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Supplemental Information

MicroRNA-29 Regulates T-Box Transcription Factors and Interferon-γ Production in Helper T Cells

David F. Steiner, Molly F. Thomas, Joyce K. Hu, Zhiyong Yang, Joshua E. Babiarz, Christopher D.C. Allen, Mehrdad Matloubian, Robert Blelloch, and K. Mark Ansel

Inventory of Supplemental Material

Figure S1 (related to Figure 1): Phenotype of cells isolated from the lymph nodes and spleens of *Dgcr8*$^{fl/fl}$ *CD4-Cre* mice.

Figure S2 (related to Figure 2): Restoring miRNA expression rescues cytokine production defects of DGCR8-deficeint cells in a cell intrinsic manner

Figure S3 (related to Figure 3) Validating miRNA gain of function by transfection in primary helper T cells

Figure S4 (related to Figure 3): Effects of miR-17~92 in DGCR8-deficient helper T cells.

Figure S5 (related to Figure 5): Deep sequencing analysis of miR-29 cluster expression in activated helper T cells.

Table S1 (related to Figure 3): small RNA sequencing reads in activated helper T cells (Excel spreadsheet). Available online.

Table S2 (related to Figure 4): Summary of *Z* scores for rescue screens (Excel spreadsheet). Available online.

Table S3 (related to Figure 5): Differential gene expression following miR-29 transfection or inhibition (Excel spreadsheet). Available online.

Supplemental Experimental Procedures and References: Additional details about procedures not included in the main text

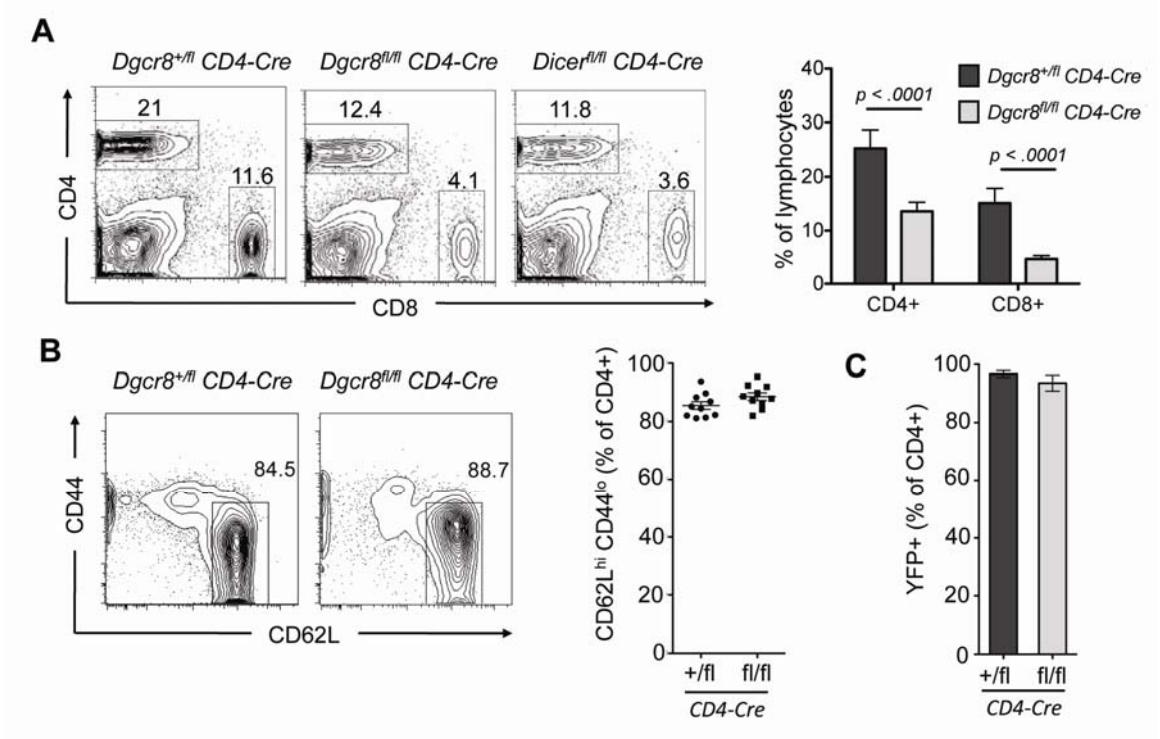

Figure S1 (related to Figure 1). Phenotype of cells isolated from the lymph nodes and spleens of *Dgcr8$^{fl/fl}$ CD4-Cre* mice.

(A) Total lymphocytes were isolated from lymph nodes and spleens of *Dgcr8$^{fl/fl}$ CD4-Cre, Dgcr8$^{+/fl}$ CD4-Cre,* or *Dicer$^{fl/fl}$ CD4-Cre* mice and immediately analyzed for surface expression of CD4 and CD8. Plots depict relative frequencies among all live lymphocytes. Bar graphs are means ± SD (n=10).

(B) The CD4^{+} cells among the freshly isolated lymphocytes obtained as in (A) from *Dgcr8$^{fl/fl}$ CD4-Cre, Dgcr8$^{+/fl}$ CD4-Cre* mice were further analyzed for cell surface expression of CD62L and CD44.

