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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∗,†,‡,‖

Dicer-deficient MHCI-restricted mature CD4CD8+ T cells show impaired Cd8 silencing and impaired induction of the CD8 lineage–specifying transcription factor Thpok. Finally, we show that the Drosha silencing machinery, 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-specific transcription factors and silencing of Cd4 and Cd8 during αβ T cell differentiation. The Journal of Immunology, 2014, 193: 4032–4042.

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 involves upregulation of lineage-specific transcription factors and transcriptional silencing of CD8 or CD4 coreceptors, respectively, in MHC class II or I (MHCI 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 MHCI- and MHCIIderestricted mature CD4CD8+ 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 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 MHCI-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-specific transcription factors and silencing of Cd4 and Cd8 during αβ T cell differentiation. The Journal of Immunology, 2014, 193: 4032–4042.

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Abbreviations used in this article:

BCCL2, EpBCL2:Dicer−/− homo; CBD, Cd4Cre:EpBCL2:Dicer−/− /BBD; DN, double-negative; DP, double-positive; LB, LckCre:EpBCL2:BBD; LBD, LckCre:EpBCL2:Dicer−/− /BBD; LD, LckCre:Dicer−/− homo/LPD; LDK, LckCre:p53−/− homo; Dicer−/− homo; miR, microRNA; q, quantitative; siRNA, short-interfering RNA; SP, single-positive; WT, wild-type.

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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 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). 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, MHCII- and MHCII-restricted mature Cd4+Cd8+ T cells. 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 requirement of Dicer in maintenance of Cd4 and Cd8 silencing in peripheral Cd4+ or Cd8αβ T cells. We also show that Dicer-deficient MHCII-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 miRs in this process during lineage commitment during Cd8 signaling.

Materials and Methods

Mice

LckCre (28), EµBcl2 (29), Dicer<sup>−/+</sup> (26), Tp53<sup>−/−</sup> (30), Cd4Cre (28), Rag2<sup>−/−</sup> (31), MhcII<sup>−/−</sup> (32), MhcII<sup>−/−</sup> (33), Ot-I<sup>−/−</sup> (34), Ot-II<sup>−/−</sup> (35), and Drosha<sup>−/−</sup> (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 CD4<sup>+</sup> CD8αβ<sup>+</sup> T cell chimeras, which were competitively reconstituted with CD4<sup>+</sup> CD8αβ<sup>+</sup> T cells from WT mice by cyclophosphamide treatment. CD4<sup>+</sup> CD8αβ<sup>+</sup> T cells from WT mice (28) were a generous gift from Dr. James Forman (Children’s Hospital of Philadelphia, Philadelphia, PA). All studies were performed in accordance with regulations and approved by 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 → TCRβ<sup>−</sup>CD2<sup>+</sup>CD<sup>+</sup>4<sup>+</sup>CD<sup>+</sup>8αβ<sup>+</sup>, followed by CD4 and CD8α gating.

Bone marrow chimeras

Single-cell suspensions were prepared from tibia and femur bone marrow. CD4<sup>+</sup> and CD8<sup>+</sup> 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′-GCCAGATGAGAAGGAGCTGG-3′, and Cd4 intron 1R, 5′-CAGAACATTCCGACACATTTAGC-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% polyvinylidene difluoride membranes (Immobilon-P; Millipore), and analyzed by immunoblot and chemiluminescence. The anti-Runx Ab was from Epitomics (number 2593-1).

Quantification of miR 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. Quantitative (q) RT-PCR was performed on a Viia7 (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’s t test was used for statistical analyses. Error bars are SEM.

