Supporting Information

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SI Materials and Methods

Mice. *Dicer*$^{fl/fl}$ mice have been previously described (1). *Dicer*$^{fl/fl}$ were crossed to CD4-Cre mice, to P14 transgenic mice, and in some experiments to B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J (Rosa26R-YFP) reporter mice (The Jackson Laboratory); in the latter, YFP expression marks all of the cells that underwent Cre-mediated recombination in vivo. Heterozygous *Dicer*$^{fl/+}$ or wild-type *Dicer*$^{+/+}$ littermates were used as controls in all of the experiments. For *Listeria monocytogenes* infection, C57BL/6 mice between 6 and 8 wk of age were used as recipients. P14 transgenic:T-cell receptor (TCR)α$^{-/-}$ mice were on the C57BL/6 background and purchased from Taconic.

Isolation, Culture, and Retroviral Transduction of Primary Mouse CD8$^+$ T Cells. Total CD8$^+$ T cells were isolated by negative selection using Dynabeads (Invitrogen) from spleen and lymph nodes (excluding mesenteric) and cultured as previously described (2). Naïve (CD62L$^+$CD44^{low}) CD8$^+$ T cells were sorted in all of the experiments where *Dicer* was deleted in vivo using CD4-Cre. Briefly, CD8$^+$ T cells were activated by anti-CD3 (clone 2C11) and anti-CD28 (clone 37.51) (BD Biosciences) at 1 μg/mL on plates precoated with 100 μg/mL goat anti-hamster IgG (MP Biomedicals). On day 2, cells were removed from stimuli and recultured at 5×10^5 per mL with recombinant human IL-2 (Hoffmann-La Roche). Effector cytotoxic T lymphocytes (CTLs) were generated using 100 U/mL (6.7 ng/mL) IL-2, and memory-like CTLs using 10 U/mL IL-2. Every day the cell concentration was adjusted to 5×10^5 per mL. For antigen presenting cells (APC) stimulation, total splenocytes were plated at 2.5×10^6 per mL with GP33 peptide with or without CpG (ODN1826), both at 1 μM. On day 2, CD8$^+$ T cells were isolated by negative selection and recultured with IL-2 as described above. When indicated, 5 ng/mL IL-12 was added to the cultures on day 2. Cells were diluted to 5×10^5 per mL every day until day 6. For retroviral transduction, 18 h after activation, CD8$^+$ T cells were centrifuged at 2,200 rpm for 1 h in the presence of fresh viral supernatant supplemented with 8 μg/mL polybrene (Sigma-Aldrich). Viral supernatants were removed and replaced with T-cell "conditioned" medium 4 h after transduction. Transduced cells were selected by sorting GFP$^+$ cells. In some experiments, two rounds of transduction were used, on day 1 and day 2, achieving between 85% and 95% transduction efficiency without sorting. Retroviral vectors were produced by transient transfection of Plat-E cells (3), and viral supernatants were collected 40 and 64 h after transfection.

Adoptive Cell Transfer and *Listeria* Infection. For the *Listeria* protection assay, 5×10^4 resting P14 transgenic memory-like CD8$^+$ T cells obtained by 6 days of culture in 10 U/mL IL-2 and transduced with pMIG-microRNA (miR)-139 or control retrovirus (RV) were transferred intravenously, followed by i.v. injection of 1×10^5 CFUs per mouse of *Listeria-gp33* (a kind gift of Rafi Ahmed, Emory University, Atlanta, GA), 24 h after cell transfer. Bacterial load in the spleen was analyzed 3 days after infection by plating serial dilutions of spleen suspension lysed in 0.5% Triton X-100 onto brain-heart infusion (BHI) broth agar plates.

