Aberrant T cell differentiation in the absence of Dicer

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Dicer is an RNaseIII-like enzyme that is required for generating short interfering RNAs and microRNAs. The latter have been implicated in regulating cell fate determination in invertebrates and vertebrates. To test the requirement for Dicer in cell-lineage decisions in a mammalian organism, we have generated a conditional allele of dicer-1 (dcr-1) in the mouse. Specific deletion of dcr-1 in the T cell lineage resulted in impaired T cell development and aberrant T helper cell differentiation and cytokine production. A severe block in peripheral CD8^+ T cell development was observed upon dcr-1 deletion in the thymus. However, Dicer-deficient CD4^+ T cells, although reduced in numbers, were viable and could be analyzed further. These cells were defective in microRNA processing, and upon stimulation they proliferated poorly and underwent increased apoptosis. Independent of their proliferation defect, Dicer-deficient helper T cells preferentially expressed interferon-γ, the hallmark effector cytokine of the Th1 lineage.

RNA interference (RNAi) is an evolutionarily conserved pathway, central to a broad spectrum of biological phenomena, including development, stem cell maintenance, transcriptional gene silencing, heterochromatin formation, and transposon silencing (1–3). RNAi is mediated by ribonucleoprotein complexes that effect translational inhibition, mRNA degradation, or transcriptional silencing (3, 4). The sequence specificity of these effector complexes is programmed by the incorporation of short interfering RNAs (siRNAs) or microRNAs (miRNAs) that anneal to target nucleic acid sequences. Dicer is a key enzyme in this pathway, because it is responsible for the cleavage of long double-stranded RNAs and short-hairpin RNAs (e.g., precursor miRNAs [pre-miRNAs]) into siRNAs and miRNAs (2, 4, 5). Dicer mutation in Caenorhabditis elegans causes defects in the developmental timing of larval stages primarily attributed to the lack of processing of lin-4 and let-7 pre-miRNAs (6). The let-7 miRNA regulates the differentiation of certain cell lineages in the worm during the transition from late larval stages to adulthood and has been proposed to play a similar role in other organisms because of its sequence conservation among several animal species (6, 7). To date, 224 mammalian miRNAs are listed in the miRNA Registry, and the expression pattern of many of these has been verified in mouse tissues through the combined effort of many investigators (8). Their functions are only now beginning to be addressed.

RESULTS

Ablation of Dicer expression and function in T lymphocytes

Despite increasing evidence that the RNAi machinery is involved in key cellular processes, the biological role of Dicer and RNAi-related pathways in mammalian cells is largely unknown at present. Dicer mutation in mice or mouse embryonic stem (ES) cells results in developmental failure (9, 10). To overcome this problem, we generated a conditional mutation of the, dicer-1 (dcr-1) gene by inserting two loxP sites in the introns that flank exons 18–20 (10). Cre recombinase can then be employed to delete an essential portion of this gene, which encodes part of the piwi/argonaute/zwille domain and the first RNaseIII domain. Insertion of the loxP sites in the intronic regions of dcr-1 does not seem to interfere with this gene’s function, because mice homozygous for the loxP-flanked (floxed) dcr-1 allele...
were obtained from heterozygous parents at the expected frequency (Table S1, available at http://www.jem.org/cgi/content/full/jem.20050678/DC1), and dcr<sup>fl/fl</sup> cells that do not express Cre did not exhibit any of the defects described for Dicer-deficient cells (reference 10 and unpublished data).

Recent evidence suggests that certain miRNAs exhibit tissue- or cell type–specific expression and may play a role in mammalian biology (11–13). To investigate the biological role of Dicer in T lymphocyte development and function, we conditionally deleted dcr<sup>1</sup> in mice using a Cre transgene under the control of the cd4 enhancer/promoter/silencer (CD4cre). We determined the efficiency of dcr<sup>1</sup> deletion by purifying T cell populations and performing Southern blot analysis. CD4cre-mediated deletion starts to peak in CD4<sup>+</sup> double-positive (DP) thymocytes, the major T cell subset in the thymus (14, 15). Consistent with this observation, most of the floxed dcr<sup>1</sup> alleles of CD4<sup>+</sup> thymocytes isolated from dcr<sup>fl/fl</sup>;CD4cre or dcr<sup>fl/fl</sup>;CD4cre mice had undergone Cre-mediated deletion (Fig. 1 A).

