate expression of Runx3, Thpok, and possibly other proteins that modulate transcription of *Cd4* and *Cd8* loci (2). Our finding that Dicer promotes expression of Runx3 and Thpok in MHC I- or II-selected αβ T cells, respectively, suggests that miRs likely are required for appropriate up-regulation of these transcription factors, and thus proper initiation of *Cd4* and/or *Cd8* silencing. Loss of miRs that bind *Runx3* and/or *Thpok* mRNAs should increase expression of their encoded proteins. Accordingly, Dicer likely controls expression of these lineage-specifying factors through miRs that enhance signaling pathways or transcriptional networks that induce Runx3 and Thpok expression, and/or through miRs that inhibit repressors of *Runx3* and *Thpok* expression. Alternatively, it is possible that the development of $CD4^+CD8^+$ αβ T cells results from indirect effects of pro-apoptotic signals generated upon miR loss, rather than direct roles of miRs in C*d4* and *Cd8* silencing.

While we have demonstrated that Dicer deficiency impairs *Cd4* transcriptional silencing, our results do not rule out additional roles for Dicer (or Drosha) in regulating post-transcriptional silencing of *Cd4* and/or *Cd8*. For example, miRs could bind to and induce degradation or block translation of *Cd4* or *Cd8* transcripts during initiation of CD4 and CD8 silencing, respectively. Consistent with this notion, positive selection decreases the half-lives of *Cd4* and *Cd8α* mRNAs (48). Yet, these changes depend on protein synthesis (48), Cd8α and Cd8β mRNAs lack conserved miR seed sequences (49), and *Cd4* reporter genes that lack the *Cd4* 3'UTR exhibit normal silencing (50), which together argue against a role for miRs in control of *Cd4* and *Cd8* silencing via direct inhibition of *Cd4* and *Cd8* mRNAs.

Notably, inactivation of Dicer or Drosha initiating in DN thymocytes leads to the failure of *Cd4* or *Cd8* silencing in less than half of positively selected $CD4^+CD8^+$ αβ T lineage cells. This could be due in part to the timing of *Dicer* or *Drosha* deletion relative to positive selection of $CD4^+CD8^+$ thymocytes and concomitant C*d4* or *Cd8* silencing. *LckCre*-mediated deletion of *Dicer* starting in DN thymocytes does not lead to a complete absence of miRs in total thymocytes (26), raising the possibility that a significant fraction of Dicer-deficient $CD4^+CD8^+$ thymocytes might undergo positive selection and CD4/CD8 lineage-commitment in the presence of miRs that promote *Cd4* and/or *Cd8* silencing. This model would be consistent with our data that *Cd4Cre*-mediated *Dicer* deletion starting in DP thymocytes is not sufficient to generate $CD4^+CD8^+$ mature thymocytes or αβ T cells. Alternatively, our data that most positively-selected αβ T lineage cells that lack Dicer or

Drosha are capable of normal *Cd4* or *Cd8* silencing may indicate that miRs serve to facilitate efficient co-receptor silencing, rather than being absolutely required. For example, miRs could function to increase the recruitment or "on-rate" of transcriptional repressors or chromatin modifiers such as histone deacetylases that mediate CD4 and CD8 co-receptor silencing. In the absence of miRs, these factors may still bind to co-receptor loci and silence transcription, but the kinetic delay in recruitment would manifest as impaired co-receptor silencing in a fraction of cells as observed. In future studies it will be critical to determine whether aberrant peripheral $CD4^+CD8^+$ cells exhibit lineage plasticity or are instead fully committed to either the CD4 or CD8 lineage but simply delayed in their differentiation (e.g., could sustained TCR signals that normally promote CD4 lineage commitment re-direct MHCI-restricted splenic $CD4^+CD8^+$ cells to the CD4 lineage?).

Identification and characterization of the *Cd4* silencer and failure to discover a *Cd8* silencer have led to acceptance in the field that distinct mechanisms control initiation of *Cd4* and *Cd8* silencing (2). Our data that the miR biogenesis machinery controls appropriate silencing of both *Cd4* and *Cd8* challenges this dogmatic view. Furthermore, our results suggest that miRs are required for appropriate expression of "master regulators" of CD4 and CD8 lineage commitment following positive selection. Specifically, Dicer deficiency uncouples the regulatory modules that mediate lineage commitment and migration out of the thymus, with phenotypically mature Dicer deficient cells capable of exiting the thymus prior to normal up-regulation of lineage-specifying factors. It will be important to determine whether miRs regulate CD4 and CD8 lineage commitment via a shared pathway, such as TCR signaling, or via distinct mechanisms. Given the role of $CD4^+$ and $CD8^+$ αβ T cell development as a paradigm for elucidating genetic and epigenetic mechanisms that control gene expression changes during cellular differentiation, elucidating Dicer- and Drosha-dependent mechanisms that control CD4 and CD8 lineage commitment should have broad relevance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Katherine Yang-Iott and Qi Xiao for technical assistance.