Isolation, Culture, and Lentiviral Transduction of Primary Human CD8$^+$ T Cells. Naïve CD8$^+$ T cells were enriched from peripheral blood mononuclear cells (PBMCs) isolated from adult peripheral blood (La Jolla Institute for Allergy and Immunology blood donor program) or buffy coats (San Diego Blood Bank) after written informed consent. Ethical approval for the use of the blood was obtained from La Jolla Institute for Allergy and Immunology's Institutional Review Board. Naïve CD8$^+$ T cells were enriched from PBMC in two steps using negative selection (Miltenyi Biotec and StemCell Technologies). Cells were activated using anti-CD3 (clone OKT3) plus anti-CD28 Abs, both at 0.1 μg/mL, on plates precoated with 100 μg/mL goat anti-mouse IgG (Jackson ImmunoResearch). In some experiments, cells were stimulated with anti-CD3/CD28-coated Dynabeads (Invitrogen) with similar results. Activated cells were transduced with lentiviral vectors on day 1 following the same protocol described above for transduction of mouse T cells with retroviral vectors. Puromycin selection (0.6 μg/mL) was started 48 h after transduction. VSV-g–pseudotyped lentiviral vectors were produced by transient transfection of 293T cells using a second-generation packaging system. The PLKO.1 vector containing shRNA against Dicer (shDicer) was a kind gift of Stefano Piccolo (University of Padua, Padua, Italy).

T-Cell Restimulation and Cytokine Production. For analysis of cytokine production by intracellular staining, human or mouse CD8$^+$ T cells were restimulated for 6 h with 10 nM PMA (phorbol 12-myristate 13-acetate) and 500 nM ionomycin (Sigma-Aldrich). Alternatively, cells were restimulated on plate-bound anti-CD3 Abs (10 μg/mL) in the presence of 10 nM PMA. Cells were fixed with 3% (wt/vol) paraformaldheyde (PFA) for 20 min, followed by permeabilization and staining in 0.5% saponin (Sigma-Aldrich) buffer. Data were acquired on Fortessa, FACSCanto, or LSRII machines (Becton Dickinson) and analyzed using FlowJo software (Tree Star).

Primary MicroRNA Constructs. Primary microRNA (pri-miRNA) constructs were generated by amplifying the endogenous miRNA hairpins plus ~200-nt–long genomic regions upstream and downstream of the mature miRNA sequences and cloned into a modified pMIG retroviral vector. pri-miRNA sequences were cloned from mouse genomic DNA by PCR and directly subcloned into the MG-155 retroviral vector (pMIG-w backbone; Addgene plasmid 26527, a gift of Dr. D. Baltimore, Department of Biology, Caltech, Pasadena, CA) after removal of the miR-155 sequence contained in the original vector with NotI and XhoI. The following forward (F) and reverse (R) primers were used to amplify pri-miRs [Primer3 software (http://biotools.umassmed.edu; http://bioinfo.ut.ee/primer3-0.4.0)]: miR-139 F: 5′-TGGCCCTGGATGGCTTTA-3′; miR-139 R: 5′-AAAGGAGAAAAGGAGCTGTGG-3′; miR-342 F: 5′-TTTATCTACACCCTAAGAGACTGACAT-3′; miR-342 R: 5′-CATGTTGGACTGAACTGCTTTC-3′; and miR-150 F: 5′-CTGCTTAGTGGCTCTACTCCTG-3′; miR-150 R: 5′-TCCCCTCTGGCTTATGTCC-3′.

Transfection of miRNA Mimics into Primary Mouse CD8$^+$ T Cells. Transfections were performed as previously described (4) using the Neon system (Invitrogen) with minor modifications. Total CD8$^+$ T cells were isolated from P14 transgenic, TCRα$^{-/-}$ mice or, alternatively, P14$^+$YFP$^+$ naïve (CD62L$^+$CD44^{low}) CD8$^+$ T cells were sorted from control and *Dicer*$^{-/-}$ P14 transgenic, ROSA26R mice. Cells were stimulated as indicated above, and on day 5 they were counted, washed with PBS, resuspended in buffer R (Neon Transfection System, Invitrogen), and transfected using 100 pmol of miRNA mimics (Dharmacon) per 3–5 × 10^5 cells in a 10-μL tip. The instrument was set at 1,500 V with three 10-ms pulses. Immediately after transfection, cells were replated in

antibiotic-free medium supplemented with fresh IL-2, returned to the incubator, and analyzed 26–28 h after transfection. Conditions were optimized using an siRNA pool against mouse CD8a.

