Dicer controls CD8+ T-cell activation, migration, and survival

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The Rnasell enzyme Dicer is required for mature microRNA production. Although extensive investigation has been carried out to determine the role of Dicer/miRNAs in the immune system, their function in mature CD8+ T cells has not been examined. We deleted Dicer in mature polyclonal and TCR transgenic CD8+ T cells using either tat-cre or the distal lck promoter, which drives cre expression after the stage of positive selection. Following antigenic challenge by a pathogen infection in vivo, Dicer-deleted CD8+ T cells failed to accumulate at the usual peak of the response. Surprisingly however, we found that deletion of Dicer in mature CD8+ T cells allowed them to respond more rapidly than control cells to TCR stimuli in vitro. In response to anti-CD3 plus anti-CD28 stimulation, Dicer-deleted T cells up-regulated CD69 faster and entered the first mitosis earlier than control T cells. In addition, activated Dicer−/− cells failed to rapidly down-regulate CD69 when removed from the TCR stimulus. As a probable consequence of this sustained CD69 expression, Dicer−/− T cells showed defective migration out of the central lymphoid organs in vivo. We identify mir-130/301, which are dramatically up-regulated following T-cell activation, as able to down-regulate CD69 expression via binding to a conserved site in the 3′ UTR of CD69 mRNA. Thus, cellular functions dependent on Dicer expression are not required for the early steps in CD8+ T-cell activation, but are essential for their survival and accumulation.

MicroRNAs are small, noncoding RNAs around 22 nucleotides in length. They inhibit target mRNA translation or promote target mRNA degradation by directly binding to the 3′ UTR of the message (1, 2). There are around 700 miRNAs that have been identified in the human genome. Each miRNA regulates up to hundreds of genes and it is estimated that the expression of many of the genes in the genome is controlled by miRNAs (1, 2). Primary miRNAs are transcribed from DNA and processed by the RNaseIII complex containing Drosha/DGCR8 into premiRNAs. After export from the nucleus to the cytoplasm, premiRNAs are further processed into mature miRNAs by another RNaseIII complex containing Dicer (1, 2). It is well established that ablation of Dicer abolishes the production of all mature miRNAs (3–5). Therefore, Dicer deficiency provides a model to determine miRNA function.

In the adaptive immune system, extensive evidence supports a critical role of Dicer/miRNAs in T and B cell differentiation, proliferation, and survival (6–9). During B cell development, Dicer deficiency leads to a complete blockade at the pro-B to pre-B transition because of deregulation of the proapoptotic gene Bim (10). CD4+ T cells exhibit defective proliferation, survival, and cytokine production in the absence of Dicer (11). In regulatory T cells (Tregs), miRNAs are essential for Treg cell homeostasis and function (12–14). Lineage-specific deletion of Dicer in Treg cells by Foxp3-cre expression leads to severe autoimmunity, similar to what is observed in Foxp3-deficient animals. Furthermore, NK T-cell development in the thymus is dramatically perturbed in the absence of Dicer (15, 16).

In conditional Dicer knockout mice in which deletion is induced early in thymocyte differentiation either by cre driven by the proximal lck promoter or by the CD4 promoter/enhancer/silencer, the peripheral CD8+ T-cell compartment is greatly reduced (11, 17). This finding demonstrates that Dicer/miRNAs play critical roles during CD8+ T-cell development. However, it also prevents further investigation of the function of Dicer/miRNA in mature CD8+ T cells using animal models in which cre is turned on early in thymocyte development.

Upon acute infection, antigen-specific naive CD8+ T cells are primed by antigen presenting cells, expand more than 1,000-fold, and differentiate into effector CD8+ T cells within 7 to 8 d (9, 18–20). The function of Dicer/miRNAs in this highly regulated dynamic process has not been addressed. Interestingly, proliferating T cells express miRNAs with shorter 3′ UTRs compared with naive T cells (21). According to this, proliferating T cells may be less susceptible to miRNA control because of the loss of miRNA binding sites. Overall, the importance of Dicer/miRNAs in CD8+ effector T-cell development remains unknown.