This work was supported by the University of Pennsylvania Training Grants in Computational Genomics HG-000046 (L.J.R.), Cell and Molecular Biology GM-07229 (B.L.B., M.E.D.) and Immunobiology of Normal and Neoplastic Lymphocytes CA-009140 (M.E.D.); NIH Intramural Research Programs of the Center for Cancer Research, NCI (A.C.C., R.B.) and NIAID (B.L.B., S.A.M.); NIH grant AI059621 (A.B.); and Leukemia and Lymphoma Society Scholar Award and NIH grants CA125195 and CA136470 (C.H.B.).

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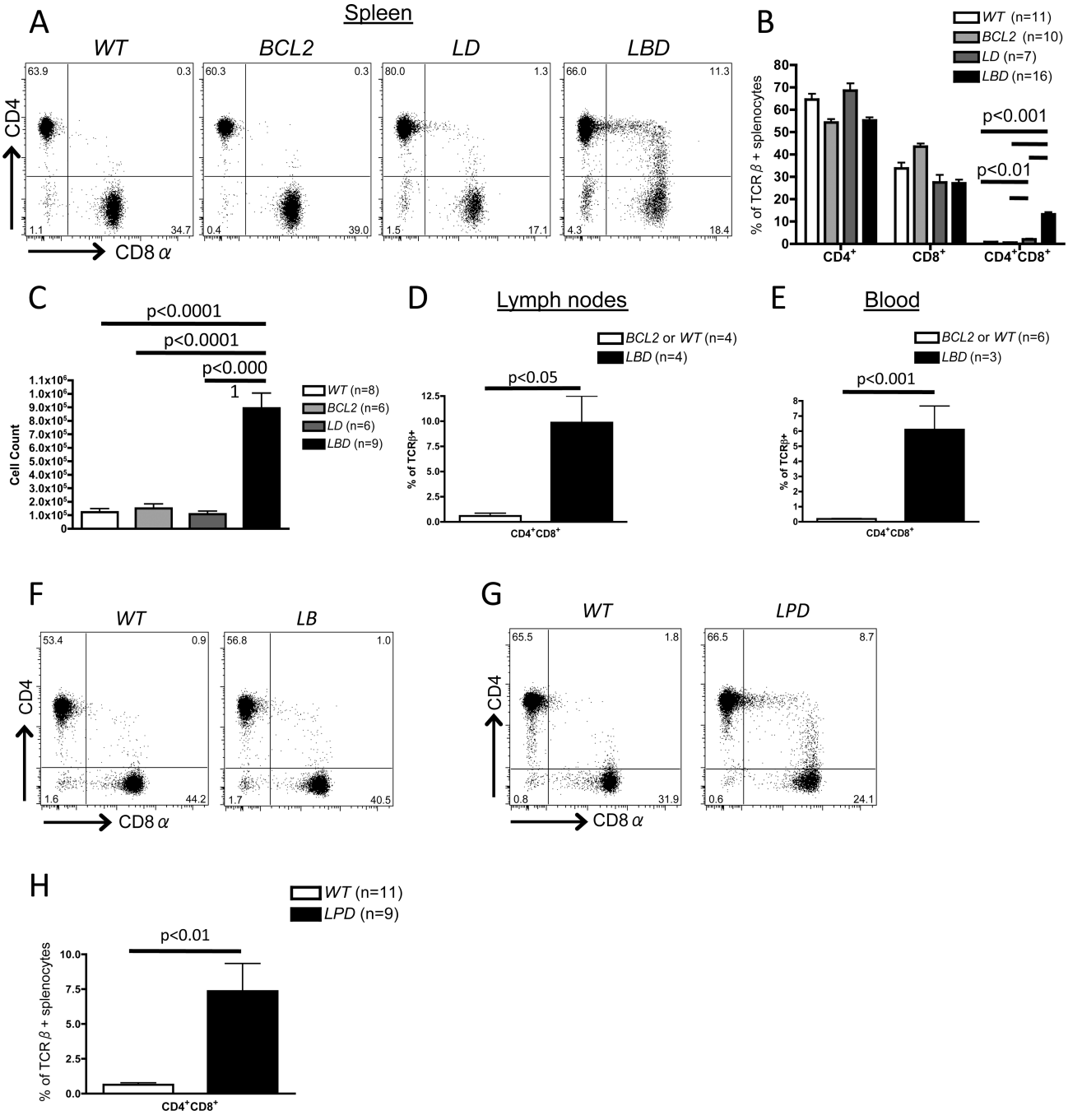