Deletion was essentially complete in peripheral CD4<sup>+</sup> cells freshly isolated from spleen and lymph nodes of dcr<sup>fl/fl</sup>;CD4cre mice (Fig. 1 B). As we previously showed using homozygous mutant ES cells, Cre-mediated excision of this conditional allele generates a Dicer-null mutation (10). Dicer protein expression was markedly reduced in purified CD4<sup>+</sup> peripheral T cells from dcr<sup>fl/fl</sup>;CD4cre mice (Fig. 1 B), and the residual Dicer protein probably derives from contaminating CD4<sup>−</sup> (non-T lineage) cells or CD4<sup>+</sup> cells with an intact floxed allele. In addition, Dicer protein may persist in some cells that have recently completed thymic development and exited to the periphery, even though deletion of dcr<sup>1</sup> has occurred.

A well-described function of Dicer is the processing of pre-miRNAs (~60-nucleotide-long hairpin RNAs) into mature ~21-nucleotide-long miRNAs (for review see reference 5). We analyzed dcr<sup>fl/fl</sup>;CD4cre T cells for the presence of four different miRNAs, miR-150, miR-21, miR-103, and miR-29, by Northern blot (Figs. 1 C, Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050678/DC1, and unpublished data).

Figure 1. Conditional gene targeting of mouse dcr<sup>−1</sup>. (A) Southern blot of BglII-digested genomic DNA isolated from the tail of a dcr<sup>fl/fl</sup>;CD4cre and dcr<sup>fl/fl</sup>;CD4cre mouse hybridized to radiolabeled 5′ probe; CD4<sup>+</sup>–enriched thymocytes from a dcr<sup>−1</sup> heterozygous mouse (fl/+;CD4cre) and a homozygous (fl/fl;CD4cre) littermate; CD4<sup>+</sup>–enriched peripheral T cells from a dcr<sup>fl/fl</sup>;CD4cre mouse freshly isolated (d0) or cultured for 5 d (d5) under nonpolarizing (THN), Th1 (TH1), or Th2 (TH2) conditions. (B) Western blot of whole-cell protein extracts derived from CD4<sup>+</sup>–enriched peripheral T cells (pooled from spleen and lymph nodes) of a heterozygous control (fl/+;CD4cre) and a homozygous (fl/fl;CD4cre) mouse. The Western blot was probed for Dicer protein using antiserum raised against a peptide of Dicer (top), and then stripped and reprobed for tubulin (bottom). (C) Northern analysis of miRNA expression in mouse T cells. Total RNA from WT (dcr<sup>fl/fl</sup>;CD4cre) and KO (dcr<sup>fl/fl</sup>;CD4cre) T cells were resolved on a denaturing polyacrylamide gel and transferred onto a nylon membrane. Samples include unfractionated thymocytes, CD4<sup>+</sup>–enriched naive peripheral T cells, and Th1 (T<sub>1</sub>, 1), and Th2 (T<sub>2</sub>, 2) conditions. (D) PhosphorImaging of a blot hybridized to a radiolabeled oligonucleotide complementary to mature miR-150. To control for equal RNA loading, a segment of the ethidium bromide–stained gel showing the transfer RNA (tRNA) bands is included (bottom panel). The arrows indicate processed mature miRNA, pre-miRNA, and tRNA. A radiolabeled Decade marker (M) consisting of 20, 30, 40, 50, 60, 70, 80, 90, 100, 150 nucleotide RNA size markers is included.

The mature form of miR-150 is present at a low level in thymocytes; its expression is increased in peripheral CD4<sup>+</sup> T cells but is down-regulated when the same T cells are differentiated under Th1 or Th2 conditions in vitro (Fig. 1 C). At each of these developmental stages, dcr<sup>fl/fl</sup>;CD4cre T cells contained much less mature miR-150 than did dcr<sup>fl/fl</sup>;CD4cre controls. Consistent with loss of Dicer function, pre-miR-150 clearly accumulated in naive CD4<sup>+</sup> and Th2 dcr<sup>fl/fl</sup>;CD4cre T cells (Fig. 1 C). Loss of mature miR-21 and increased amounts of pre-miR-21 were also observed in dcr<sup>fl/fl</sup>;CD4cre Th1 and Th2 cells (Fig. S1). Additionally, loss of mature miR-103 can be seen upon ablation of Dicer in T cells (Fig. S1), and a similar pattern was observed upon Northern analyses of miR-29 expression (unpublished data).