Results

Suppressing apoptosis of Dicer-deficient thymocytes results in generation of Cd4<sup>+</sup>Cd8<sup>+</sup> mature αβ T cells

Although previous reports suggested that Dicer does not control lineage commitment of Cd4<sup>+</sup> and Cd8αβ<sup>+</sup> 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<sup>−/+</sup> (LBD), LckCre: Dicer<sup>−/+</sup> (LD), EµBcl2: Dicer<sup>−/+</sup> (BCL2), and Dicer<sup>−/+</sup> (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 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<sup>+</sup>Cd8<sup>+</sup> 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<sup>+</sup>Cd8<sup>+</sup> αβ T cells was not simply due to loss of Cd4<sup>+</sup> or Cd8<sup>+</sup> cells because LBD mice exhibited a ~10-fold increase in total number of splenic Cd4<sup>+</sup>Cd8<sup>+</sup> cells as compared with control mice (Fig. 1C). We also observed increased frequencies of Cd4<sup>+</sup>Cd8<sup>+</sup> 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-sufficient, LckCre:EmBCL2 (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 CD24loTCRβhi. We found that CD4+CD8+ αβ T cells were CD24loTCRβhi (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 peripheral CD4+CD8+ αβ T cells.

**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 CD24loTCRβ+ splenocytes of WT, BCL2, LD, or LBD mice. (B) Average percentages of CD4+, CD8+, and CD4+CD8+ cells among CD24loTCRβ+ splenocytes of WT, BCL2, LD, or LBD mice. (C) Total numbers of CD4+CD8+CD24loTCRβ+ 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 CD24loTCRβ+ splenocytes of WT or LckCre:EqBCL2 mice. (G) Representative CD4 and CD8 staining on CD24loTCRβ+ splenocytes of WT or LPD mice. (H) Average frequency of CD4+CD8+ cells among CD24loTCRβ+ splenocytes of WT or LPD mice. (B–E and H) Each experiment was performed at least three independent times.

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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 unmask 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 CD24loTCRhi 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+TCRβhi mature thymocytes in LPD mice relative to WT mice (Fig. 2D, 2E). In addition, we found that 5% of CD24+TCRβhi 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 LD and LBD thymocytes, we quantified the expression of two miRs that are highly expressed in thymocytes (miR-181a and let-7c) (44). Postselection (CD24+TCRβhi) thymocytes from LD and LBD 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 LD and LBD 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>fl</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>fl/fl</sup> mice exhibit near complete deletion of Dicer<sup>fl</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:E<sub>µ</sub>BCL2: Dicer<sup>fl/fl</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 MHCII-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 MHCII by transferring bone marrow cells from LBD or BCL2 mice into irradiated MHCII<sup>−/−</sup> or MHCII<sup>−/−</sup> recipient mice. To determine whether Dicer is required for appropriate initiation of Cd8 silencing during development of MHCII-restricted Cd4<sup>+</sup> αβ T cells, we analyzed irradiated MHCII<sup>−/−</sup> mice (32) reconstituted with LBD or BCL2 bone marrow cells. It has been shown that BCL2 expression in MHCII<sup>−/−</sup> mice allows development of small numbers of splenic Cd8<sup>+</sup> T cells (46). We found the same in MHCII<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 MHCII<sup>−/−</sup> mice reconstituted with LBD bone marrow, whereas only 2% of Cd24<sup>+</sup>TCRβ<sup>+</sup> mature thymocytes were Cd4<sup>+</sup>Cd8<sup>+</sup> in MHCII<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 MHCII<sup>−/−</sup> mice reconstituted with LBD bone marrow, whereas only 0.5% of splenic αβ T cells expressed both Cd4 and Cd8 in MHCII<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 MHCII<sup>−/−</sup> mice reconstituted with LBD 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 MHCII-restricted αβ T cells.