Figure 1. Generation of aberrant peripheral CD4$^+$CD8$^+$ cells in Dicer deficient mice expressing a *BCL2* transgene or lacking *Trp53*

A, Representative CD4 and CD8 staining on CD24^{lo}TCRβ$^+$ splenocytes of *WTBCL2LD*, or *LBD* mice. *B*, Average percentages of CD4$^+$CD8$^+$, and CD4$^+$CD8$^+$ cells amongst CD24^{lo}TCRβ$^+$ splenocytes of *WTBCL2LD*, or *LBD* mice. *C*, Total numbers of CD4$^+$CD8$^+$CD24^{lo}TCRβ$^+$ splenocytes in mice of the indicated genotypes. *D* and *E*, Frequency of CD4$^+$CD8$^+$ cells among TCRβ$^+$ cells in lymph nodes (*D*) or blood (*E*) of *LBD* or control mice. *F*, Representative CD4 and CD8 staining on CD24^{lo}TCRβ$^+$ splenocytes of

WT or *LckCre:EμBCL2* mice. *G*, Representative CD4 and CD8 staining on $CD24^{lo}TCR\beta^{+}$ splenocytes of *WT* or *LPD* mice. *H*, Average frequency of $CD4^{+}CD8^{+}$ cells amongst $CD24^{lo}TCR\beta^{+}$ splenocytes of *WT* or *LPD* mice. *B, C, D, E,* and *H,* The total numbers of mice analyzed are indicated. *B, C, D, E, F,* and *H,* each experiment was performed at least 3 independent times.

