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The microRNA Biogenesis Machinery Modulates Lineage Commitment during αβ T Cell Development

Levi J. Rupp,*†‡ Brenna L. Brady,*†‡ Andrea C. Carpenter,§ Maria Elena De Obaldia,‖ Avinash Bhandoola,‖ Remy Bosselut,§ Stefan A. Muljo,§ and Craig H. Bassing*†‡‖

Differentialiation of CD4+ helper and CD8+ cytotoxic αβ T cells from CD4+CD8+ thymocytes involves upregulation of lineage-specifying transcription factors and transcriptional silencing of CD8 or CD4 coreceptors, respectively, in MHC class II or I (MHCII or I)–restricted thymocytes. In this study, 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 MHCII- and MHCII-restricted mature CD4+CD8+ thymocytes. Expression of the antiapoptotic BCL2 protein or inactivation of the p53 proapoptotic protein rescues these thymocytes from apoptosis, increasing their frequency and permitting accumulation of CD4+CD8+ αβ T cells in the periphery. Dicer-deficient MHCII-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 $\text{Cd8}$ 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 $\text{Cd4}$ and $\text{Cd8}$ during αβ T cell differentiation. The Journal of Immunology, 2014, 193: 000–000.

The generation of distinct cellular lineages from multipotent progenitor cells involves differentiation programs that couple upregulation 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+ thymocytes (17, 18), implying that Thpok for Runx3 and the Zbtb7b silencer in initiation of CD4+ and CD8+ lineage commitment is required to prevent Runx3 and the Zbtb7b silencer (14, 16). Despite requirement for Runx3 and the Zbtb7b 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 coreceptor 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 to 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). Although 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 in this study that inactivation of Dicer starting in DN thymocytes impairs Cd4 and Cd8 silencing, leading to generation of positively selected, MHC-I- and MHCII-restricted mature CD4+CD8+ thymocytes. Expression of the antiapoptotic BCL2 protein or inactivation of the p53 proapoptotic 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 role of Dicer in maintenance of Cd4 and Cd8 silencing in peripheral CD4+ or CD8+ αβ T cells. We also show that Dicer-deficient MHC-I-restricted αβ T cells exhibit impaired transcriptional silencing of Cd4 and impaired expression of the Cd4-silencing transcription factor Runx3, whereas 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 coreceptor silencing and lineage commitment during αβ T cell differentiation.

Materials and Methods

Mice

LckCre (28), EμBCL2 (29), Dicer−/− (26), Tp53flox/flox (30), Cd4Cre (28), Rag1−/− (31), MHC1−/− (32), MHCII−/− (33), OT-I (34), OT-Ⅱ (35), and Drosophila (36) mice have been described previously. Mice were maintained under specific pathogen-free conditions at the Children’s Hospital of Philadelphia (CHOP) or the National Institutes of Health. Unless otherwise indicated, studies were conducted on littermate or age-matched mice between 4-8 wk of age. All studies were performed in accordance with the Children’s Hospital of Philadelphia or National Institute of Allergy and Infectious Diseases/National Institutes of Health 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) → TCRβCD24+, followed by CD4 and CD8 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 rad in two 450-rad doses 4 h apart) prior to retro-orbital injection. Mice were analyzed 8–10 wk later.

Quantitative real-time PCR

RNA isolation and cDNA generation were as described previously (37). Primers: Cd4 Exon 1F, 5'-GCGAGTGAAGGACAGTG-3', and Cd4 intron 1R, 5'-CAGACATCTTGGCCACATTAGC-3'. Primers for Zbtb7b were described previously (38). Primers for Rorc and Foxo1 were purchased from Life Technologies (murine TaqMan assays).

Western blot

Cell pellets were lysed in 1% SDS buffer (v/v) 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 Ab was from Epitomics (number 2593-1).

Quantification of miRlevels

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. Quantitative (q) RT-PCR was performed on a ViiA 7 (Applied Biosystems) instrument using TaqMan Universal Master Mix (Applied Biosystems), according to the 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 t test was used for statistical analyses. Error bars are SEM.

