

# 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<sup>+</sup> T cell development was observed upon *dcr-1* deletion in the thymus. However, Dicer-deficient CD4<sup>+</sup> 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- $\gamma$ , the hallmark effector cytokine of the Th1 lineage.**

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Abbreviations used: CD4cre, Cre transgene under the control of the *cd4* enhancer/promoter/silencer; CFSE, carboxyfluorescein diacetate succinimidyl ester; *dcr-1*, *dicer-1*; DN, double-negative; DP, double-positive; ES, embryonic stem; floxed, *loxP*-flanked; miRNA, microRNA; PI, propidium iodide; pre-miRNA, precursor microRNA; RNAi, RNA interference; siRNA, short interfering RNA; SP, single positive; YFP, yellow fluorescent protein.

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/zwillie 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

S.A. Muljo, K.M. Ansel, and C. Kanellopoulou contributed equally to this work.

The online version of this article contains supplemental material.

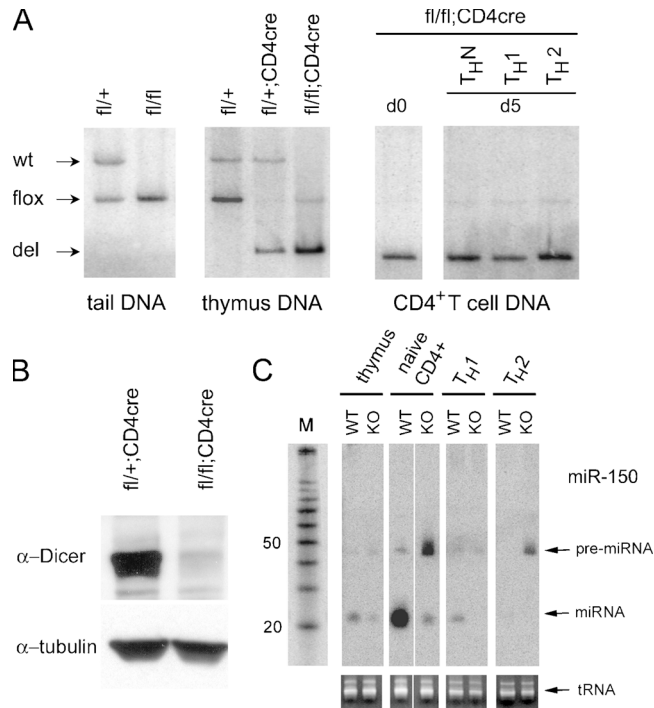
were obtained from heterozygous parents at the expected frequency (Table S1, available at <http://www.jem.org/cgi/content/full/jem.20050678/DC1>), and *dcr<sup>fl/fl</sup>* cells that do not express Cre did not exhibit any of the defects described for Dicer-deficient cells (reference 10 and unpublished 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-1* in mice using a Cre transgene under the control of the *cd4* enhancer/promoter/silencer (CD4cre). We determined the efficiency of *dcr-1* deletion by purifying T cell populations and performing Southern blot analysis. CD4cre-mediated deletion starts to peak in CD4<sup>+</sup>8<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-1* alleles of CD4<sup>+</sup> thymocytes isolated from *dcr<sup>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, as well as in differentiated T cells expanded for 5 d in culture (Fig. 1 A). 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). 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-1* 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).