Western Blot. Whole-cell lysates were prepared in RIPA buffer from either resting $CD8^+$ T cells or after a 6-h restimulation with PMA and ionomycin. Cyclosporin A was added as indicated 30 min before restimulation. Lysates were cleared by centrifugation for 15 min at 16,000 rcf and proteins were resolved by SDS/PAGE. The following primary Abs were used: anti-mouse perforin (clone CB5.4; Abcam), anti-Dicer (clone H-212; Santa Cruz Biotechnology), anti-mouse Runx3 (a kind gift of Yoram Groner, Weizmann Institute of Science, Rehovot, Israel), anti-human perforin (clone 2d4; a kind gift of Gillian Griffiths, Cambridge Institute for Medical Research, Cambridge, UK), anti-mouse actin (Sigma-Aldrich), and anti-GAPDH (clone FF26A/F9; BioLegend). HRP-conjugated secondary Abs were from Sigma-Aldrich.

RNA Isolation and Quantitative RT-PCR for miRNA Detection. Total RNA was isolated with TRIzol reagent (Invitrogen) followed by purification with the miRNeasy Kit (Qiagen). Alternatively, total RNA was isolated using the mirVana RNA Isolation Kit (Invitrogen) according to the manufacturer's instructions. SYBR Green or TaqMan real-time PCR was used to analyze mRNA and miRNA expression. For standard quantitative RT-PCR of mRNAs, cDNA was generated using the SuperScript III Kit (Invitrogen) and oligo-dT primers. Human *DICER* mRNA was quantified using FastStart Universal SYBR Green (Roche) and previously described primers (F primer: 5′-TGGGTCCTTTCTTTGGACTG-3′; R primer: 5′-CTGGTTTGCAGAGTTGACCA-3′). *DICER* expression was normalized to actin. Mouse *Prf1* and *Dicer* mRNA were quantified using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and the following primer/probe sets (all from Applied Biosystems): Mm00812512_m1 (*Prf1*), Mm00521731_m1 [*Dicer1*, targeted on the *floxed* exon 23 (1)], and Mm01205647_g1 (*actin*, *beta*). miR-150 and miR-139-5p and -3p were quantified using TaqMan microRNA assays (Applied Biosystems) and normalized to the levels of miR-191, chosen because of its high expression levels and independency of T-cell activation status (Dataset S1). A Dicer-independent small nucleolar RNA (RNU6B) was used to normalize miR expression levels between control and Dicer-deficient cells.

Cytotoxic Assay. Total $CD8^+$ T cells were isolated from P14 transgenic mice, transduced with empty RV or miR-139-RV, and cultured for 6 days in high IL-2. On day 6, target EL4 cells were labeled with 3 μM CellTrace Violet for 20 min at 37 °C and subsequently pulsed with 1 μM gp33 peptide for 2 h at 37 °C. Serial dilutions of effector CTLs were incubated with target cells in 96-well U-bottom plates for 6 h. Samples were stained with anti-CD8 PerCP-Cy5.5 Ab (BioLegend) and the Fixable Viability Dye eFluor780 (eBioscience), followed by fixation in 3% PFA. Samples were then acquired on an LSRFortessa cell analyzer (BD Biosciences) using the High Throughput Sampler platform. Target cells were identified by gating on $CD8^-$ CellTrace $Violet^+$ cells, and dead targets were identified as positive for the viability dye. Target cells incubated without effectors were used as a reference of spontaneous cell death (min), whereas Triton X-100 was added to target cells to calculate the maximum cell death (usually 100%). The percentage of specific lysis was calculated as follows: (X sample − min)/(max − min) × 100. Technical duplicates were run for each effector:target dilution.