In contrast to miR-150 and miR-21, the precursors of miR-103 and miR-29 do not accumulate upon Dicer ablation. Because CD4cre-mediated deletion of dcr<sup>1</sup> begins in DP thymocytes, we asked whether Dicer was involved in subsequent stages of thymocyte development by examining the percentages of single-positive (SP) CD4<sup>+</sup> and CD8<sup>+</sup> thymocytes in dcr<sup>fl/fl</sup>;CD4cre mice. Mature miR-150 and, to a lesser extent, miR-103 were reduced in thymus of KO mice compared with control. This finding suggests that some, al-
CD4cre (left column) and 

were normal compared with 

controls (unpublished data). Furthermore, flow cytometric analyses showed that percentages of thymic subsets in 

were normal compared with littermate controls (Fig. 2 A and Fig. S2). This finding suggests that Dicer may be required for the maturation and/or maintenance of peripheral T cells.

To address this possibility, we analyzed the expression of a yellow fluorescent protein (YFP)-based Cre reporter allele in 

and in 

mice compared with controls (Fig. 2 A and Fig. S2). This reduction was reflected by an overall decrease of 

peripheral T cells (Fig. 2 B). In contrast, generation of 

mature B cells was unaffected in the mutants, as expected (Fig. 2 B and unpublished data). This finding suggests that Dicer may be required for the maturation and/or maintenance of peripheral T cells.

This finding suggests that Dicer deficiency is also disadvantageous in peripheral CD4+ T cells and may account for their approximately twofold reduction in spleen, lymph nodes, and blood. Compared with the other subsets, peripheral CD8+ T cells showed the greatest reduction of YFP+ cells in the CD8 SP fraction of 

mice but not in controls (Fig. 3 D and Fig. S3), indicating that in the CD8+ T cell lineage there is a strong counterselection of cells that have expressed Cre. Taken together with the overall reduction in 

and blood, these data support the notion that Dicer is required for optimal maturation and homeostasis of peripheral T lymphocytes, particularly those of CD8+ cytotoxic T cell lineage.

Defective helper T cell proliferation and survival

We isolated Dicer-deficient CD4+ T lymphocytes to assess their ability to proliferate and differentiate in response to antigen and cytokine signals. Naive CD4+ T cells can differentiate into two effector helper T cell subsets that are defined by the cytokines that they produce. Th1 cells are typically defined as...
Th cells that produce IFNγ but not IL-4, whereas Th2 cells are defined as Th cells that produce IL-4 but not IFNγ (17). The genes encoding these cytokines are reciprocally regulated during differentiation, being remodeled at the chromatin level for robust transcription in the appropriate lineage but undergoing silencing in the inappropriate lineage (18). Proper regulation of Th1/Th2 lineage decisions and cytokine gene activation and silencing are crucial for effective immune function and prevention of autoimmunity or allergy (19).

T cells proliferate during Th1 and Th2 differentiation in vitro, resulting in expansion of cell numbers by a factor of as much as 30 in 4 d (Fig. 4 A). Dicer-deficient T cells expanded only four- to sixfold when cultured under the same conditions (Fig. 4 A). To determine whether this reduction in viable cell recovery was the result of increased cell death, decreased proliferation, or both, we stained cells with Annexin V and propidium iodide (PI) to assess apoptosis (Annexin V+/PI−) and cell death (Annexin V+/PI+) at days 1 and 2 of culture. This analysis revealed increased percentages of both apoptotic and dead cells in the Dicer-deficient versus control cultures (Fig. 4 B). In addition, analysis of cell division by dilution of the intracellular dye carboxyfluorescein diacetate succinimidyl ester (CFSE) revealed that Dicer-deficient T cells proliferated more slowly than control cells (Fig. 4 C and Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20050678/DC1). Control T cells began dividing between 24 and 44 h of stimulation, but a large majority of Dicer-deficient T cells had not yet divided at 44 h. By 69 h, most Dicer-deficient T cells had divided. However, they continued to proliferate more slowly than control cells, even after 87 h in culture (Fig. 4 C and Fig. S4, A and B). Similar reductions in proliferation and viability were also observed when T cells were cultured under Th1 or Th2 conditions (Fig. S4, C and D). Poor cell growth was also observed when Dicer was absent in Schizosaccharomyces pombe (20), the chicken DT40 B cell line (21), and mouse ES cells (10). In Drosophila cells, both proliferation and survival can be regulated by the miRNA bantam (22).