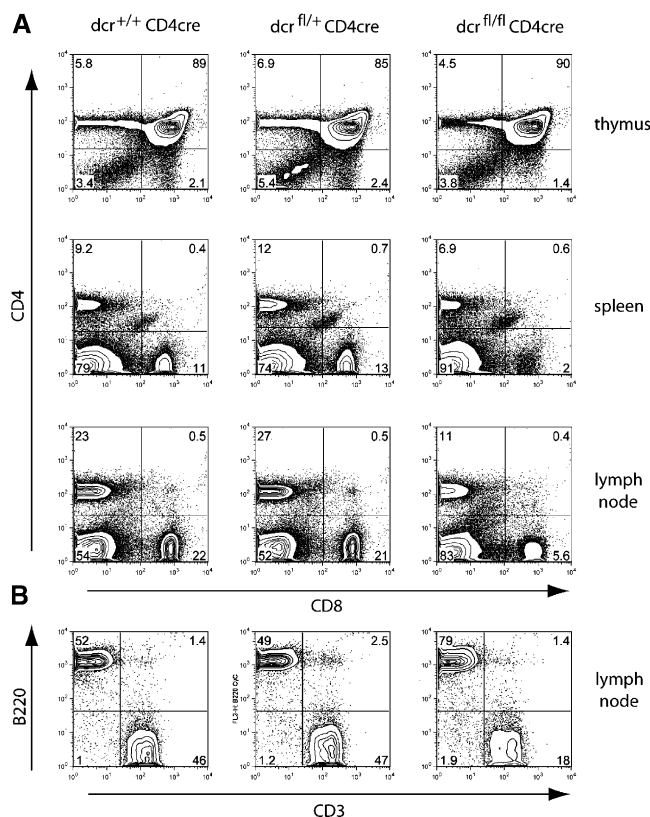
The mature form of *miR-150* is present at a low level in thymocytes; its expression is increased in peripheral CD4<sup>+</sup> T cells but is down-regulated when the same T cells are differentiated under Th1 or Th2 conditions in vitro (Fig. 1 C). At each of these developmental stages, *dcr<sup>fl/fl</sup>;CD4cre* T cells contained much less mature miR-150 than did *dcr<sup>fl/+</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).



**Figure 1. Conditional gene targeting of mouse *dcr-1*.** (A) Southern blot of *BglI*-digested genomic DNA isolated from the tail of a *dcr<sup>fl/+</sup>;CD4cre* and *dcr<sup>fl/fl</sup>;CD4cre* mouse hybridized to radiolabeled 5' probe; CD4<sup>+</sup>-enriched thymocytes from a *dcr-1* 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 (T<sub>H</sub>N), Th1 (T<sub>H</sub>1), or Th2 (T<sub>H</sub>2) 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/+</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>H</sub>1) and Th2 (T<sub>H</sub>2) cultures (d 5). Shown is the PhosphorImage 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.

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-1* 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-



**Figure 2. Reduction of the mature T cell compartment in the absence of Dicer.** Flow cytometric analysis of lymphocytes from *dcr<sup>fl/+</sup>*; CD4cre (left column) and *dcr<sup>fl/fl</sup>*; CD4cre littermates (right column). (A) Contour plots depict CD4 (y-axis) versus CD8 (x-axis) staining profiles in thymus (top panels), spleen (middle panels), and lymph nodes (bottom panels). Percentages of double-negative (DN), DP, and SP cells are indicated in the corner of each quadrant. When the data are available, the average  $\pm$  SD is shown for certain subsets ( $n = 4$ , except for lymph nodes, where  $n = 3$ ). (B) Contour plots depict B220 (y-axis) versus CD3 (x-axis) staining profiles in lymph nodes. Percentages of cells in each quadrant are indicated. Data are representative of three independent experiments.

beit incomplete, loss of Dicer activity occurs in the thymus (Fig. 1 C). However, total numbers of thymocytes in *dcr<sup>fl/+</sup>*; CD4cre mice were normal compared with littermate controls (unpublished data). Furthermore, flow cytometric analyses showed that percentages of thymic subsets in *dcr<sup>fl/+</sup>*; CD4cre mice were normal compared with littermate controls (Fig. 2 A and Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20050678/DC1>).

Because CD4cre-mediated deletion is not efficient before the DP stage of thymocyte development (Fig. 3 C), and mature miRNAs persist after Dicer ablation (Figs. 1 C and Fig. S1 B), the function of Dicer in T cells before this stage cannot be addressed.