Illumina Sequencing and Bioinformatics Analysis. Total RNA was extracted as described above from the following samples: (a: $n = 2$ replicates) effector CTLs obtained by stimulation of total splenocytes from P14 transgenic:$TCR\alpha^{-/-}$ mice with gp33 peptide followed by culture in 100 U/mL IL-2 for 6 days; (b: $n = 2$ replicates) effector CTLs obtained by stimulation of total splenocytes from P14 transgenic:$TCR\alpha^{-/-}$ mice with gp33 plus CpG peptide followed by culture in 100 U/mL IL-2 plus IL-12 for 6 days; (c: $n = 2$ replicates) effector CTLs from P14 transgenic: $Dicer^{fl/fl}$ mice stimulated with aCD3 plus aCD28 Abs, transduced with pMIG-Cre-ires-GFP, and cultured for 6 days in 100 U/mL IL-2; (d: $n = 2$ replicates) effector CTLs from P14 transgenic: $Dicer^{fl/fl}$ mice stimulated with aCD3 plus aCD28 Abs, transduced with pMIG-*empty*-ires-GFP, and cultured for 6 days in 100 U/mL IL-2; (e: $n = 2$ replicates) effector CTLs from P14 transgenic: $Dicer^{fl/+}$ or $Dicer^{+/+}$ mice stimulated with aCD3 plus aCD28 Abs, transduced with pMIG-Cre-ires-GFP, and cultured for 6 days in 100 U/mL IL-2; and (f: $n = 1$ replicate) $KLRG1^+CD127^-$ and $KLRG1^-CD127^+$ $CD8^+CD44^{high}$ cells sorted from the spleen of C57BL/6 mice 8 days after lymphocytic choriomeningitis virus (LCMV)-Armstrong infection. Small RNA libraries were constructed and barcoded using the TruSeq Small RNA Kit (Illumina) according to the manufacturer's instructions and size-selected on polyacrylamide gels. RNA and DNA quality control was done using an Agilent 2100 Bioanalyzer, and libraries were quantified by quantitative PCR (Kapa Biosystems). Samples were sequenced on an Illumina HiSeq 2000 instrument. For bioinformatics analysis, Cutadapt (http://code.google.com/p/cutadapt) was used to remove adapter sequences. Bowtie (http://bowtie-bio.sourceforge.net/index.shtml) was used to map all of the reads longer than 15 nt to the mm9 genome without allowing mismatches. If a read had multiple mappable positions, then one was selected randomly. MicroRNA annotations were extracted from the miRBase (*Mus musculus*) database (www.mirbase.org). The number of reads falling into each miRNA locus was quantified using BEDTools (http://bedtools.readthedocs.org/en/latest). To count a read as an miRNA read, we required that it had the correct strand information and resided completely within the annotated miRNA region. Read counts were normalized based on the total number of million mapped reads and the average miRNA length (0.022 kb) and expressed as reads per kb per million (RPKM) values. Of the reads aligned to the mouse genome, 47–67% mapped to miRNA loci in 10 Dicer-sufficient samples, whereas only 12% and 21% of reads mapped to miRNAs in the 2 Dicer-deficient samples.

1. Harfe BD, McManus MT, Mansfield JH, Hornstein E, Tabin CJ (2005) The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. *Proc Natl Acad Sci USA* 102(31):10898–10903.
2. Pipkin ME, et al. (2010) Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity* 32(1):79–90.
3. Morita S, Kojima T, Kitamura T (2000) Plat-E: An efficient and stable system for transient packaging of retroviruses. *Gene Ther* 7(12):1063–1066.
4. Steiner DF, et al. (2011) MicroRNA-29 regulates T-box transcription factors and interferon-γ production in helper T cells. *Immunity* 35(2):169–181.

