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

Nu Zhang and Michael J. Bevan1

Department of Immunology and The Howard Hughes Medical Institute, University of Washington, Seattle, WA 98195

The RNaseIII 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−/− 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 naïve 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 naïve 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 naïve 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 Rosa-YFPfl OT-1 (Dicer−) and Dicerfl Rosa-YFPf 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 Dicerfl donor population. However, in the mice that received Dicerfl T cells, YFP+ CD8+ OT-1 T cells were absent although, in the same recipients, the YFP− OT-1 cells

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1To whom correspondence should be addressed. E-mail: mbevan@u.washington.edu.

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crossed Dicer+/f mice with mice in which the distal lck promoter results in YFP expression from 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 population (Fig. 2C). 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. S4). 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.

Dicer−/− CD8+ T Cells Show Enhanced Activation and Proliferation and Prolonged CD69 Expression upon In Vitro Stimulation. To further understand 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.

responded robustly in all organs examined (Fig. 1 B and C and Fig. S1A). To exclude the possibility that Dicer deficiency changed the kinetics of CD8+ effector T-cell expansion, other time-points postinfection were also examined and no YFP+ OT-1 T cells were found for Dicer−/− cells (Fig. S1 B and C). These data clearly demonstrate that Dicer is essential for CD8+ effector T-cell expansion upon infectious antigenic challenge in vivo.

Characterizing Dicer Deletion in Mice Transgenic for Distal Lck-cre. To confirm and extend the results from the tat-cre system, we crossed Dicer−/− mice with mice in which the distal lck promoter drives cre expression (dLck-cre mice). As shown in Fig. 2 A and B, the distal lck promoter results in YFP expression from the Rosa-YFP reporter locus only at the single positive stage of thymocyte development. In the peripheral mature T-cell compartment, dLck-cre resulted in YFP expression in around 50% of CD4− T cells and 80% of CD8− T cells (Fig. 2B). By PCR analysis, the level of Dicer mRNA in sorted YFP+ Dicer+/f 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.

![Fig. 1. Dicer is essential for the CD8+ T-cell response to pathogen in vivo.](#)

(A) Two days after in vitro culture, YFP expression in tat-cre treated OT-1 cells was examined. FACs profiles were pregated on 7AAD− live cells. (B and C) Seven days after LM-ova infection, host mice were killed and donor OT-1 cells in the spleen, lymph node (LN), and liver were analyzed. The number of YFP+ cells was calculated based on total organ cellularity and the percentage of YFP+ cells. Each symbol in C represents an individual mouse.

![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− naive 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 postimmunization, 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.

![Fig. 4.](#)
Surface CD69 Retention and Defective Migration of Dicer<sup⊥</sup>−<sup⊥</sup> T Cells in Vivo. CD69 expression inhibits S1P1 function and the migration of activated T cells out of central lymphoid organs in response to a gradient of S1P (24). Following an acute infection, antigen-specific CD8<sup+</sup> T cells up-regulate CD69 expression but by around 3 d postinfection, they down-regulate CD69 expression and start to migrate out of central lymphoid organs, such as lymph nodes and splenic white pulp (26). The defective down-regulation of CD69 on Dicer<sup⊥</sup>−<sup⊥</sup> CD8<sup+</sup> T cells that we observed in vitro prompted us to investigate the migration of antigen-activated Dicer<sup⊥</sup>−<sup⊥</sup> CD8<sup+</sup> T cells in vivo. To observe early events of CD8<sup+</sup> T-cell activation in vivo, a larger number of OT-I cells was transferred to congenic recipients, and adoptive host mice were challenged with a virus that results in faster antigen presentation. CD45.2 Dicer<sup⊥</sup>−<sup⊥</sup> and Dicer<sup⊥</sup>−<sup⊥</sup> OT-1 cells were transferred into CD45.1 host mice followed by vesicular stomatitis virus-ova infection. As expected, at day 3.5 postinfection, we saw defective accumulation of Dicer<sup⊥</sup>−<sup⊥</sup> cells in all of the tissues examined, including the spleen and liver (Fig. 5B). At this time point, control OT-1 cells had lost surface CD69 expression but a substantial percentage of Dicer<sup⊥</sup>−<sup⊥</sup> OT-1 cells still retained CD69 expression (Fig. 5 B and C). Consistent with the hypothesis that CD69 expression inhibits Dicer<sup⊥</sup>−<sup⊥</sup> T-cell migration, the accumulation of Dicer<sup⊥</sup>−<sup⊥</sup> OT-1 cells was more defective in the blood and liver compared with the spleen and lymph nodes (11- to 15-fold vs. 3- to 4-fold decrease compared with control Dicer<sup⊥</sup>−<sup⊥</sup> cells, respectively) (Fig. 5D).

MicroRNA-130/301 Regulates CD69 Expression. Examination of the 3′UTR of CD69 mRNA revealed several conserved miR binding sites. One of them is the site for miR-130/301, and this sequence is entirely conserved across a range of species (Fig. 6A). There are four members of the mir-130/301 family in the mouse genome: miR-130a, miR-130b, miR-301a, and miR-301b (www.mirbase.org). To determine the role of miR-130/301 in CD69 regulation, we examined the expression pattern of miR-130/301 during CD8<sup+</sup> T-cell activation. CD8<sup+</sup> 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<sup+</sup> 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<sup+</sup> T cells (Fig. 4C). To directly test the hypothesis that miR-130/301 down-regulate CD69 expression in activated CD8<sup+</sup> T cells, we transfected activated Dicer<sup⊥</sup>−<sup⊥</sup> CD8<sup+</sup> 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<sup⊥</sup>−<sup⊥</sup> CD8<sup+</sup> 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<sup⊥</sup>−<sup⊥</sup> CD8<sup+</sup> T cells. Taken together, these data suggest that miR-130/301 expression is dramatically enhanced after T-cell activation and down-regulate CD69 expression in CD8<sup+</sup> 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<sup+</sup> T-cell differentiation by studying Dicer deficient cells. It had been previously unclear whether Dicer/miRNAs are critically involved in CD8<sup+</sup> 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. 2B). Considering the requirement for Dicer in Treg homeostasis and function (12, 14, 27), the WT CD4+ T cells in Dicerf/f/dLck-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 lymphopoietic 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 further 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.](image)