Mature SP thymocytes exit the thymus as naive CD4<sup>+</sup> helper T lymphocytes or CD8<sup>+</sup> cytotoxic T lymphocytes. Flow cytometric analyses of lymphocytes in spleen and lymph nodes showed a marked reduction of the percentage

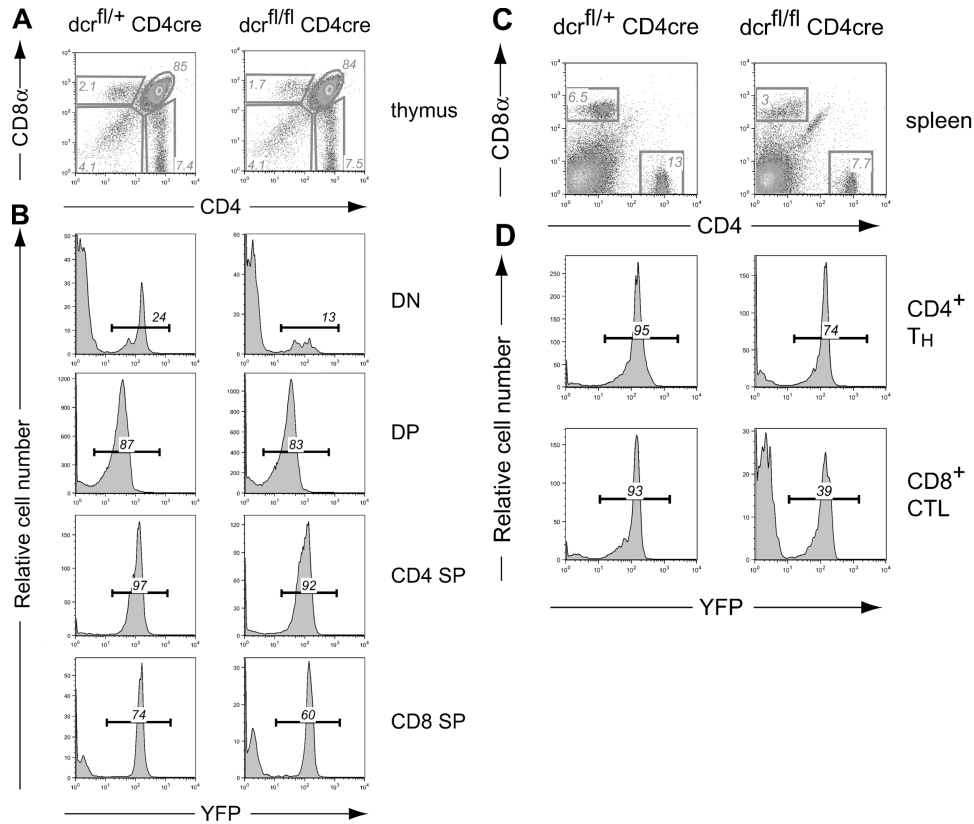
of CD8<sup>+</sup> T cells (fourfold reduction on average in spleen,  $P < 0.01$ ) and a smaller reduction in CD4<sup>+</sup> T cells (twofold reduction on average in spleen,  $P < 0.01$ ) in *dcr<sup>fl/fl</sup>*; CD4cre mice compared with littermate controls (Fig. 2 A and Fig. S2). This reduction was reflected by an overall decrease of CD3<sup>+</sup> peripheral T cells (Fig. 2 B). In contrast, generation of B220<sup>+</sup> 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.

To address this possibility, we analyzed the expression of a yellow fluorescent protein (YFP)-based Cre reporter allele in *dcr<sup>fl/+</sup>*; CD4cre mice and in *dcr<sup>fl/+</sup>*; CD4cre littermate controls. The R26R-YFP reporter allele consists of a YFP cDNA preceded by a floxed transcriptional stop cassette inserted into the ubiquitously expressed *rosa26* locus (16). Before Cre expression, YFP is not expressed; after Cre is expressed in a cell, the transcriptional stop cassette is excised, and YFP is expressed in that cell (16). The percentage of cells expressing YFP correlates with the percentage of cells that have expressed Cre. It is not known whether YFP expression in this system correlates with deletion of the floxed *dcr-1* gene and reduction in mature miRNA expression. This analysis showed that the extent of Cre-mediated deletion (i.e., the percentage of YFP<sup>+</sup> cells) is comparable in all subsets in *dcr<sup>fl/fl</sup>*; CD4cre; R26R-YFP thymus compared with control (Fig. 3 C). The modest reduction in the proportion of YFP<sup>+</sup> cells in the CD8 SP fraction of *dcr<sup>fl/fl</sup>*; CD4cre; R26R-YFP thymus compared with control suggests that reduced Dicer expression impairs production and/or survival of CD8 SP thymocytes (Fig. 3 C). Similarly, the proportion of YFP<sup>+</sup> CD4<sup>+</sup> T cells in the periphery is reduced in *dcr<sup>fl/fl</sup>*; CD4cre; R26R-YFP mice compared with controls (Fig. 3 D and Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20050678/DC1>).