Results

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

Although 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−/− (LBD), LckCre:Dicer−/− (LBD), EμBCL2: Dicer−/− (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 antiapoptotic 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 reported previously (37). We show in this study that SP thymocyte numbers are also rescued in LBD mice (Supplemental Fig. 1A, 1B), although peripheral αβ T cell numbers are reduced in LBD mice relative to controls (Supplemental Fig. 1C, 1D), likely because Eμ activity (and therefore transgenic BCL2 expression) declines as αβ T cells mature (Supplemental Fig. 1E). 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 LD and LBD 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, 1B), corresponding to ∼15% of splenic αβ T cells in 4- to 8-wk-old animals. This population was absent from WT and BCL2 mice (Fig. 1A, 1B). The increased frequency of CD4+CD8+ αβ T cells was not simply due to loss of CD4+ or CD8+ cells because LBD mice exhibited a ∼10-fold increase in total number of splenic CD4+CD8+ cells as compared with 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 with WT and BCL2 mice (Fig. 1D, 1E). In addition, CD4+CD8+ αβ T cells were absent in Dicer-deficient, LckCre: EpBCL2 (LB) mice (Fig. 1F), indicating that this phenotype of LBD mice is not due to combined expression of Cre and BCL2.

Normally, preselection DP thymocytes are CD24hiTCRβlo cells, become CD24hiTCRβhi cells after upregulation of TCRβ expression during positive selection, and then downregulate CD24 expression to become CD24loTCRβhi SP mature thymocytes (40); mature peripheral αβ T cells are similarly CD24hiTCRβhi. We found that CD4+CD8+ αβ T cells were CD24hiTCRβhi (Fig. 1A, 1B), suggesting that they are mature postselection 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+CD24loTCRβhi αβ T cells did not express Rag1 or Rorc, two markers of immature DP thymocytes (Supplemental Fig. 2A, 2B). Splenic CD4+CD8+CD24loTCRβhi αβ T cells also exhibited higher expression of Foxo1 (Supplemental Fig. 2C), which is upregulated 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 postselection αβ T cells and are not simply immature preselection 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 postselection 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:p53flox/flox:Dicerflox/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, 1H). This finding indicates that inactivation of the proapoptotic 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 postselection thymocytes. To determine whether initiation of CD4/CD8 silencing is impaired in LBD mice, we analyzed CD4 and CD8 expression on pre- and postselection LBD and control thymocytes. We found that 18% of CD24loTCRαβ 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, 2B). The increased frequency of CD4+CD8+ cells was not simply due to loss of CD4+ or CD8+ cells because LBD mice exhibited a ~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+TCRαβ mature thymocytes in LPD mice relative to WT mice (Fig. 2D, 2E). In addition, we found that 5% of CD4+CD8+TCRαβ mature thymocytes in LD mice express both CD4 and CD8. Although this frequency is above the levels observed in mature thymocytes of WT and BCL2 mice (Fig. 2A, 2B), 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). Postselection (CD24loTCRαβ) thymocytes from LBD and LD mice exhibited >80% reduction in miR-181a and let-7c levels (Supplemental Fig. 3A, 3B), indicating comparable and substantial inactivation of Dicer in postselection thymocytes of both LBD and LD mice. Furthermore, the levels of each miR were reduced similarly among postselection CD4+, CD8+, or CD4+CD8+ thymocytes from LBD and LD mice (Supplemental Fig. 3A, 3B), indicating that Dicer is required for normal initiation of CD4/CD8 silencing in only a subset of postselection 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 used a genetic approach. The Cd4Cre transgene drives expression of Cre recombinase and deletion of Dicer<sup>flx</sup> 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<sup>flx/flx</sup> mice exhibit near complete deletion of Dicer<sup>flx</sup> alleles, low expression of miRs, and phenotypes indicative of Dicer inactivation (27, 39, 45). On the basis of these observations, we reasoned that Cd4Cre-mediated deletion of Dicer starting in CD4<sup>+</sup>CD8<sup>+</sup> 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 coreceptor silencing in CD4<sup>+</sup> and CD8<sup>+</sup> αβ T cells. Thus, we generated and analyzed Cd4Cre:Eμ.BCL2: Dicer<sup>flx/flx</sup> (CBD) mice. In striking contrast to LBD mice, CBD mice had neither mature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes (Fig. 3A, 3B) nor CD4<sup>+</sup>CD8<sup>+</sup> splenic αβ T cells (Fig. 3C, 3D). 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<sup>+</sup>CD8<sup>+</sup> αβ T cells in LBD mice could make up MHCI-restricted cells with impaired Cd4 silencing, MHCI-restricted cells with impaired Cd8 silencing, or both. To address this issue, we restricted the ability of thymocytes to develop on MHCI or MHCI by transferring bone marrow cells from LBD or BCL2 mice into irradiated MHCI<sup>−/−</sup> or MHCI<sup>−/−</sup> recipient mice.