Upon stimulation, naive CD4+ T cells produce IL-2, a key regulator of T cell growth and survival. We considered the possibility that the defective expansion of Dicer-deficient T cells was caused by an inability to produce IL-2. However, secreted IL-2 levels in Dicer-deficient and control cultures were comparable after primary stimulation (Fig. 4 D), and addition of exogenous IL-2 to the growth media did not rescue the proliferation defect of Dicer-deficient T cells (Fig.
experiments. (D) ELISA analysis of secreted IL-2 in culture supernatants of CFSE in daughter cells. Data are representative of three independent experiments described in (A). Cell divisions can be visualized by serial twofold dilution panels) mice are indicated within the quadrants. (C) Fluorescence profile of AnnexinV staining of apoptotic T cells after culture for the indicated times in ThN conditions as described in (A). Percentages of apoptotic (AnnexinV+ PI-) and dead (AnnexinV+ PI+) cells in control (CTRL, left) versus KO (right panels) mice are indicated within the quadrants. (D) Fluorescence profile of control (CTRL, black histogram) or KO (gray histogram) T cells labeled with CFSE at day 0 and cultured for the indicated times in ThN conditions as described in (A). Cell divisions can be visualized by serial twofold dilution of CFSE in daughter cells. Data are representative of three independent experiments. (D) ELISA analysis of secreted IL-2 in culture supernatants of dcr−/−CD4cre (open squares) versus dcr+/−CD4cre (gray squares) T cells stimulated with plate-bound anti-CD3 and anti-CD28 for the indicated times. Averages of triplicate cultures ± SD are shown.

Figure 4. Decreased proliferation and increased apoptosis of Dicer-deficient T cells. (A) Expansion of T cell populations expressed as fold increase from seeding number at day 0 to live cell number at day 4 of culture in Th1 (T1) or Th2 (T2) conditions. Purified spleen and lymph node CD4+ T cells from control (CTRL, dcr+/−, dcr+/−CD4cre or dcr+/−;CD4cre) or KO (dcr−/−CD4cre) mice were stimulated with plate-bound anti-CD3 and anti-CD28 for 2 d and then cultured in IL-2-containing medium for another 2 d (ThN condition), Th1 cultures also contain IL-12 and anti-IL-4 blocking antibody. Th2 cultures contain exogenous IL-4, anti-IL-12, and anti-IFN-γ. Averages of three independent experiments ± SD are shown. (B) AnnexinV+PI− cells were cultured under polarizing Th1 conditions for 5 d, the percentage of IFN-γ–producing cells was similar in Dicer-deficient and control cultures (Fig. 5 A). However, at early time points a difference was evident: after only 54 h, 78% of Dicer-deficient cells attained the ability to express high levels of IFN-γ, compared with 59% of control T cells (Fig. 5 C). In nonpolarizing conditions, both the proportion of cells expressing IFN-γ and the amount of IFN-γ detected per cell were dramatically increased in Dicer-deficient cells (Fig. 5 C).

Initially, differentiated Th1 cells retain the ability to express IL-2 upon restimulation (Fig. 5 D). However, although IFN-γ expression is reinforced by repeated rounds of stimulation, the ability to produce IL-2 declines progressively (23). This loss of IL-2 expression upon restimulation was also accelerated in the absence of Dicer. The majority of Dicer-deficient T cells had lost the ability to produce IL-2 within 54 h of culture in Th1 conditions (Fig. 5 D). Both increased IFN-γ and decreased IL-2 production occurred independently of the number of cell divisions, indicating that Dicer separately regulates T cell growth and differentiation (Fig. 4, C and D). When percentages of cytokine-expressing cells are plotted as a function of the number of cell divisions after restimulation, the numbers of IFN-γ+ cells are consistently higher (whereas the numbers of IL-2+ cells are lower) in Dicer-deficient cell cultures compared with control T cell cultures (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20050678/DC1).
Furthermore, increased secretion of IFNγ could be detected by ELISA as early as 24 h after the first stimulation of Dicer-deficient CD4+ T cells (Fig. 5 E), before the onset of cell division. These data indicate that Dicer-deficient CD4+ T cells are compromised in their ability to repress IFNγ production and are predisposed to become Th1 cells. Even after two consecutive rounds of activation under Th2 conditions, 18% of Dicer-deficient T cells produced IFNγ but not IL-4, compared with 0.8% in the control culture (Fig. 6).