This finding suggests that Dicer deficiency is also disadvantageous in peripheral CD4<sup>+</sup> T cells and may account for their approximately twofold reduction in spleen, lymph nodes, and blood. Compared with the other subsets, peripheral CD8<sup>+</sup> T cells showed the greatest reduction of YFP<sup>+</sup> cells in *dcr<sup>fl/fl</sup>*; CD4cre; R26R-YFP mice but not in controls (Fig. 3 D and Fig. S3), indicating that in the CD8<sup>+</sup> T cell lineage there is a strong counterselection of cells that have expressed Cre. Taken together with the overall reduction in CD4<sup>+</sup> and CD8<sup>+</sup> T cell numbers in spleen, lymph nodes, and blood, these data support the notion that Dicer is required for optimal maturation and homeostasis of peripheral T lymphocytes, particularly those of CD8<sup>+</sup> cytotoxic T cell lineage.

### Defective helper T cell proliferation and survival

We isolated Dicer-deficient CD4<sup>+</sup> T lymphocytes to assess their ability to proliferate and differentiate in response to antigen and cytokine signals. Naive CD4<sup>+</sup> 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



**Figure 3. Counterselection of Dicer-deficient T cells.** Flow cytometric analysis of lymphocytes from  $dcr^{fl/+}$ ;CD4cre;R26R-YFP (left), and  $dcr^{fl/fl}$ ;CD4cre;R26R-YFP littermates (right). Dot plots depict CD8 (y-axis) versus CD4 (x-axis) staining profiles in (A) thymus and (B) spleen. Percentages of

DN, DP, and SP cells are indicated beside each gate. Histograms depict relative cell number (y-axis) versus Cre-induced YFP fluorescence (x-axis) within the DN, DP, and SP thymocyte fractions (C), or SP splenocyte fractions (D). Percentages of YFP-expressing cells are indicated.

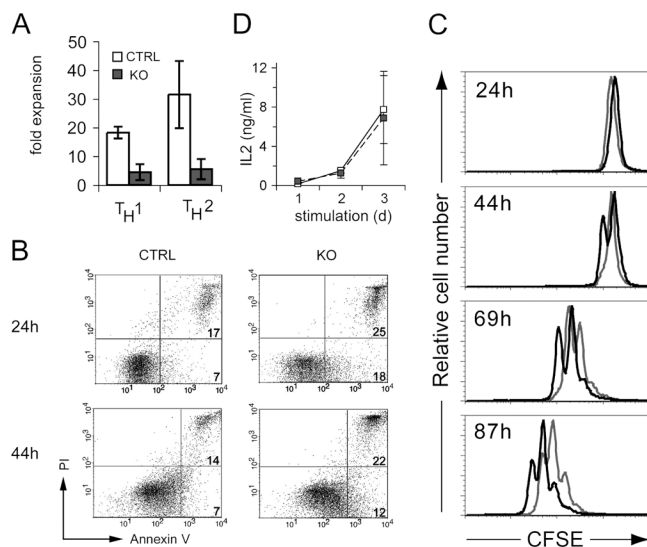
Th cells that produce IFN $\gamma$  but not IL-4, whereas Th2 cells are defined as Th cells that produce IL-4 but not IFN $\gamma$  (17). The genes encoding these cytokines are reciprocally regulated during differentiation, being remodeled at the chromatin level for robust transcription in the appropriate 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 AnnexinV and propidium iodide (PI) to assess apoptosis (AnnexinV<sup>+</sup> PI<sup>-</sup>) and cell death (AnnexinV<sup>+</sup> PI<sup>+</sup>) 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-defi-

cient 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<sup>+</sup> 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.