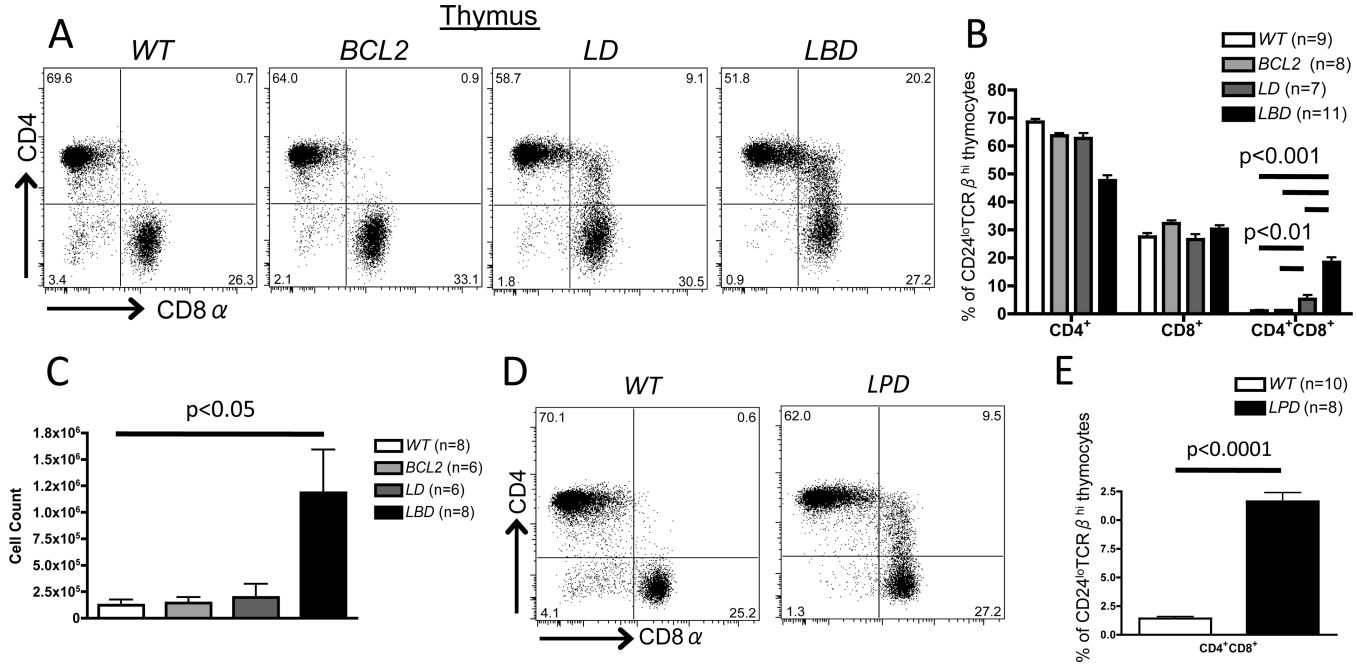

Figure 2. Impaired initiation of co-receptor silencing in mature thymocytes of Dicer deficient mice expressing a *BCL2* transgene or lacking *Trp53*

A, Representative CD4 and CD8 staining on $CD24^{lo}TCR\beta^{hi}$ mature thymocytes of *WTBCL2LD*, or *LBD* mice. *B*, Average percentages of $CD4^{+}CD8^{+}$and $CD4^{+}CD8^{+}$ cells amongst $CD24^{lo}TCR\beta^{hi}$ mature thymocytes of *WTBCL2LD*, or *LBD* mice. *C*, Total numbers of $CD4^{+}CD8^{+}CD24^{lo}TCR\beta^{hi}$ mature thymocytes in mice of the indicated genotypes. *D*, Representative CD4 and CD8 staining on $CD24^{lo}TCR\beta^{hi}$ mature thymocytes of *WT* or *LPD* mice. *E*, Average frequency of $CD4^{+}CD8^{+}$ cells amongst $CD24^{lo}TCR\beta^{+}$ mature thymocytes of *WT* or *LPD* mice. *B, C*, and *E*, the numbers of mice analyzed are indicated; each experiment was performed at least 3 independent times.