To address this critical question without significantly perturbing CD8+ T-cell development in the thymus, we used two experimental systems to conditionally delete Dicer in mature CD8+ T cells: in vitro treatment with a tat-cre fusion protein, and the distal lck promoter to drive cre expression only after thymocyte-positive selection. Our results from both systems clearly demonstrate that Dicer is essential for the CD8+ effector T-cell response. Furthermore, Dicer/miRNAs also control CD8+ T-cell activation, proliferation, migration, and accumulation during acute infection.

**Results**

Dicer Is Essential for CD8+ Effector T-Cell Responses in Vivo. To examine the function of Dicer/miRNAs in the CD8+ T-cell response to foreign antigen without deleting Dicer during thymocyte differentiation, we treated naive CD8+ T cells from mice bearing floxed Dicer alleles and the Rosa-YFP reporter (22) with tat-cre fusion protein. The basic peptide derived from HIV-TAT significantly promotes the cellular uptake of cre recombinase (23). In this system, Dicer is deleted from peripheral mature CD8+ T cells, avoiding any complications from T-cell development defects caused by the absence of Dicer (11, 17). In short, OT-1 T cells specific for an epitope of ovalbumin were purified from Dicerfl/fl Rosa-YFPfl OT-1 (Dicer−/−) and Dicerfl/+ Rosa-YFPfl OT-1 (Dicer+/−) mice. The cells were incubated with tat-cre for 1 h in vitro followed by extensive washing. Treated cells were either left in culture for 2 d to assess the extent of recombination, or low numbers of treated cells were transferred into congenically marked recipient mice that were subsequently infected with Listeria monocytogenes ova (LM-ova). As shown in Fig. 1A, a significant percentage of tat-cre–treated OT-1 cells expressed YFP after 2 d in vitro culture with anti-CD3/CD28 or with IL-7.

In the recipient mice at 7 d postinfection with LM-ova, YFP+ OT-1 T cells were present at roughly their starting frequency among the control Dicer+/− donor population. However, in the mice that received Dicer−/− T cells, YFP+ CD8+ OT-1 T cells were absent although, in the same recipients, the YFP+ OT-1 cells...
characterized the defects in accumulation of Dicer−/−CD8+ T cells observed following infectious challenge in vivo, an in vitro experiment was performed. For this, Dicer−/− or Dicer+/+ CD8+ T cells were mixed at a 1:1 ratio with congenically marked B6 CD8+ T cells and subjected to real-time PCR analysis. The level of Dicer mRNA in sorted YFP+ Dicer−/− dLck-cre (Dicer−/−) CD8+ T cells was less than 3% of that in WT CD8+ T cells (Fig. 2C). Interestingly, even YFP+ CD8+ T cells from Dicer−/− mice expressed a significantly lower level of Dicer mRNA than control CD8+ T cells (Fig. 2C), suggesting that the Dicer locus is more accessible to the action of cre than the Rosa-YFP locus. Dicer−/− mice contain an apparently normal CD4+ T-cell population and a slight decrease in the size of the CD8+ T-cell compartment (Fig. 2B). Phenotypically, the peripheral CD8+ T cells in Dicer−/− and control mice were similar for CD44 and CD62L expression (Fig. S2). Reduced CD8+ Response in Dicer−/− Mice. Consistent with the results from the tat-cre experiments with OT-1 cells (Fig. 1), a dramatically reduced CD8+ effector T-cell response was observed in Dicer−/− mice 7 d following infection with LM-ova. Both tetramer staining (Fig. 3A) and intracellular cytokine staining (Fig. 3B) revealed a 10- to 20-fold reduction in the CD8+ T-cell response compared with the response in Dicer+/+ Rosa-YFP dLck-cre (Dicer+/+) control mice. In addition, among the tetramer-positive cells, there was a decreased frequency of YFP+ cells, suggesting that in these mice, CD8+ T cells that retain Dicer have a selective advantage in expansion following immunization (Fig. 3A). Even in the tetramer-negative population, an increased percentage of CD8+ T cells were YFP+ compared with control immunized or naive mice (Figs. 2B and 3A). These results suggest that CD8+ effector T-cell expansion is defective in polyclonal endogenous responses and in TCR transgenic responses in the absence of Dicer.

Fig. 2. Characterization of Dicer deletion driven by expression of dLck-cre. (A) Thymus and (B) lymph nodes from 7-wk-old Dicer−/− Rosa-YFP dLck-cre (Dicer−/−) and Dicer+/+ Rosa-YFP dLck-cre (Dicer+/+) were analyzed by flow cytometry. (C) RNA was isolated from CD8+CD62L−CD44+ naïve cells from control and Dicer−/− mice, and from YFP+ and YFP− OT-1 CD8+ splenocytes from Dicer−/− mice and subjected to real-time PCR analysis.