In addition, 15% of Dicer-deficient cells expressed both IFNγ and IL-4, compared with 3% in the control culture. The failure of Dicer-deficient Th cells to silence IFNγ production under Th2 growth conditions led us to test whether the Th2 cultures could be induced to express IFNγ by restimulating them and further culturing under Th1 growth conditions for 5 more days. In the control culture, this switch yielded only 5% IFNγ+IL-4− Th1-like cells, indicating that most cells had already committed to the Th2 lineage. Indeed, 39% of the control cells remained IFNγ+IL-4− Th2 cells, whereas only 6% were induced to coexpress IFNγ and IL-4 when switched to Th1 conditions. The Dicer-deficient Th2 culture behaved very differently: after 5 d in Th1 growth conditions, ~87% of the remaining cells were IFNγ+IL-4− Th1-like cells, and nearly all of the IL-4−
cells also expressed IFNγ. These data reinforce the notion that Dicer is required to repress the Th1 genetic program and raise the possibility that Dicer deficiency may also impair stable commitment to the Th2 lineage. Alternatively, lack of Dicer may render terminal Th2 differentiation so inefficient that a residual pool of uncommitted cells remains after 5 d of culture in Th2 conditions, and these cells expanded preferentially as Th1 cells when switched to Th1 growth conditions.

DISCUSSION

The data presented here demonstrate that Dicer and the endogenous RNAi machinery regulate diverse aspects of T cell biology, including basic cellular processes such as proliferation and survival as well as cell lineage decisions and cytokine production during helper T cell differentiation. Although Cre-mediated inactivation of dicer was essentially complete, and very little Dicer protein was detected, residual and presumably functional mature miRNAs were still found in peripheral CD4+ T cells from dcr-1/CD4cre mice, with an even higher level being detected in the thymus. Thus, the half-life of mature miRNAs in developing T cells seems to exceed that of Dicer protein, and the decrease in peripheral T cell numbers in dcr-1/CD4cre mice occurs in the context of reduced, but not abolished, miRNA function. Complete deficiency of RNAi pathway in these cells may well produce a more pronounced or even distinct phenotype.

Expression profiling of miRNAs coupled with target prediction and validation may uncover specific roles for particular miRNAs in T cell differentiation and effector functions. A wide range of potential miRNA targets can be envisioned, among them cytokines, transcription factors, cell surface receptors, and signaling proteins. However, an attractive possibility is that miRNAs target specific key mRNAs, such as those encoding the cytokine IFNγ itself or the transcription factor T-bet. T-bet is a major regulator of Th1 differentiation and Ifnγ gene expression and is itself induced by IFNγ receptor signaling (17). Even minor perturbation of this positive feedback loop could account for the substantial Th1-skewing of Dicer-deficient T cell differentiation.

Th1 and Th2 lineage decisions are also subject to negative feedback control. For example, introduction of GATA3 into Th1 cells is sufficient to inhibit the expression of IFNγ and STAT4, a key transcription factor in Th1 differentiation (24, 25). It is possible that the expression or activity of proteins that would usually restrain Th1 differentiation may be compromised in Dicer-deficient T cells. Abundance of Gata3 transcripts is reduced by ∼30% in Dicer-deficient Th2 cultures and could contribute to the failure of these cells to repress IFNγ expression.

In addition to cytokine and transcription factor feedback loops, helper T cell differentiation and Th1/Th2 lineage commitment are controlled by epigenetic mechanisms of gene activation and silencing (26, 27). Dicer may participate in either or both of these pathways. Dicer-generated siRNAs direct the silencing of genes during helper T cell lineage commitment. Although dcr-1/CD4cre mice, with the neomycin-resistance cassette (neo) is flanked by Flp recognition target sites and can be recombined by Flp recombinase, a founder dcr-1/CD4cre chimera mouse was bred to a Flp deleter transgenic mouse (53). Genotypes were confirmed by Southern blotting as described (10). Routinely, mice were genotyped using PCR assays of DNA from tail biopsies. Sequences of primers are available upon request.

To generate T cell–specific KO mice, CD4cre transgenic mice (14) were bred to R26R-YFP mice (16), mice used for experiments in this study were of the C57BL/6 background and were analyzed between 4 and 8 wk of age. This mouse colony was managed and archived with the help of Mausoleum v3.1, a Java application written by H.E. Stöffler. Mice were cared for in accordance with institutional guidelines.