To determine whether Dicer is required for appropriate initiation of Cd8 silencing during development of MHCI-restricted CD4<sup>+</sup> αβ T cells, we analyzed irradiated MHCI<sup>−/−</sup> mice (32) reconstituted with LBD or BCL2 bone marrow cells. It has been shown that BCL2 expression in MHCI<sup>−/−</sup> mice allows development of small numbers of splenic CD8<sup>+</sup> T cells (46). We found the same in MHCI<sup>−/−</sup> mice reconstituted with BCL2 or LBD bone marrow cells (Fig. 4C). However, we also observed that ∼15% of CD24<sup>hi</sup> TCRβ<sup>hi</sup> mature thymocytes aberrantly expressed both Cd4 and Cd8 in MHCI<sup>−/−</sup> mice reconstituted with LBD bone marrow, whereas only 2% of CD24<sup>hi</sup> TCRβ<sup>hi</sup> mature thymocytes were CD4<sup>+</sup>CD8<sup>+</sup> in MHCI<sup>−/−</sup> mice reconstituted from BCL2 cells (Fig. 4A, 4B). We also found that ∼15% of splenic αβ T cells were CD4<sup>+</sup>CD8<sup>+</sup> in MHCI<sup>−/−</sup> mice reconstituted with BLD bone marrow cells, whereas only 0.5% of splenic αβ T cells expressed both Cd4 and Cd8 in MHCI<sup>−/−</sup> mice reconstituted with BCL2 bone marrow (Fig. 4C, 4D). Although we cannot rule out that the CD4<sup>+</sup>CD8<sup>+</sup> αβ T cells in MHCI<sup>−/−</sup> mice reconstituted with BCL2 cells developed from CD8<sup>+</sup> cells that failed to silence Cd4, the substantial population of CD4<sup>+</sup>CD8<sup>+</sup> 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 MHCI-restricted αβ T cells.

To determine whether Dicer is required for appropriate initiation of Cd4 silencing during development of MHCI-restricted CD8<sup>+</sup> αβ T cells, we analyzed irradiated MHCI<sup>−/−</sup> mice (33) reconstituted with bone marrow from LBD or BCL2 mice. As previously shown (33, 46), a small population of CD4<sup>+</sup> αβ T cells does develop in MHCI<sup>−/−</sup> mice, although the vast majority of cells are CD8<sup>+</sup> (Fig. 4E). We found that ∼16% of CD24<sup>hi</sup> TCRβ<sup>hi</sup> mature thymocytes expressed both Cd4 and Cd8 in MHCI<sup>−/−</sup> mice reconstituted with LBD bone marrow, but only ∼4% of these cells were