Fig. 3. Defective polyclonal CD8+ T-cell responses in Dicer−/− mice. Dicer−/− and Dicer+/+ mice were infected with 2,000 cfu LM-ova i.v. and 7 d post-immunization, the CD8+ effector T-cell response was determined. (A) Tetramer staining of splenocytes for Kb/ova-specific cells. (B) Splenocytes were restimulated with ova peptide and the YFP+ CD8+ population was analyzed by intracellular cytokine staining.
indicating Dicer+/- CD8+ T cells from naive B6 mice were stimulated with anti-CD3 and anti-CD28 for 2 d, washed, and transferred to IL-2 for a further 3 d. As shown in Fig. 6B, miR-130/301 family members have very similar expression patterns during the course of in vitro activation (miR-301a and miR-301b only have one nucleotide difference and are detected by a common pair of primers), with minimal levels of expression in naive CD8+ T cells. After 2 d of αCD3/CD28 stimulation, a slight increase was observed (Fig. 6B). However, upon transfer into IL-2 cultures, there was a dramatic up-regulation of miR-130/301 expression levels (Fig. 6B). This miRNA expression pattern correlates very well with the kinetics of surface CD69 down-regulation observed in WT CD8+ T cells (Fig. 4C). To directly test the hypothesis that miR-130/301 down-regulate CD69 expression in activated CD8+ T cells, we transfected activated Dicer+/- CD8+ T cells with different miR-130/301 oligos and scrambled control oligos. As shown in Fig. 6C, miR-130b and miR-301a slightly but consistently down-regulated CD69 expression in Dicer+/- CD8+ T cells compared with scrambled oligo controls. Moreover, combining miR-130b and miR-301a oligos resulted in a more substantial down-regulation of CD69 expression in Dicer+/- CD8+ T cells. Taken together, these data suggest that miR-130/301 expression are dramatically enhanced after T-cell activation and down-regulate CD69 expression in CD8+ T cells.

Discussion

The discovery of miRNAs has added another layer of complexity to the regulation of gene expression. We wished to begin an investigation of the role of miRNAs in effector CD8+ T-cell differentiation by studying Dicer deficient cells. It had been previously unclear whether Dicer/miRNAs are critically involved in CD8+ effector T-cell differentiation following antigenic challenge be-
cause of the requirement for Dicer during CD8+ T-cell development in the thymus. Thus, deletion of Dicer at the early double-negative stage of thymocyte development (with proximal lck-cre) or at the double-negative to double-positive transition (with CD4-cre) results in decreased numbers of mature CD4+ and CD8+ T cells (11, 17). Here, we used tat-cre and dLck-cre, to show that the CD8+ effector T-cell response to infectious challenge in vivo is completely abolished in the absence of Dicer/miRNAs. Induction of Dicer deletion by tat-cre in mature CD8+ T cells avoids any complications from defective T-cell development in the absence of Dicer. Distal Lck-cre is activated after thymocyte-positive selection and only a proportion of single positive thymocytes contain detectable cre activity (Fig. 2A). In the periphery, the majority of mature CD8+ T cells are cre+ but only about 50% of CD4+ T cells are cre+, as judged by recombination at the Rosa-YFP reporter locus (Fig. 2A). Considering the requirement for Dicer in Treg homeostasis and function (12, 14, 27), the WT CD4+ T cells in Dicerf/fdLck-cre mice likely contribute to the healthy status of the mice. Therefore, dLck-cre induced deletion has a minimal impact on CD8+ T-cell development and maintains a healthy environment for Dicer−/− CD8+ T cells for our research. The results presented here convincingly support a critical role for Dicer during CD8+ effector T-cell development.