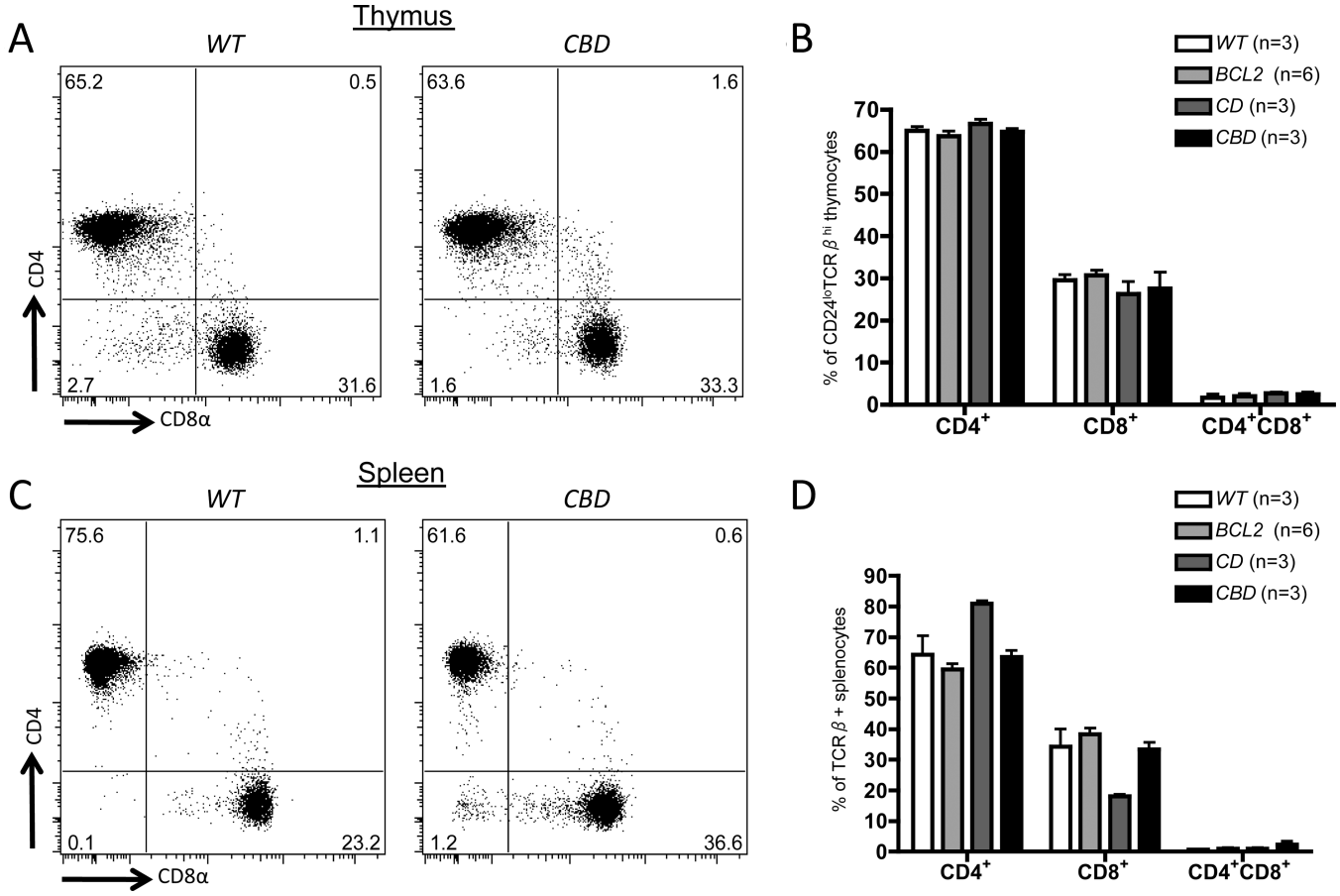

Figure 3. Normal initiation of *Cd4* and *Cd8* silencing in thymocytes and T cells with Dicer deletion initiating in DP thymocytes

A and *C*, Representative CD4 and CD8 staining on mature thymocytes (*A*) or $CD24^{lo}TCR\beta^{+}$ splenocytes (*C*) of *WT* or *CBD* mice. *B* and *D*, Average percentages of $CD4^{+}CD8^{+}$and $CD4^{+}CD8^{+}$ cells amongst mature thymocytes (*B*) or $CD24^{lo}TCR\beta^{+}$ splenocytes (*D*) of *WTBCL2CD*, and *CBD* mice. *B* and *D*, the numbers of mice analyzed are indicated. Each experiment was performed at least 3 independent times.