(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.](image)

(A) Alignment of CD69 mRNA 3′ UTR to show the conserved miR-130/301 binding site. (B) Expression of microR-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.
MicroR-17 to 92 family members target the proapoptotic gene Bim in both T and B lymphocytes. Indeed, we do observe a substantial increase in Bim protein level in both naïve and activated Dicer−/− T cells (Fig. S5B). However, we also observe an increase in the expression of the antiapoptotic gene Bcl-2 in both naïve and activated Dicer−/− T cells (Fig. S5B). Because the mouse Bcl-2 gene has a 5′-kb 3′ UTR, which contains more than 10 conserved miRNA binding sites, it is not surprising that the Bcl-2 gene may be regulated by miRNAs. In fact, it has been shown that Bcl-2 may be regulated by miR-181a during thymocyte development (31). At present, we do not have a simple and straightforward explanation for the survival defects in Dicer−/− CD8+ T cells following in vitro and in vivo stimulation.

Dicer−/− CD8+ T cells retain surface expression of CD69 following TCR stimulation much longer than control cells, both in vitro and in vivo. In addition, Dicer−/− CD8+ T cells are defective in their migration out of lymphoid organs during acute infection. Because CD69 negatively regulates T-cell migration, it is probable that Dicer controls CD8+ T-cell migration through CD69 expression. However, in tumor cells, more than 10 miRNAs have been shown to control cell migration and invasion (32). It is highly possible that other molecules in addition to CD69 are targets of miRNAs/Dicer and regulate CD8+ T-cell migration. In the 3′ UTR of the CD69 message, five conserved miRNA binding sites, including miR-130/301, are identified. We were able to show that forced expression of miR-130/301 in Dicer−/− CD8+ T cells partially reverses the CD8+ T-cell migration defect. Other miRNAs may directly control CD69 expression, such as miR-181a (31), or additional miRNAs may control other molecules upstream of CD69 in activated CD8+ T cells. Thus, the defects in CD69 retention and migration may be a combined effect from multiple miRNAs and targets in Dicer−/− CD8+ T cells.

Dicer−/− CD8+ T cells show enhanced early proliferation upon TCR stimulation in vitro (Fig. 4 A and B), and this may be regulated by mechanisms distinct from the CD69 retention phenotype for the following reasons. First, the enhanced activation and proliferation phenotype has a bystander effect on control cells in the same culture, although the prolonged CD69 expression phenotype is Dicer−/− cell intrinsic (Fig. 4 A and B). Second, other activation markers are not greatly perturbed when CD69 expression is retained on Dicer−/− CD8+ T cells. MicroR-181a targets several phosphatases leading to enhanced TCR sensitivity during thymocyte development (2, 28) and is then down-regulated in several phosphatases leading to enhanced TCR sensitivity during thymocyte development (2, 28) and is then down-regulated in mature CD8+ T cells (35). At present, we do not have a simple and straightforward explanation for the survival defects in Dicer−/− CD8+ T cells following in vitro and in vivo stimulation.

In Vitro Activation and Proliferation. CD8+ T cells were purified using a CD8 isolation kit (Miltenyi), labeled with 5 μM CFSE (Invitrogen), and stimulated with plate-bound αCD3 (ebiosis) plus 1 μg/mL soluble αCD28 (ebiosis) for 2 d. Cells were washed and cultured in 100 U/ml IL-2 (ebiosis), 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, CD25, CD44, TCRi, CD69, CD45.2 (ebiosis and BD) or with K5-kiNFkEtetrimer (Fred Hutchinson Cancer Research Center shared resources for Immunology, Seattle, WA). For intracellular staining, cells were prepared with the Cytotox/Cytopern kit (BD) in the presence of brefeldin A (BD) and stained as described in ref. 2. Blocking reagents for TNF-α (clone MP6-XT2) and type I IFN (B18R) were purchased from ebiosis. Cells were analyzed using a FACSCanto (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 miR-specific primers from the MicroRNA assay kit (Applied Biosystems) using the mir reverse-transcription kit (Applied 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 Transfer. 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 100 nM (Exiqon) using DharmaFect (Dharmacon) following the manufacturer’s manual. Mature miRNA sequences are: Scramble SS: 5′-GTACCAAGAGAAUAACAUACAU-3′; scramble AS: 5′-UGGGCGUAUAGGUGUUCAAGUA-3′; miR-130b SS: 5′-GGCUCUUCCUGUG-UGCAUC-3′; miR-130b AS: 5′-CAGUGCAAAUGGAGGCGAU-3′; miR-30a SS: 5′-TTTACGUUAUUGGACAUAG-3′ and miR-30a AS: 5′-CAGUG-CAAAUGUAUGGACAAAG-3′. Fourteen hours after transfection, cells were washed and FITC-positive cells were sorted on FACSaria (BD) and cultured in 100 U/ml IL-2 for another 66 h.

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