**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 ( $T_{H1}$ ) or Th2 ( $T_{H2}$ ) conditions. Purified spleen and lymph node  $CD4^+$  T cells from control (CTRL,  $dcr^{fl/+}$ ,  $dcr^{fl/+};CD4cre$  or  $dcr^{+/+};CD4cre$ ) or KO ( $dcr^{fl/fl};CD4cre$ ) 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 $\gamma$ . Averages of three independent experiments  $\pm$  SD are shown. (B) AnnexinV staining of apoptotic T cells after culture for the indicated times in ThN conditions as described in (A). Percentages of apoptotic (AnnexinV<sup>+</sup> PI<sup>-</sup>) and dead (AnnexinV<sup>+</sup> PI<sup>+</sup>) cells in control (CTRL, left) versus KO (right panels) mice are indicated within the quadrants. (C) Fluorescence profile of control (CTRL, black histogram) or KO (gray histogram) T cells labeled with CFSE at d 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  $dcr^{fl/+};CD4cre$  (open squares) versus  $dcr^{fl/fl};CD4cre$  (gray squares) T cells stimulated with plate-bound anti-CD3 and anti-CD28 for the indicated times. Averages of triplicate cultures  $\pm$  SD are shown.

4 C and unpublished data). It remains a possibility that Dicer-deficient T cells failed to respond normally to IL-2 because of some perturbation of IL-2 receptor signaling.

### Dysregulated helper T cell differentiation and cytokine gene regulation

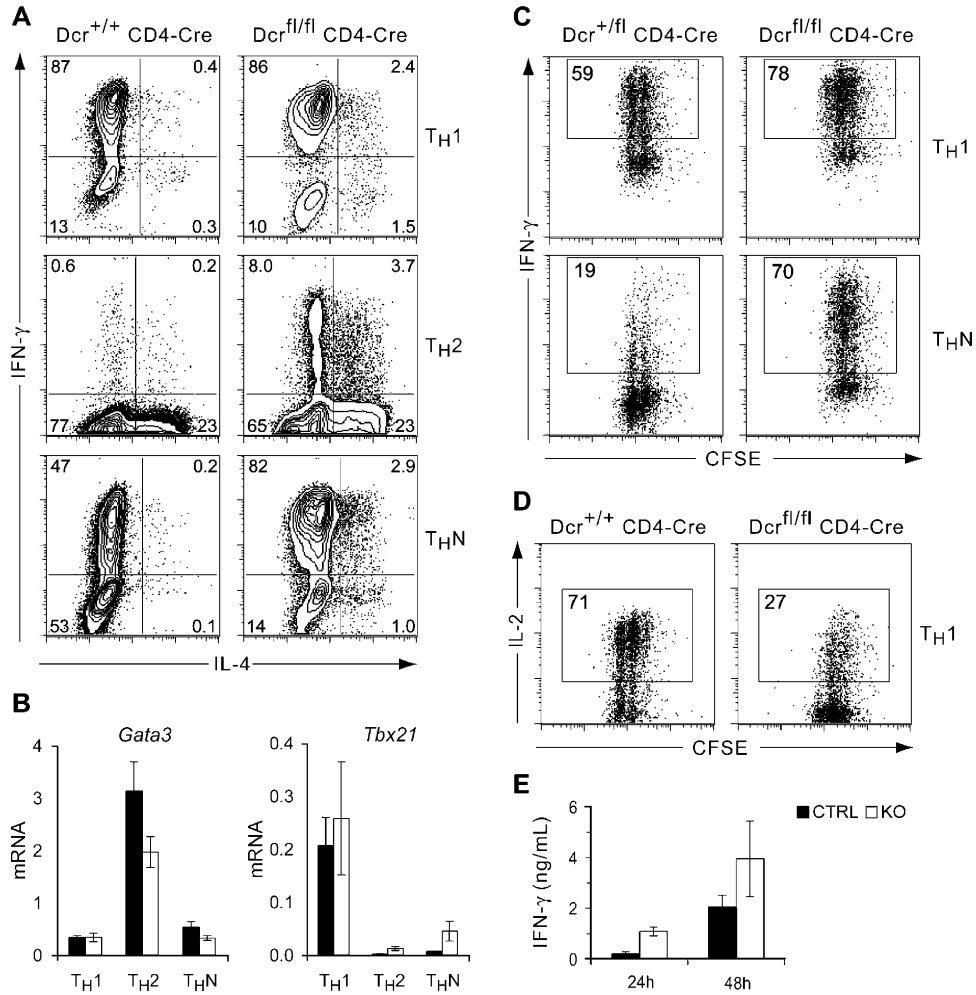
Dicer-deficient  $CD4^+$  T cells cultured under Th1 conditions appropriately produced large amounts of IFN $\gamma$  upon restimulation (Fig. 5 A). Consistent with robust Th1 differentiation, these cells also up-regulated expression of the Th1 master transcription factor T-bet (encoded by *Tbx21*) at the transcriptional level (Fig. 5 B). Culture of Dicer-deficient T cells in Th2 conditions induced differentiation of IL-4-producing cells (Fig. 5 A) and up-regulation of *Gata3*, a key regulator of Th2 differentiation (Fig. 5 B). Thus, Dicer is not