What are the molecular mechanisms underlying the defective accumulation of Dicer−/− CD8+ effector T cells following antigen challenge? Dicer is essential for mouse and human ES cell proliferation and differentiation (3, 4, 28). Dicer is important for the survival of many different cell types, including developing T and B lymphocytes, although it is clear that stem cells of the lympho-ietic system can go through many rounds of division without Dicer (10, 17). Upon TCR stimulation, Dicer−/− CD8+ T cells are activated and enter the cell cycle with faster kinetics compared with control cells in vitro (Fig. 4A). However, proliferating Dicer−/− CD8+ T cells failed to accumulate in vivo upon acute infection (Figs. 1 and 3). Proliferation itself may be defective in Dicer−/− CD8+ T cells, given the facts that several classic cell cycle-related genes contain multiple conserved miRNA binding sites (16), and several highly conserved miRNAs have demonstrated roles in cell cycle regulation or oncogenesis, such as miR-21, the miR-17 to 92 cluster, the miR-15b/16 cluster, and the miR-290 to 295 cluster (29). Their roles in the expansion phase of mature CD8+ T cells responding to antigen await future investigation. The survival of mature T cells is mainly controlled by Bcl-2 family members (30).

Fig. 5. Prolonged CD69 surface expression and defective migration of Dicer−/− CD8+ T cells responding to infection in vivo. (A) YFP expression profiles of OT-1 T cells before transfer. (B) At day 3.5 postinfection, donor OT-1 cells in spleen and liver, gated as CD8+CD45.2+, were analyzed by flow cytometry. (C) Percentage of CD69+ cells among the OT-1 T-cell population is shown for various organs. (D) Percentage of YFP+ OT-1 cells among total lymphocyte population from each organ. Each point represents an individual mouse.

Fig. 6. MicroR-130/301 regulates CD69 expression in activated CD8+ T cells. (A) Alignment of CD69 mRNA 3′ UTR to show the conserved miR-130/301 binding site. (B) Expression of miR-130/301 in WT CD8+ T cells during in vitro activation. Purified WT CD8+ T cells were activated with αCD3/CD28 for 2 d, washed, and transferred to IL-2 for up to another 3 d. At the indicated time points, cells were harvested. RNA was extracted and subjected to real-time PCR analysis. (C) FACS profile of sorted FITC+ cells post culture, gated on 7-AAD− live cells.
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the same culture, although the prolonged CD69 expression phe-

cantly reversed the CD69 retention phenotype. Other miRNAs

been shown to control cell migration and invasion (32). It is highly

expression. However, in tumor cells, more than 10 miRNAs have

activation markers are not greatly perturbed when CD69 expres-

forced expression of miR-130/301 in Dicer

CD28 (eBioscience) for another 2 d. Cells were washed and cultured in 100 U/mL IL-2 (eBio-

science), 5 ng/mL IL-15 (Peprotech) or 5 ng/mL IL-7 (Peprotech) for another 1 to 3 d and analyzed by flow cytometry.

Flow Cytometry. Single-cell suspensions were prepared from the spleen, lymph nodes, and liver after perfusion of the animal at the indicated time-points postinfection. Cells were typically stained with antibodies specific for CD8, CD4, CD45, CD44, TCR, CD69, CD45.2 (eBioscience and BD) or with K3-SIINFEKL tetramer (Fred Hutchinson Cancer Research Center shared resources for Immune Monitoring, Seattle, WA). For intracellular staining, cells were prepared with the Cytofix/Cytoperm kit (BD) in the presence of brefeldin A (BD) and stained as usual with anti-IFN-γ, TNF-α, anti-CD45.2, anti-CD8, and anti-Bcl-2. Anti-IFN-γ and BD). Blocking reagents for TNF-α receptor (clone M-6X-2T2) and type I IFNs (B18R) were purchased from eBioscience. Cells were analyzed using a FACS-

Canto (BD) and analyzed using FLOWJO (TreeStar) software.

Real-Time PCR. Total RNA containing miRNAs was purified by mirVana RNA isolation kit (Ambion) according to the manufacturer’s instructions. Complementary DNA was generated by an oligo dT primer. Real-time PCR primers were purchased from the MicroRNA assay kit (Applied Biosystems) using the mir reverse-transcription kit (Ap-

plied Biosystems). All real-time PCR reactions were performed in triplicate wells using the 7300 real-time PCR system (Applied Biosystems). For Dicer mRNA quantitation, total RNA was purified by TRIzol (Invitrogen) and cDNA was generated by an oligo dT primer. Real-time PCR primers were purchased from Applied Biosystems (for Dicer, the assay number is Mm00521731_m1 and for Actin B, the part number is 4352933E).