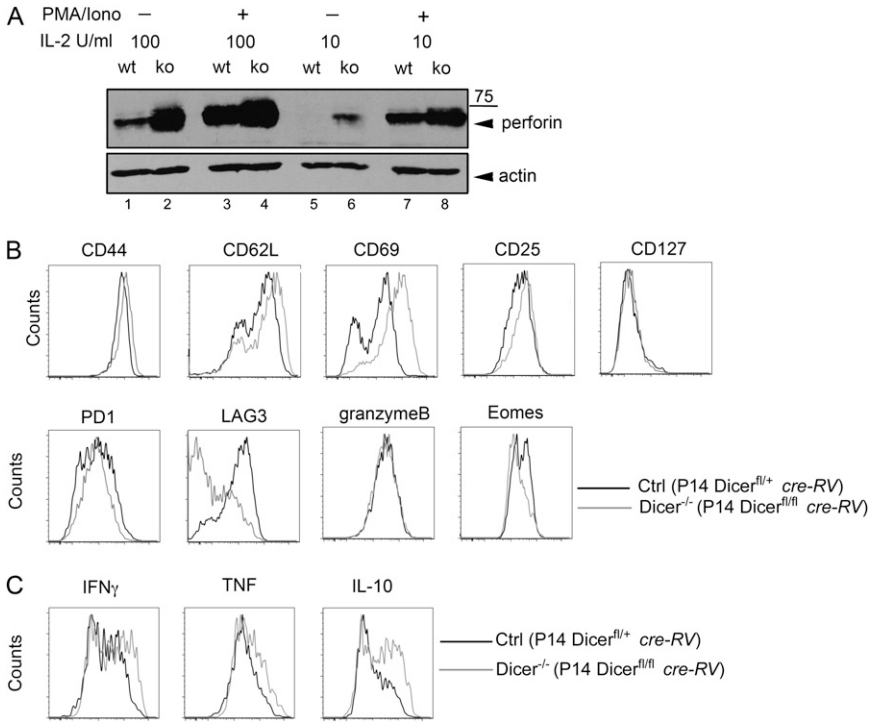

Fig. S1. (*A*) Perforin expression analyzed by Western blot on day 6 in P14$^+$:*Dicer*$^{fl/+}$cd4-Cre control (ctrl) and P14$^+$:*Dicer*$^{fl/fl}$cd4-Cre knockout (ko) CTLs, cultured with the indicated doses of IL-2. Cells were resting or restimulated with PMA/ionomycin as indicated. (*B* and *C*) Activated CD8$^+$ T cells from P14$^+$:*Dicer*$^{fl/+}$ and P14$^+$:*Dicer*$^{fl/fl}$ mice were transduced with a retroviral vector, either empty (ires-GFP) or encoding Cre recombinase (Cre-ires-GFP) on days 1 and 2 poststimulation. On day 2, cells were removed from TCR stimulation and recultured with IL-2. (*B*) Flow cytometry analysis of the indicated molecules on day 6 resting CTLs. (*C*) Intracytoplasmic staining for IFN-γ, TNF, and IL-10 on control and Dicer-deficient CTLs obtained as in *A* and restimulated for 6 h with anti-CD3 plus PMA. Black, controls; gray, Dicer knockout. Results are representative of at least three independent experiments.

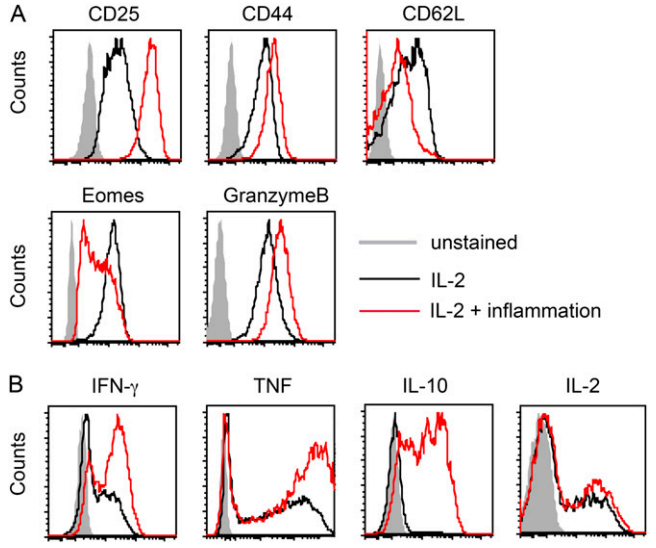

Fig. S2. Total splenocytes were isolated from P14 transgenic:TCRα$^{-/-}$ mice and stimulated with gp33 peptide with or without CpG. On day 2, CD8$^+$ T cells were purified by negative selection and recultured with IL-2 only (IL-2) or IL-2 plus IL-12 (IL-2 + inflammation). (*A*) Expression of the indicated molecules on resting CTLs derived with and without inflammation, analyzed on day 6 poststimulation. (*B*) Expression of the indicated cytokines in the same cells as in *A*, analyzed after a 6-h stimulation with anti-CD3 plus PMA. Results are representative of three independent experiments.