required for differentiation of the Th1 and Th2 lineages. However, the abundance of *Gata3* mRNA was  $\sim$ 30% lower in Dicer-deficient Th2 cultures than in controls (Fig. 5 B,  $P < 0.05$ ), and inefficient differentiation of IL-4-producing Th2 cells was observed in some cases (unpublished data). Most strikingly, however, repression of IFN $\gamma$  expression was markedly impaired in Dicer-deficient Th2 cultures, even when the proportion of IL-4-producing cells was equal to control cells (Fig. 5 A). Dicer-deficient Th2 cultures contained substantially increased proportions of both IFN $\gamma^+$ IL-4<sup>-</sup> Th1-like cells and cells expressing both IFN $\gamma$  and IL-4.

IFN $\gamma$  expression was also increased in Dicer-deficient cells cultured in the absence of exogenous cytokines and blocking antibodies (nonpolarizing conditions; ThN) (Fig. 5 A). This increased IFN $\gamma$  expression was accompanied by up-regulation of *Tbx21* transcripts, indicating increased differentiation of Th1 cells in Dicer-deficient ThN cultures (Fig. 5 B). Increased IL-4 production was also occasionally seen in restimulated Dicer-deficient cells from ThN cultures (Fig. 5 A and unpublished data). Although this phenomenon was not observed consistently, we cannot rule out the possibility that IL-4 expression is also dysregulated and that this dysregulation is masked by the predominant Th1 phenotype of Dicer-deficient T cells.

Although the rate of cell division was decreased, differentiation-associated changes in cytokine production occurred more rapidly in the absence of Dicer. When  $CD4^+$  T cells were cultured under polarizing Th1 conditions for 5 d, the percentage of IFN $\gamma$ -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 $\gamma$ , compared with 59% of control T cells (Fig. 5 C). In nonpolarizing conditions, both the proportion of cells expressing IFN $\gamma$  and the amount of IFN $\gamma$  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 $\gamma$  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 $\gamma$  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 $\gamma^+$  cells are consistently higher (whereas the numbers of IL-2<sup>+</sup> 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>).