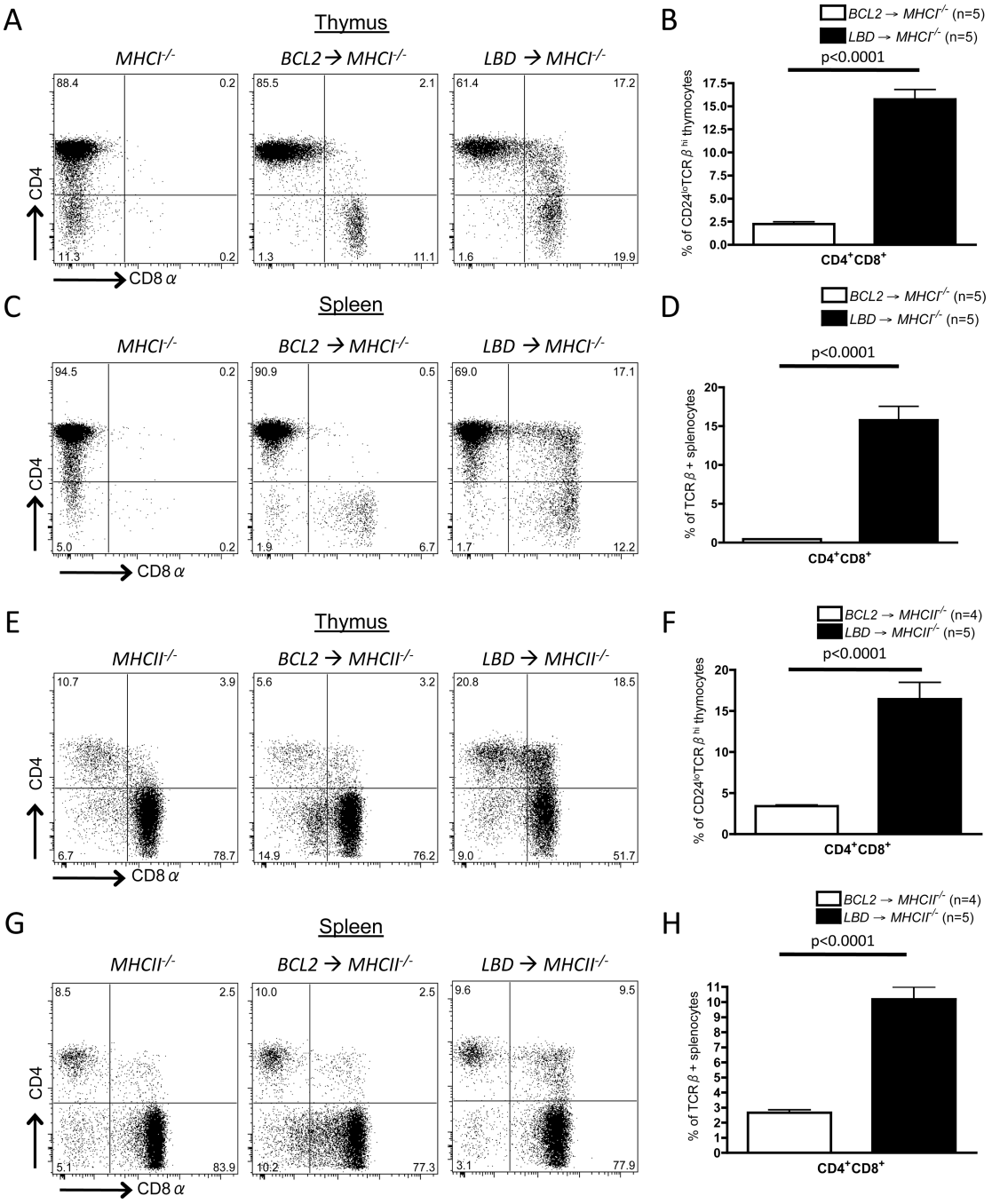

Figure 4. Dicer is required for appropriate initiation of *Cd4* silencing in MHCI-restricted cells and *Cd8* silencing in MHCII-restricted cells

A-D, Representative CD4 and CD8 staining on mature thymocytes (*A*) or CD24^{lo}TCRβ^{+} splenocytes (*C*) of *MHCI*$^{-/-}$ mice reconstituted with *BCL2* or *LBD* bone marrow. Average frequencies of CD4^{+}CD8^{+} cells amongst mature thymocytes (*B*) or CD24^{lo}TCRβ^{+} splenocytes (*D*) of *MHCI*$^{-/-}$ mice reconstituted with *BCL2* or *LBD* bone marrow. *E-H*, Representative CD4 and CD8 staining on mature thymocytes (*E*) or CD24^{lo}TCRβ^{+} splenocytes (*G*) of *MHCII*$^{-/-}$ mice reconstituted with *BCL2* or *LBD* bone marrow. Average

frequencies of $CD4^+CD8^+$ cells among mature thymocytes (*F*) or $CD24^{lo}TCR\beta^+$ splenocytes (*H*) of $MHCII^{-/-}$ mice reconstituted with *BCL2* or *LBD* bone marrow. *B, D, F* and *H*, The numbers of mice analyzed are shown. The experiment was performed twice with at least 4 recipient mice per group; a representative experiment is shown.