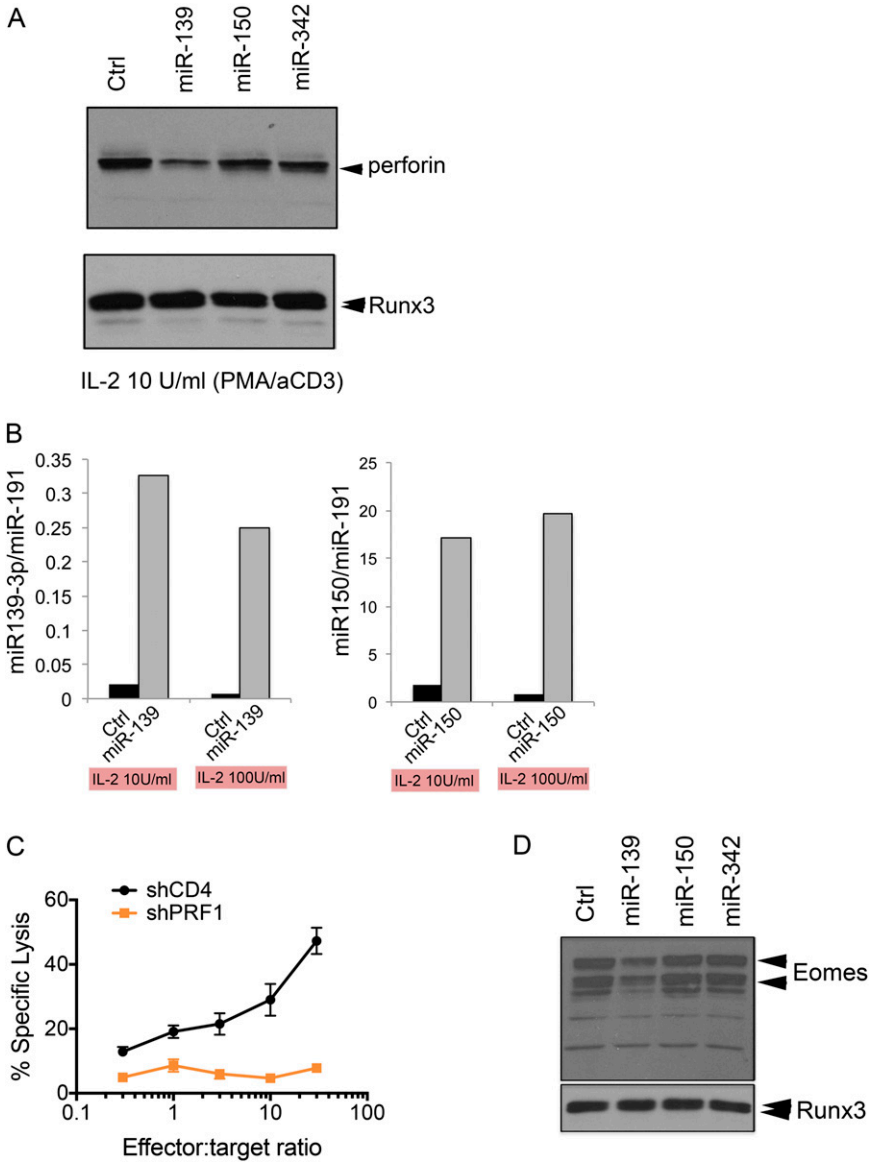

Fig. S3. (*A*) Expression of perforin in memory CTLs derived from P14 transgenic:TCR$\alpha^{-/-}$ mice, transduced with the indicated miR-expressing RV and cultured for 6 days in low IL-2. On day 6, cells were restimulated for 4 h with anti-CD3 plus PMA. "Ctrl" refers to mock-transduced cells. (*B*) Real-time analysis of miR-139-3p (*Left*) and miR-150 (*Right*) expression in CTLs transduced with miR-139– or miR-150–expressing retroviral vectors and differentiated with 10 or 100 U/mL IL-2 (as indicated below the graph). Mock-transduced cells are used as a reference. (*C*) Cytotoxic assay performed with effector CTLs derived from P14 transgenic:TCR$\alpha^{-/-}$ mice, either mock-transduced (shCD4) or transduced with an shRNA targeting perforin (shPRF). As target cells, gp33-loaded EL4 cells were used. Mean +/– standard error is shown. Results are representative of three independent experiments. (*D*) Western blot analysis of Eomes (eomesodermin) expression in resting effector CTLs derived from P14 transgenic:TCR$\alpha^{-/-}$ mice and transduced with the indicated miR RVs.