**Figure 5. Helper T cell differentiation in the absence of Dicer.** (A) Intracellular FACS analysis of IL-4 (x-axis) and IFN $\gamma$  (y-axis) expression by cells cultured under Th1 (top), Th2 (middle), or ThN (bottom panels) conditions for 5 d, and then restimulated with phorbol ester (phorbol 12-myristate 13-acetate) and ionomycin. Percentages of cells in each quadrant are indicated. Data are representative of three independent experiments. (B) In one experiment, the abundance of the real-time fluorescent RT-PCR reactions were normalized to *Hprt1* mRNA abundance. Indicated mRNAs (*Gata3*, *Tbx21*) were measured in cells cultured as in A but left unstimulated. Data are presented as the average relative abundance  $\pm$

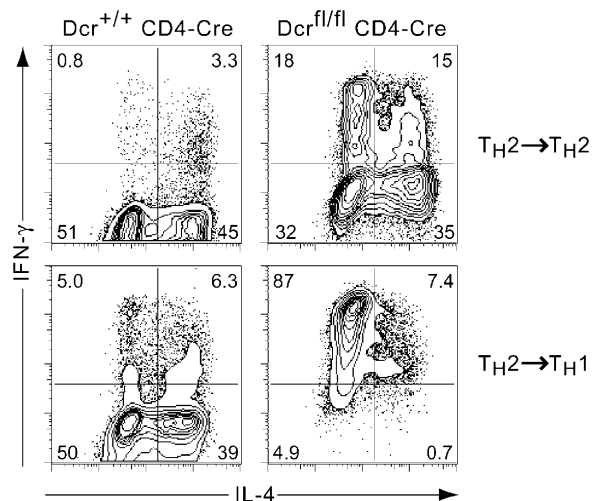
SD for triplicate reactions, and are representative of two independent experiments. (C and D) CD4<sup>+</sup> T cells were labeled with CFSE (x-axis), stimulated for 47 h under Th1 or ThN conditions, “rested” in IL-2-containing medium for 7 h, and restimulated for intracellular FACS analysis of IFN $\gamma$  (C) and IL-2 (D) expression (y-axis). The percentage of cytokine-expressing cells is indicated in each box. (E) ELISA analysis of secreted IFN $\gamma$  in culture supernatants of *dcr<sup>fl/fl</sup>*;CD4cre (CTRL, black columns) and *dcr<sup>fl/fl</sup>*;CD4cre (KO, open columns) T cells stimulated with plate-bound anti-CD3 and anti-CD28 for the indicated times. Averages of triplicate cultures  $\pm$  SD are shown.

Furthermore, increased secretion of IFN $\gamma$  could be detected by ELISA as early as 24 h after the first stimulation of Dicer-deficient CD4<sup>+</sup> T cells (Fig. 5 E), before the onset of cell division.

These data indicate that Dicer-deficient CD4<sup>+</sup> T cells are compromised in their ability to repress IFN $\gamma$  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 IFN $\gamma$  but not IL-4, compared with 0.8% in the control culture (Fig. 6).

In addition, 15% of Dicer-deficient cells expressed both IFN $\gamma$  and IL-4, compared with 3% in the control culture. The failure of Dicer-deficient Th cells to silence IFN $\gamma$  pro-

duction under Th2 growth conditions led us to test whether the Th2 cultures could be induced to express IFN $\gamma$  by re-stimulating them and further culturing under Th1 growth conditions for 5 more days. In the control culture, this switch yielded only 5% IFN $\gamma$ <sup>+</sup>IL-4<sup>-</sup> Th1-like cells, indicating that most cells had already committed to the Th2 lineage. Indeed, 39% of the control cells remained IFN $\gamma$ <sup>-</sup>IL-4<sup>+</sup> Th2 cells, whereas only 6% were induced to coexpress IFN $\gamma$  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 IFN $\gamma$ <sup>+</sup>IL-4<sup>-</sup> Th1-like cells, and nearly all of the IL-4<sup>+</sup>



**Figure 6. Defective IFN $\gamma$  repression in Dicer-deficient Th2 cultures.** CD4<sup>+</sup> T cells were cultured under Th2 conditions for 5 d, and the resulting cells were stimulated again with anti-CD3 and anti-CD28 and cultured for an additional 5 d under Th2 conditions (Th2 $\rightarrow$ Th2, top) or Th1 conditions (Th2 $\rightarrow$ Th1, bottom panels). IL-4 and IFN $\gamma$  expression was analyzed by intracellular FACS after restimulation with phorbol ester and ionomycin. The percentage of cytokine-expressing cells is indicated in each quadrant.