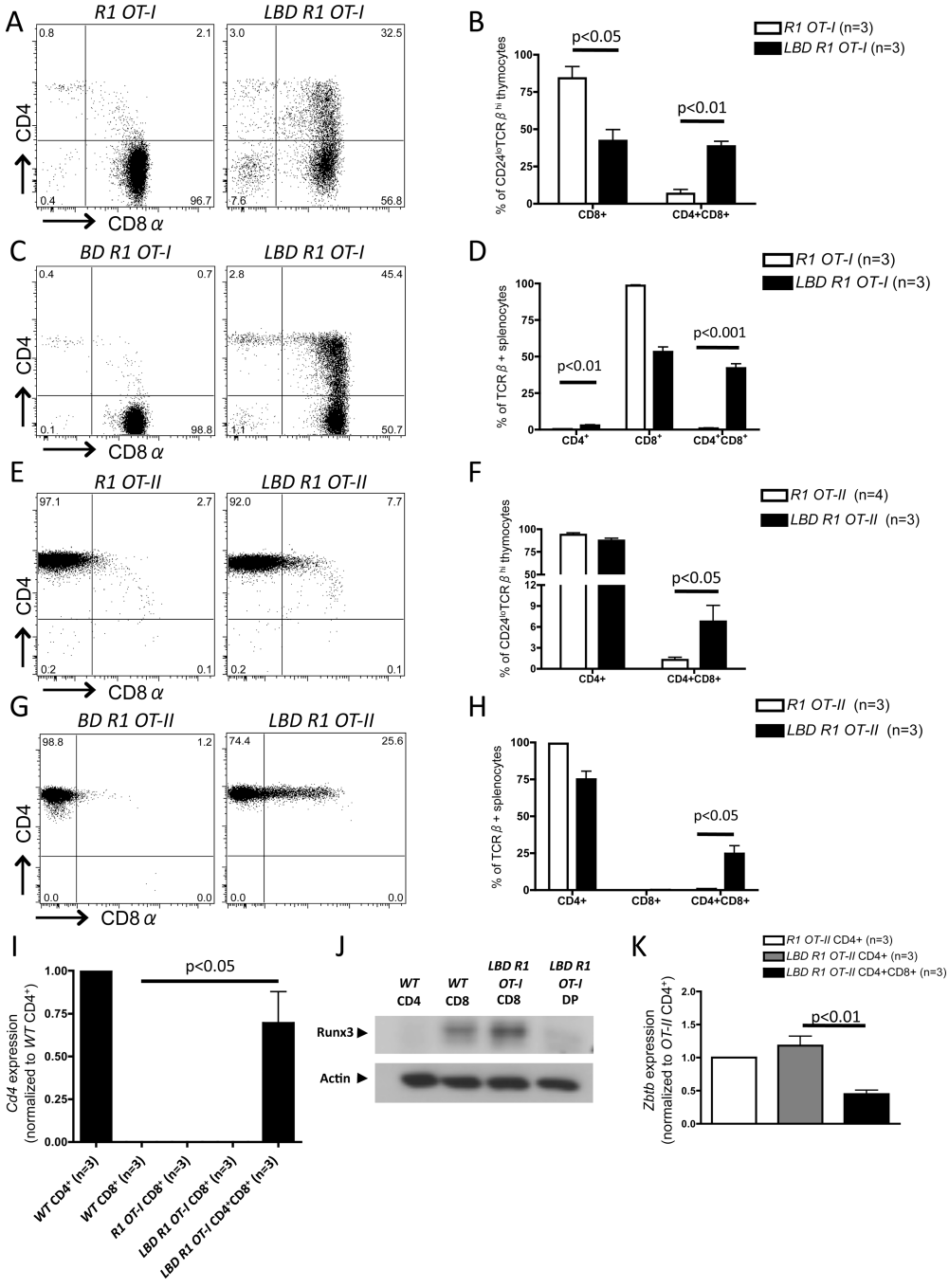

Figure 5. Dicer regulates *Cd4* and *Cd8* silencing and expression of *Runx3* and *Zbtb7b* in positively-selected αβ T cells

A, Representative CD4 and CD8 staining on $CD24^{lo}TCR\beta^{hi}$ mature thymocytes of *R1 OT-I* and *LBD R1 OT-I* mice. *B*, Average percentages of $CD4^{+}CD8^{+}$, and $CD4^{+}CD8^{+}$ cells amongst $CD24^{lo}TCR\beta^{hi}$ mature thymocytes of *R1 OT-I* and *LBD R1 OT-I* mice. *C*, Representative CD4 and CD8 staining on $CD24^{lo}TCR\beta^{+}$ splenocytes of *BD R1 OT-I* and *LBD R1 OT-I* mice. *D*, Average percentages of $CD4^{+}CD8^{+}$, and $CD4^{+}CD8^{+}$ cells amongst $CD24^{lo}TCR\beta^{+}$ splenocytes of *R1 OT-I* and *LBD R1 OT-I* mice. *E*, Representative CD4 and

CD8 staining on $CD24^{lo}TCR\beta^{hi}$ mature thymocytes of *R1 OT-II* and *LBD R1 OT-II* mice. *F*, Average percentages of $CD4^+CD8^+$, and $CD4^+CD8^+$ cells amongst $CD24^{lo}TCR\beta^{hi}$ mature thymocytes of *R1 OT-II* and *LBD R1 OT-II* mice. *G*, Representative CD4 and CD8 staining on $CD24^{lo}TCR\beta^+$ splenocytes of *BD R1 OT-II* and *LBD R1 OT-II* mice. *H*, Average percentages of $CD4^+CD8^+$, and $CD4^+CD8^+$ cells amongst $CD24^{lo}TCR\beta^+$ splenocytes of *R1 OT-II* and *LBD R1 OT-II* mice. *I*, qRT-PCR for primary (un-spliced) *Cd4* transcripts in sorted splenic populations from *WTR1 OT-I*, or *LBD R1 OT-I* mice. *J*, Runx3 Western blot in sorted splenic $CD4^+$ or $CD8^+$ cells from *WT* mice and $CD8^+$ or $CD4^+CD8^+$ DP) cells from *LBD R1 OT-I*. Three independent replicates were performed; a representative blot is shown. *K*, *Zbtb7b* qRT-PCR in sorted populations from *R1 OT-II* or *LBD R1 OT-II* mice. *B, D, F, H, I*, and *K*, The numbers of mice analyzed are indicated. At least 3 independent experiments were performed in each case.