Mature miRNA Transfection. Purified CD8+ T cells from Dicer−/− mice were stimulated by plate bound anti-CD3 plus 1 μg/mL soluble anti-CD28 for 2 d in antibiotic free RPMI. One day postactivation, cells were transfected with different FITC-labeled mature LNA-miRNA at 10 nM (Exiqon) using DharmaFect (Dharmacon) following the manufacturer’s manual. Mature miRNA sequences are: Scramble 5′-GTACACAGAGAUCACGACU 3′; scramble AS; 5′-UGGCGCUAGUGAGCUGACU 3′; miR-130b 5′-GCUCUUCCUGCGU-

GAUCUC 3′; miR-130b AS: 5′-CAGUGAAUAGUAGAAGGCAU 3′; miR-

301a AS: 5′-TTTCAGAAUUGAUCAGUACU 3′ and miR-301a AS: 5′-CAGUG-

CAUGGAUUGAUCAGA 3′. Fourteen hours after transfection, cells were washed and FITC-positive cells were sorted on FACS Aria (BD) and cultured in 100 U/mL IL-2 for another 66 h.

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Fig. S1. Defective CD8$^+$ effector T-cell response in Dicer$^{-/-}$ cells. After tat-cre treatment, Dicer$^{f/+}$ and Dicer$^{f/+}$ OT-1 cells were transferred into CD45.1 hosts followed by *Listeria monocytogenes* ova (LM-ova) infection. (A) Seven days postinfection, the OT-1 T-cell response was examined by intracellular cytokine staining and flow cytometry. FACS profiles were pregated on donor OT-1 T cells. (B) Eleven days postinfection, the OT-1 T-cell response in spleen, lymph node (LN), and liver was examined. (C) Percentage of YFP$^+$ cells in OT-1 cells was shown for indicated time-points postinfection. Each symbol represents an individual mouse.

Fig. S2. Normal CD44 and CD62L expression on Dicer$^{-/-}$ T cells. Lymph node cells were prepared from 7-wk-old naive Dicer$^{f/+}$ and Dicer$^{-/-}$ mice. FACS profiles were pregated on CD4$^+$ or CD8$^+$ T lymphocytes.
Fig. S3. The bystander effect on cocultured B6 cells is independent of TNF-α and type I IFNs. (A) Freshly isolated Dicer<sup>+/−</sup> and Dicer<sup>−/−</sup> splenocytes were stimulated with 5 μg/mL αCD3/CD28 for 4 h. TNF-α production was examined by intracellular cytokine staining. FACS profiles were pregated on CD44<sup>low</sup>CD8<sup>+</sup> cells. (B) Dicer<sup>+/−</sup> and Dicer<sup>−/−</sup> CD8<sup>+</sup> T cells (CD45.2) were mixed in a 1:1 ratio with CD45.1 B6 CD8<sup>+</sup> T cells, and stimulated with αCD3/CD28 in the presence or absence of anti–TNF-α (5 μg/mL) or type I IFN blocking reagent (100 ng/mL vaccinia virus-encoded neutralizing type I IFNR B18R). Two days after stimulation, CD45.1 and CD45.2 viable cells were analyzed for CD69 and CD25 expression.

Fig. S4. Comparable expression of CD25, CD122 and CD127 on Dicer<sup>−/−</sup> CD8<sup>+</sup> T cells following in vitro stimulation. Dicer<sup>+/−</sup> and Dicer<sup>−/−</sup> CD8<sup>+</sup> T cells were labeled with CFSE and stimulated with αCD3/CD28. Two days after stimulation, cells were washed and transferred to medium containing 5 ng/mL IL-7. One day later, viable cells were analyzed. The numbers represent mean fluorescence intensity.
Fig. S5. Decreased survival of Dicer−/− CD8+ T cells following in vitro stimulation. (A) Dicer+/− and Dicer−/− CD8+ T cells (CD45.2) were mixed in a 1:1 ratio with CD45.1 B6 CD8+ T cells, labeled with CFSE, and stimulated with αCD3/CD28. After 2 d, cells were washed and transferred to medium containing the indicated cytokines. One day later, viable cells were analyzed. (B) Bim and Bcl-2 expression were examined on freshly isolated (Upper) or αCD3 activated (Lower) CD8+ T cells from Dicer+/+ and Dicer−/− mice.