cells also expressed IFN $\gamma$ . 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 *dcr-1* was essentially complete, and very little Dicer protein remained, residual and presumably functional mature miRNAs were still found in peripheral CD4<sup>+</sup> T cells from *dcr<sup>fl/fl</sup>;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<sup>fl/fl</sup>;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 func-

tions. 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 IFN $\gamma$  itself or the transcription factor T-bet. T-bet is a major regulator of Th1 differentiation and *Ifn $\gamma$*  gene expression and is itself induced by IFN $\gamma$  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 IFN $\gamma$  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  $\sim$ 30% in Dicer-deficient Th2 cultures and could contribute to the failure of these cells to repress IFN $\gamma$  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 chromatin-based transcriptional silencing at centromeres and other loci in fission yeast (28–30), and recent reports have provided evidence that this mechanism may be conserved in mammalian cells (10, 21, 31, 32). The failure of Dicer-deficient Th2 cells to silence IFN $\gamma$  expression and the reversal of IFN $\gamma$  and IL-4 expression patterns in polarized Th2 cultures switched into Th1 conditions suggest that the RNAi pathway may also participate in the epigenetic silencing of relevant genes during helper T cell lineage commitment.

## MATERIALS AND METHODS

**Conditional gene targeting.** Gene targeting of *dcr-1* was performed by homologous recombination in Bruce-4 ES cells derived from C57BL/6 mice as previously described (10). A heterozygous *dcr<sup>neo/+</sup>* 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 *dcr-1* allele by Flp recombinase. Thus, to generate *dcr<sup>fl/+</sup>* offspring, a founder *dcr<sup>neo/+</sup>* chimeric 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<sup>fl/+</sup>* mice and progeny were intercrossed. Then, R26R-YFP mice (16) were bred to *dcr<sup>fl/+</sup>;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. Stöfler. Mice were cared for in accordance with institutional guidelines.

**RNA analyses.** Northern analyses were performed as previously described (10). Quantitative transcript analysis by 5' nuclease fluorogenic 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  $\mu$ g 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<sup>+</sup> 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<sup>+</sup> T cells was analyzed by FACS to confirm the expected proportion of cells expressing the CD62L<sup>hi</sup> 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.) using AnnexinV-Alexa488 and PI was used according to manufacturer's instructions. T cell populations from thymus, spleen, and lymph nodes were analyzed by flow cytometry using monoclonal antibodies directly coupled to fluorochromes: CD4-FITC, CD8 $\alpha$ -CyChrome, B220-CyChrome, and CD3 $\epsilon$ -PE antibodies (BD Biosciences). FACS data were analyzed using FlowJo software (Tree Star).

**IL-2 ELISA.** 10<sup>5</sup> freshly isolated CD4<sup>+</sup> T cells were activated in 200  $\mu$ l 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 anti-hamster 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 (JES6-5H4; BD Biosystems) followed by streptavidin-horseradish peroxidase conjugate (Zymed Laboratories). Chromogenic development with TMB<sup>+</sup> substrate (DakoCytomation) was terminated by addition of sulfuric acid to 0.5 N. Optical density at 450 $\lambda$  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 miR-103 is reduced upon ablation of Dicer in T cells. Fig. S2 summarizes in a bar graph the average percentages  $\pm$  SD of some T cell subsets in the thymus and spleen as in Fig. 2. Fig. S3 is an extension of Fig. 3 showing data from lymph nodes and blood. Fig. S4 is a graphical representation of the data in Fig. 4 C and includes additional data on proliferation and viability under different Th growth conditions. Fig. S5 is a graphical representation of the data in Fig. 5, C and D. Table S1 shows the Mendelian transmission of our floxed *dcr-1* allele for the first 70 pups obtained during the initial breedings. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20050678/DC1>.

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