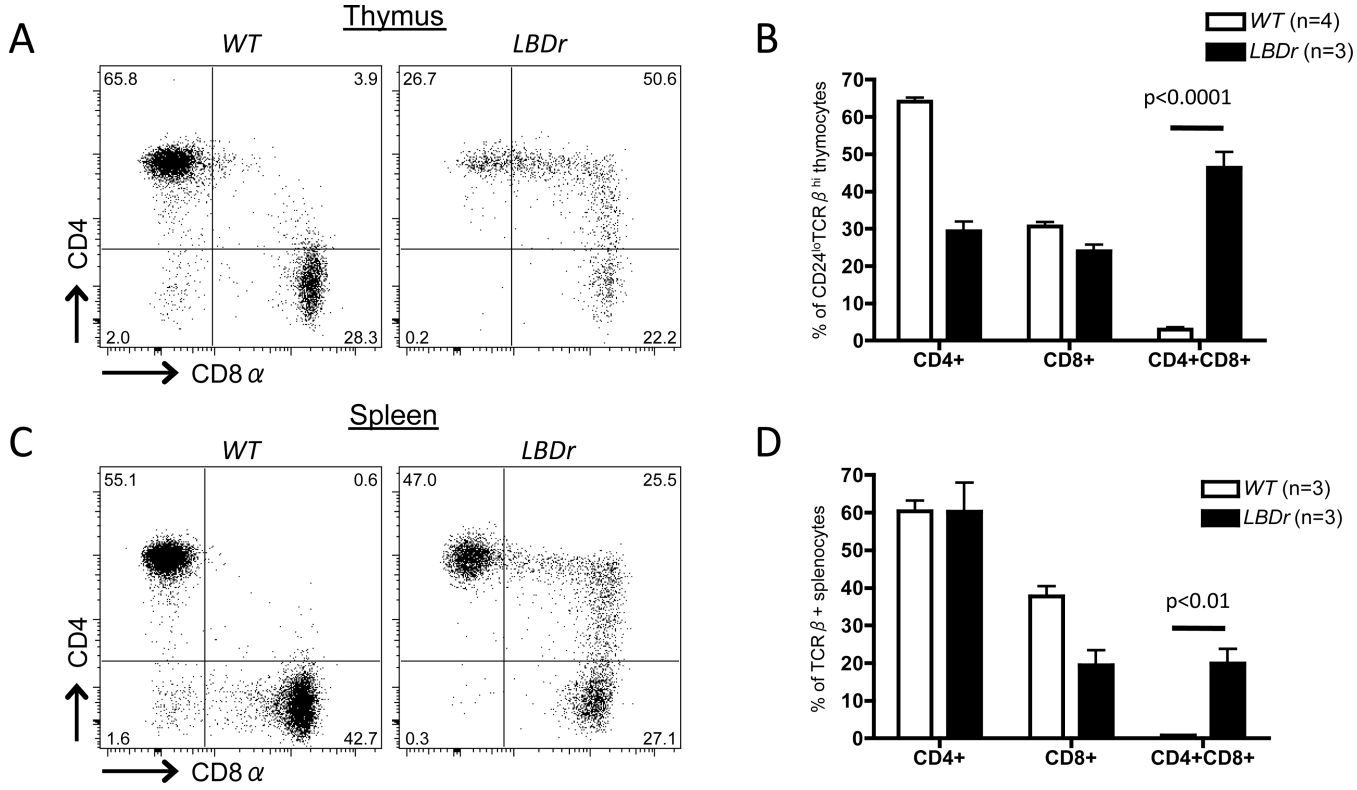

Figure 6. Drosha is required for appropriate initiation of *Cd4* and *Cd8* silencing after positive selection

Representative CD4 and CD8 staining on $CD24^{lo}TCR\beta^{hi}$ mature thymocytes (*A*) or $TCR\beta^{+}$ splenocytes (*C*) of *WT* or *LBDr* mice. Average percentages of $CD4^{+}CD8^{+}$, and $CD4^{+}CD8^{+}$ cells among mature thymocytes (*B*) or $TCR\beta^{+}$ splenocytes (*D*) of *WT* and *LBDr* mice. *B* and *D*, The numbers of mice analyzed are indicated; each experiment was performed at least 3 independent times.