| MATERIALS AND METHODS |
|------------------------|
| Conditional gene targeting. Gene targeting of dicer was performed by homologous recombination in Bruce-4 ES cells derived from C57BL/6 mice as previously described (10). A heterozygous dcr+/Es cell clone was injected into blastocysts to derive chimeric mice. The neomycin-resistance cassette (neo) is flanked by Flp recognition target sites and can be removed from the conditional dicer allele by Flp recombinase. Thus, to generate dcr−/+ offspring, a founder dcr−/+ chimera mouse was bred to a Flp deleter transgenic mouse (33). Genotypes were confirmed by Southern blotting as described (10). Routinely, mice were genotyped using PCR assays of DNA from tail biopsies. Sequences of primers are available upon request. To generate T cell–specific KO mice, CD4cre transgenic mice (14) were bred to dcr−/+ mice and progeny were intercrossed. Then, R26R-YFP mice (16) were bred to dcr−/+;CD4cre mice. Mice used for analyses only harbor one allele of R26R-YFP and one allele of CD4cre. All the mice used for experiments in this study were of the C57BL/6 background and were analyzed between 4 and 8 wk of age. This mouse colony was managed and archived with the help of Mausoleum v3.1, a Java application written by H.E. Stöffler. Mice were cared for in accordance with institutional guidelines.

RNA analyses. Northern analyses were performed as previously described (10). Quantitative transcript analysis by 5′ nucleic acid fluorescence real-time RT-PCR was performed as described (27).
Western analysis. Lysates from T cells were prepared by lysis in NETN buffer (containing protease inhibitors), and 10 μg of lysate were loaded per lane. Western blot analysis of Dicer was performed as previously described (10).

Th1/Th2 differentiation and FACS analyses. Purification of CD4+ T cells from spleen and lymph nodes, induction of Th1/Th2 differentiation, labeling with CFSE, and restimulation for flow cytometric analysis of intracellular cytokine staining were performed as described previously (27). Before each in vitro differentiation experiment, the starting population of CD4+ T cells was analyzed by FACS to confirm the expected proportion of cells expressing the CD69hi naive T cell phenotype. For CFSE experiments, unstimulated cells were analyzed to determine the fluorescence intensity of undivided cells. The Vybrant apoptosis assay kit #2 (Molecular Probes, Inc.) was used to mark apoptosis. Bound IL-2 was detected using biotin anti–IL-2 (JES6-5H4; BD Biosystems) in PBS. The plates were coated with anti–IL-2 (JES6-1A12; BD Biosystems) in PBS. Immulon4 microtiter plates (Thermo Fisher Scientific, Inc.) were coated with biotin anti–CD3 and biotin anti–CD28 (145.2C11; BD Biosystems). For measurement of IL-2, supernatants diluted in blocking buffer were incubated in the coated wells for 4 h at 37°C. Supernatants were collected and subjected to ELISA. Bound IL-2 was detected using streptavidin-horseradish peroxidase conjugate (Zymed Laboratories) were coated with anti–IL-2 (JES6-1A12; BD Biosystems) in PBS. T cell populations from thymus, spleen, and lymph nodes were analyzed by flow cytometry using monoclonal antibodies directly coupled to fluorochromes: CD4-FITC, CD8α-CyChrome, CD25-CyChrome, and CD3ε-PE antibodies (BD Biosciences). FACS data were analyzed using FlowJo software (Tree Star).

IL-2 ELISA. 105 freshly isolated CD4+ T cells were activated in 200 μl medium with hamster anti-CD3 (145.2C11) and hamster anti-CD28 (37.51; BD Biosciences) in 96-well tissue culture plates (Costar) coated with goat-antihamster IgG (ICN Biomedicals, Inc.). For measurement of IL-2, supernatants were collected and subjected to ELISA. Immulon4 microtiter plates (Thermo Labsystems) were coated with anti–IL-2 (JES6-1A12; BD Biosystems) in PBS. After washing with PBS-T (0.01% Tween-20), plates were incubated with blocking buffer (PBS + 2% bovine serum albumin). Stimulated T cell supernatants diluted in blocking buffer were incubated in the coated wells for 4 h at 37°C. Recombinant IL-2 (BD Biosystems) was used as a standard for quantitation. Bound IL-2 was detected using biotin anti–IL-2 (JES6-5F4; BD Biosystems) followed by streptavidin-horseradish peroxidase conjugate (Zymed Laboratories). Chromogenic development with TMB substrate (DakoCytochemicals) was terminated by addition of sulfuric acid to 0.5 N. Optical density at 450 nm was measured on a Spectramax 96-well spectrophotometer ( Molecular Devices Corporation). Standard curves and IL-2 concentrations were calculated using Softmax Pro software ( Molecular Devices Corporation).

Online supplemental material. Fig. S1 shows that pre-miR-21 accumulates in Dicer-deficient T cells and mature miR-21 is absent. Additionally, mature miR103–105

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