![FIGURE 3](http://www.jimmunol.org/) 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<sup>+</sup>TCRβ<sup>+</sup> splenocytes (C) of WT or CBD mice. (B and D) Average percentages of CD4<sup>+</sup>, CD8<sup>+</sup>, and CD4<sup>+</sup>CD8<sup>+</sup> cells among mature thymocytes (B) or CD24<sup>+</sup>TCRβ<sup>+</sup> splenocytes (D) of WT, BCL2, CD, and CBD mice. The numbers of mice analyzed are indicated. Each experiment was performed at least three 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 CD4<sup>+</sup>TCR<beta><sup>+</sup> splenocytes (C) of MHCI<sup>−/−</sup> mice reconstituted with BCL2 or LBD bone marrow. Average frequencies of CD4<sup>+</sup>CD8<sup>+</sup> cells among mature thymocytes (B) or CD4<sup>+</sup>CD8<sup>+</sup> splenocytes (D) of MHCI<sup>−/−</sup> mice reconstituted with BCL2 or LBD bone marrow. (E-H) Representative CD4 and CD8 staining on mature thymocytes (E) or CD4<sup>+</sup>TCR<beta><sup>+</sup> splenocytes (G) of MHCII<sup>−/−</sup> mice reconstituted with BCL2 or LBD bone marrow. Average frequencies of CD4<sup>+</sup>CD8<sup>+</sup> cells among mature thymocytes (F) or CD4<sup>+</sup>CD8<sup>+</sup> splenocytes (H) of MHCII<sup>−/−</sup> 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 four recipient mice per group. A representative experiment is shown.
CD4+CD8+ in MHCII−/− mice reconstituted with BCL2 cells (Fig. 4E, 4F). 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, 4H). 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 MHCII-restricted αβ T cells.

On the basis of our analyses of MHCII−/− 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 MHCII- 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 MHCII-restricted OT-I αβ TCR transgene, which normally promotes positive selection of only CD4+ 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 R1 OT-II mice) to prevent TCRβ and TRα 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, 5B) and αβ T cells (Fig. 5C, 5D) 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 MHCII-restricted αβ TCR transgene. Similarly, we found that positively selected LBD R1 OT-II mature thymocytes (Figs. 5E, 5F) and αβ T cells (Fig. 5G, 5H) 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 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 coreceptor transcriptional silencing, we conducted qRT-PCR analyses of primary (unspliced) 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+ and WT CD4+ cells (Fig. 5I). In contrast, we were unable to detect primary Cd4 transcripts in CD8+ cells of WT, R1 OT-I, or LBD R1 OT-I mice (Fig. 5I). These results demonstrate that Dicer is required for appropriate transcriptional silencing of Cd4 in MHCII-restricted αβ T cells.

Following positive selection, Runx3 expression is upregulated in MHCII-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 MHCII-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 with 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 MHCII-restricted αβ T cells.