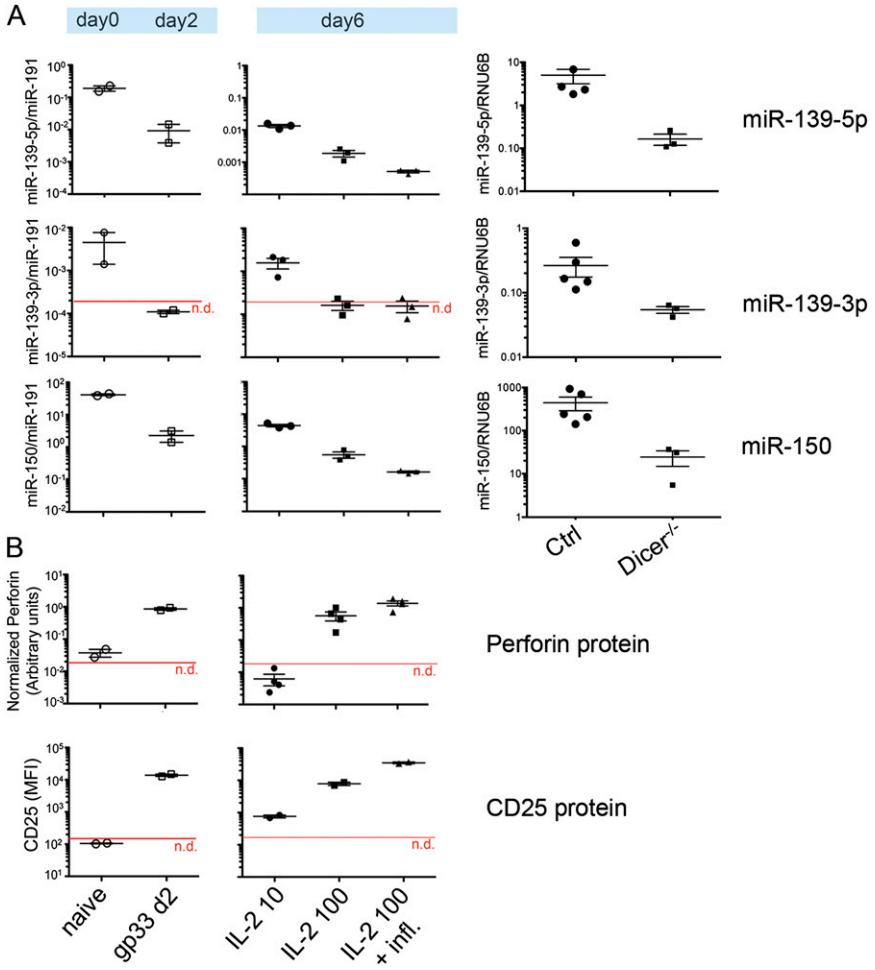

Fig. S4. (*A*) (*Left*) Expression of miR-139-5p, miR-139-3p, and miR-150 in wild-type naïve P14 transgenic:TCRα$^{-/-}$ CD8^{+} T cells, or after 2 days of stimulation of whole splenocytes with gp33 ("day 2") and of memory and effector CTLs derived with low (IL-2 10 U), high (IL-2 100 U), or IL-2 + inflammation (CpG and IL-12). Data from two experiments are shown for naïve and day 2 cells and from three independent experiments for day 6 cells. (*Right*) Expression of miR-139-5p, miR-139-3p, and miR-150 in wild-type and Dicer-deficient CTLs obtained by stimulation of purified CD8^{+} T cells from P14 transgenic *Dicer*$^{+/-}$ (Ctrl) and P14 transgenic *Dicer*$^{-/-}$, followed by transduction with Cre-expressing vector. miRNA expression was analyzed by real-time PCR after 6 days of culture in high IL-2. Mean +/− standard error is shown; n.d. not detected. (*B*) Quantification of perforin and CD25 expression by Western blot and flow cytometry analysis, respectively, in wild-type CD8^{+} T cells differentiated as in *A*. MFI, mean fluorescence intensity.