To determine whether Dicer is required for appropriate initiation of Cd4 silencing during development of MHCII-restricted Cd4<sup>+</sup> αβ T cells, we analyzed irradiated MHCII<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 MHCII<sup>−/−</sup> mice, although the vast majority of cells are Cd8<sup>+</sup> (Fig. 4E). We found that ~16% of Cd24<sup>+</sup>TCRβ<sup>+</sup> mature thymocytes expressed both Cd4 and Cd8 in MHCII<sup>−/−</sup> mice reconstituted with LBD bone marrow, but only ~4% of these cells were...
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 CD24loTCRb+ splenocytes (C) of MHCI-/- mice reconstituted with BCL2 or LBD bone marrow. Average frequencies of CD4+CD8+ cells among mature thymocytes (B) or CD24loTCRb+ splenocytes (D) of MHCI-/- mice reconstituted with BCL2 or LBD bone marrow. (E–H) Representative CD4 and CD8 staining on mature thymocytes (E) or CD24loTCRb+ splenocytes (G) of MHCII-/- mice reconstituted with BCL2 or LBD bone marrow. Average frequencies of CD4+CD8+ cells among mature thymocytes (F) or CD24loTCRb+ 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 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 could not 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 MHC-I- 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 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 Rag−/− background (LBD R1 OT-I and LBD R1 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, 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 (Fig. 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 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 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+ cells 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 Thpok 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 Thpok in MHCII-restricted cells. To test this hypothesis, we performed qRT-PCR analyses for Zbtb7b mRNA (because we were unable to isolate enough cells for Western blot with available Thpok Abs) 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 I- 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 whether Dicer-dependent siRNAs and/or miRs regulate initiation of Cd4 and Cd8 silencing, we generated and analyzed LckCre:EbxBCL2: Drosha−/− (LDb) mice because the Drosha RNA endonuclease is required for production of miRs but not siRNAs (47). We observed that ~40% of Cd24LTCRβ+ mature thymocytes in LDb 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 LDb 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 Cd24LTCRβ+Cd4+Cd8− immature thymocytes activates intracellular signals that upregulate TCRβ expression and down-regulate Cd4 expression as these cells differentiate into lineage-committed Cd24LTCRβ+Cd4+ or Cd24LTCRβ+Cd8+ mature thymocytes that exit the thymus as mature Cd4+ or Cd8+ αβ T cells (40). Our detection of Cd24LTCRβ+Cd4+Cd8+ mature thymocytes in mice with Drosha inactivation 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
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 CD24loTCRbhi mature thymocytes of R1 OT-I and LBD R1 OT-I mice. (B) Average percentages of CD4+, CD8+, and CD4+CD8+ cells among CD24loTCRbhi mature thymocytes of R1 OT-I and LBD R1 OT-I mice. (C) Representative CD4 and CD8 staining on CD24loTCRbhi splenocytes of BD R1 OT-I and LBD R1 OT-I mice. (D) Average percentages of CD4+, CD8+, and CD4+CD8+ cells among CD24loTCRbhi splenocytes of R1 OT-I and LBD R1 OT-I mice. (E) Representative CD4 and CD8 staining on CD24loTCRbhi mature thymocytes of R1 OT-II and LBD R1 OT-II mice. (F) Average percentages of CD4+, CD8+, and CD4+CD8+ cells among CD24loTCRbhi mature thymocytes of R1 OT-II and LBD R1 OT-II mice. (G) Representative CD4 and CD8 staining on CD24loTCRbhi splenocytes of BD R1 OT-II and LBD R1 OT-II mice. (H) Average percentages of CD4+, CD8+, and CD4+CD8+ cells among CD24loTCRbhi 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 (Figure legend continues)
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, although our data do not exclude another mechanism, miR bio-genesis 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 (37). Thus, our observations indicate that suppressing apoptosis should be standard practice when analyzing and interpreting phenotypes of Dicer- or Drosha-deficient cells.

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 and Cd8 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 Cd4⁺Cd8⁺ αβ T lineage cells. This could be in part because of the timing of Dicer or Drosha deletion relative to positive selection of Cd4⁺Cd8⁺ 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...
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 corereceptor 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 corereceptor silencing. In the absence of miRs, these factors may still bind to corereceptor loci and silence transcription, but the kinetic delay in recruitment would manifest as impaired corereceptor 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 sustain commitment in the presence of miRs that promote Cd4 and/or 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 upregulation 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.

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Disclosures

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References