Analogous to Runx3 upregulation in MHCII-restricted cells, positive selection of MHCII-restricted cells induces Tpok expression, which drives Cd4 lineage commitment and facilitates Cd8 silencing (8–10). Given that Runx3 expression was impaired in Dicer-deficient MHCII-restricted αβ T cells, we hypothesized that Dicer might similarly control expression of Tpok in MHCII-restricted cells. To test this hypothesis, we performed qRT-PCR analyses for Zrb7b mRNA (because we were unable to isolate enough cells for Western blot with available Tpok Abs) in sorted cells from LBD R1 OT-II and control mice. We found a lower level of Zrb7b 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 Zrb7b 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, Tpok, or other factors that control Cd4 and Cd8 expression. To determine whether Dicer-dependent siRNAs and/or miRs regulate initiation of Cd4 and Cd8 silencing, we generated and analyzed LekCre:Es3BcI2:Droshaflx/flox (LBDr) mice because the Drosha RNA endonuclease is required for production of miRs but not siRNAs (47). We observed that ~40% of Cd24hiTCRβ+ mature thymocytes in LBD mice were CD4+CD8+ (Fig. 6A, 6B), 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 LBD mice were CD4+CD8+ (Fig. 6C, 6D), indicating that ectopic expression of Bcl2 throughout development of Drosha-deficient αβ T cells permits the accumulation of mature postselection splenic Cd4+CD8+ αβ T cells. Because 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 miR(s) 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 Cd24hiTCRβhiCD4+CD8+ immature thymocytes activates intracellular signals that upregulate TCRβ expression and down-regulate Cd24 expression as these cells differentiate into lineage-committed Cd24loTCRβloCD4+ or Cd24loTCRβhiCD8+ mature thymocytes that exit the thymus as mature Cd4+ or Cd8+ αβ T cells (40). Our detection of Cd24hiTCRβhiCd4+ Cd8+ mature thymocytes in mice with Dicer or Drosha inactivation starting in bone marrow indicates that apoptosis of thymocytes that exit the thymus as mature CD4+ or CD8+ T cells, we conclude that miR(s) likely control the appropriate initiation of Cd4 and Cd8 silencing during αβ T cell differentiation.
FIGURE 5. Dicer regulates Cd4 and Cdh silencing and expression of Runx3 and Zbtb7b in positively-selected αβ T cells. (A) Representative CD4 and CD8 staining on CD24loTCRb hi mature thymocytes of R1 OT-I and LBD R1 OT-I mice. (B) Average percentages of CD+4, CD8+, and CD4+CD8+ cells among CD24loTCRb hi mature thymocytes of R1 OT-I and LBD R1 OT-I mice. (C) Representative CD4 and CD8 staining on CD24loTCRb splenocytes of BD R1 OT-I and LBD R1 OT-I mice. (D) Average percentages of CD4+, CD8+, and CD4+CD8+ cells among CD24loTCRb splenocytes of R1 OT-I and LBD R1 OT-I mice. (E) Representative CD4 and CD8 staining on CD24loTCRb mature thymocytes of R1 OT-II and LBD R1 OT-II mice. (F) Average percentages of CD4+, CD8+, and CD4+CD8+ cells among CD24loTCRb mature thymocytes of R1 OT-II and LBD R1 OT-II mice. (G) Representative CD4 and CD8 staining on CD24loTCRb splenocytes of BD R1 OT-II and LBD R1 OT-II mice. (H) Average percentages of CD4+, CD8+, and CD4+CD8+ cells among CD24loTCRb splenocytes of R1 OT-II and LBD R1 OT-II mice. (I) qRT-PCR for primary (unspliced) Cd4 transcripts in sorted splenic populations from WT, R1 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
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 TCR 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, although 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 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 Cd4Cd8αβ T cells results from indirect effects of proapoptotic signals generated upon miR loss, rather than direct roles of miRs in Cd4 and Cd8 silencing.

Although we have demonstrated that Dicer deficiency impairs Cd4 transcriptional silencing, our results do not rule out additional roles for Dicer (or Drosha) in regulating posttranscriptional 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′-untranslated region 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 Cd4Cd8αβ T lineage cells. This could be in part because of the timing of Dicer or Drosha deletion relative to positive selection of Cd4Cd8αβ thymocytes and concomitant Cd4 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

![Image](https://example.com/image.png)

**FIGURE 6.** Drosha is required for appropriate initiation of Cd4 and Cd8 silencing after positive selection. Representative Cd4 and Cd8 staining on CD24hi mature thymocytes (A) or TCRβ hi splenocytes (C) of WT or LBDr mice. Average percentages of Cd4+, Cd8+, and Cd4+Cd8+ cells among mature thymocytes (B) or TCRβ hi splenocytes (D) of WT and LBDr mice. (B and D) The numbers of mice analyzed are indicated; each experiment was performed at least three independent times.

**CD4+CD8+ (DP) cells from LBD R1 OT-1.** 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 three independent experiments were performed in each case.
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 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 or lineage but simply delayed in their differentiation (e.g., could sustain plasticity or are instead fully committed to either the CD4 or CD8 lineage commitment should have broad relevance.

Dicer- and Drosha-dependent mechanisms that control CD4 and CD8 lineage commitment should have broad relevance. We thank Katherine Yang-Iott and Qi Xiao for technical assistance.

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Disclosures

The authors have no financial conflicts of interest.

References