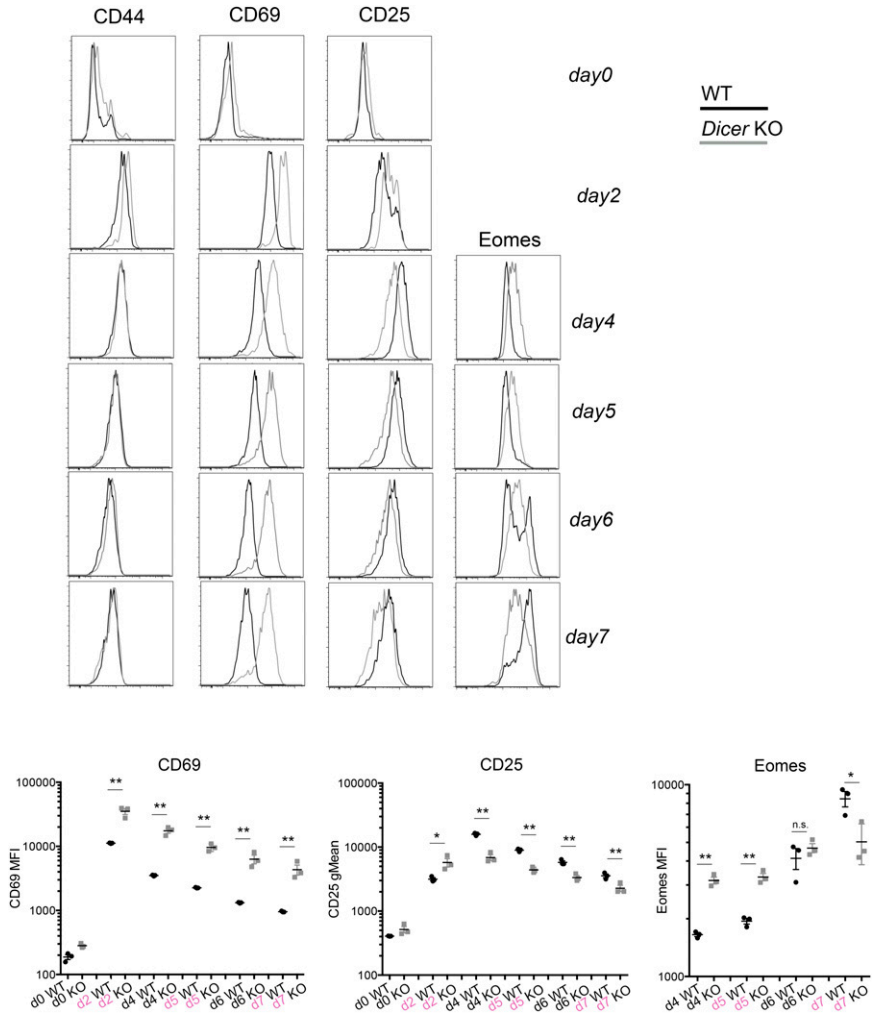

Fig. S5. Time course analysis of the expression of CD69, CD25, and Eomes in wild-type and Dicer-deficient cells, isolated from *Dicer*$^{+/+}$:*Cd4*-Cre-R26R and *Dicer*$^{fl/fl}$:*Cd4*-Cre-R26R mice, activated with anti-CD3 plus anti-CD28, and on day 2 recultured with 100 U/mL IL-2. (*Upper*) CD25 and CD69 expression was analyzed by surface staining starting from day 2, whereas Eomes expression was analyzed by intracellular staining starting from day 4. Graphs show geometric mean (gMean) fluorescence intensity values of the indicated markers at all time points analyzed (*Lower*). Three wild-type and three knockout mice were analyzed at each time point. *$P < 0.05$; **$P < 0.01$; n.s., not significant.

Dataset S1. Small RNA sequencing dataset

Dataset S1

(Sheet 1) RPKM of each miRNA in all of the biological replicates examined. (Sheet 2) Fold change of miRNA expression in CTLs differentiated in vitro from P14 transgenic mice with gp33 and 100 U IL-2 in the presence or absence of inflammation. miRNAs down-regulated by inflammation are highlighted in red; miRNAs up-regulated by inflammation are in blue. The percentage of miRNAs down-regulated or up-regulated by inflammation is calculated for all miRNAs (above) or focusing only on low-expressed miRNAs (100 < RPKM < 1000). When a cutoff of twofold is used to define differentially expressed miRNAs, only five miRNAs (out of 171) resulted in down-regulation by inflammation. However, a less stringent analysis in which all miRNAs whose expression changed at least 1.5-fold were considered revealed that in each biological replicate 25% of miRNAs were down-regulated by inflammatory stimuli whereas 10% of miRNAs were up-regulated. When we focused our analysis only on miRNAs with moderate-to-low expression (100 < RPKM ≤ 1,000), we observed that up to 41% and 50% of all considered miRNAs were down-regulated by inflammation by at least 1.5-fold, as opposed to only 10% and 13% being up-regulated, in the two different biological replicates. (Sheet 3) Fold change of miRNA expression in effector (KLRG1^{+}CD127^{-}) and memory precursor (KLRG1^{-}CD127^{+}) CTLs isolated from mice infected with LCMV-Armstrong 8 days after infection.