(C) CD4^{+} cells from *Dgcr8$^{fl/fl}$ CD4-Cre R26-YFP* or *Dgcr8$^{+/fl}$ CD4-Cre R26-YFP* mice were analyzed for YFP expression (with YFP used as a marker for Cre expression and in this case, *Dgcr8* inactivation).

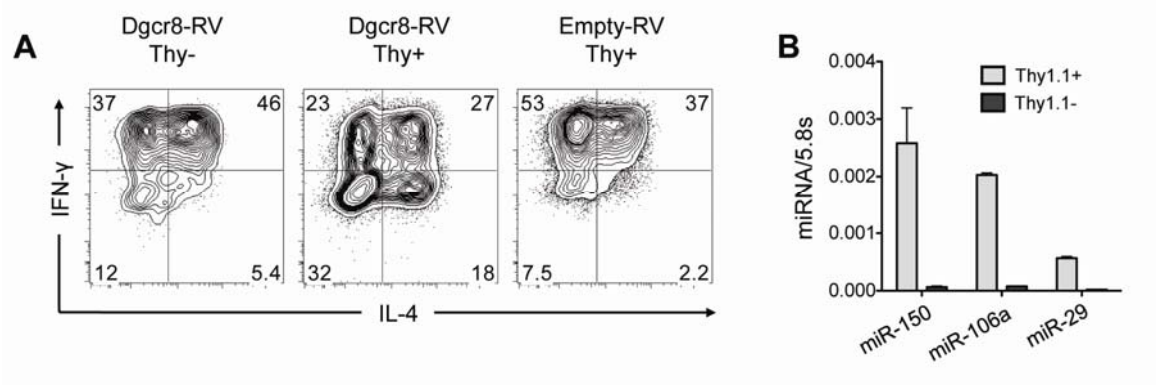

Figure S2 (related to Figure 2). Restoring miRNA expression rescues cytokine production defects of DGCR8-deficeint cells in a cell intrinsic manner
(A) CD4$^+$ T cells isolated from *Dgcr8$^{fl/fl}$ CD4-Cre R26-YFP* mice were transduced with MSCV-Dgcr8-Thy1.1 (Dgcr8-RV) or MSCV-Thy1.1 (Empty-RV) after 48 hours of stimulation. Cytokine analysis was performed on day 5 using Thy1.1 (Thy) expression to distinguish transduced and untransduced cells. Data are representative of 6 independent experiments.
(B) miRNA expression following re-introduction of DGCR8. CD4$^+$ T cells isolated from *Dgcr8$^{fl/fl}$ CD4-Cre R26-YFP* mice were transduced with MSCV-Dgcr8-Thy1.1 on day 2. Transduced (Thy1.1$^+$) and untransduced (Thy1.1$^-$) cells were isolated by FACS on day 5 and miRNA expression was analyzed by qPCR. Error bars represent range for replicate qPCR reactions.

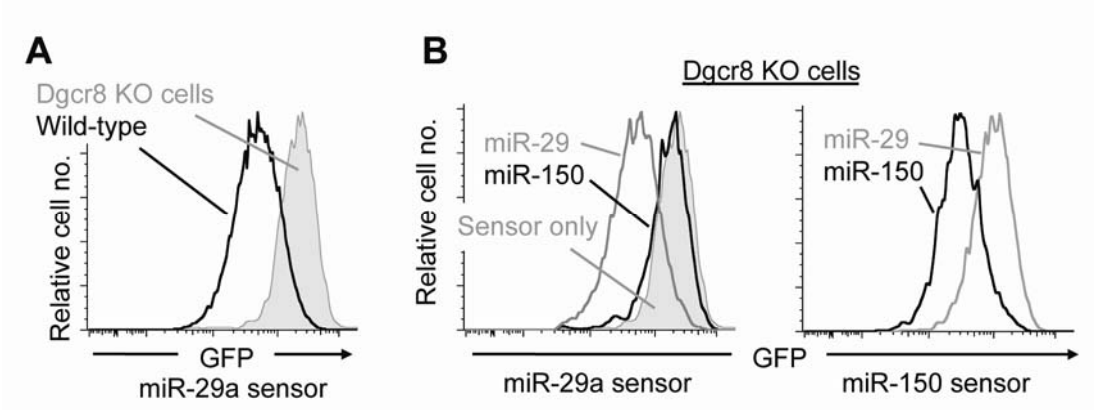

Figure S3 (related to Figure 3). Validating miRNA gain of function by transfection in primary helper T cells
(A) CD4$^+$ T cells were isolated from *Dgcr8$^{fl/fl}$ CD4-Cre* or wildtype mice and transduced with miR-29a sensor retrovirus on day 2 (see methods for sensor description). Sensor GFP expression was analyzed on day 5. To control for possible differences in transduction efficiency between cultures, analysis was limited to cells with equal expression of human-CD25 that is independently transcribed from the sensor retrovirus and is not affected by miRNA activity.
(B) *Dgcr8$^{fl/fl}$ CD4-Cre R26-tdRFP* CD4$^+$ T cells were transduced with mir-29a sensor (left panel) or miR-150 sensor (right panel) on day 2. Cells were transfected with miRNA oligonucleotides on day 4 and analyzed 24 hours later as in (A).

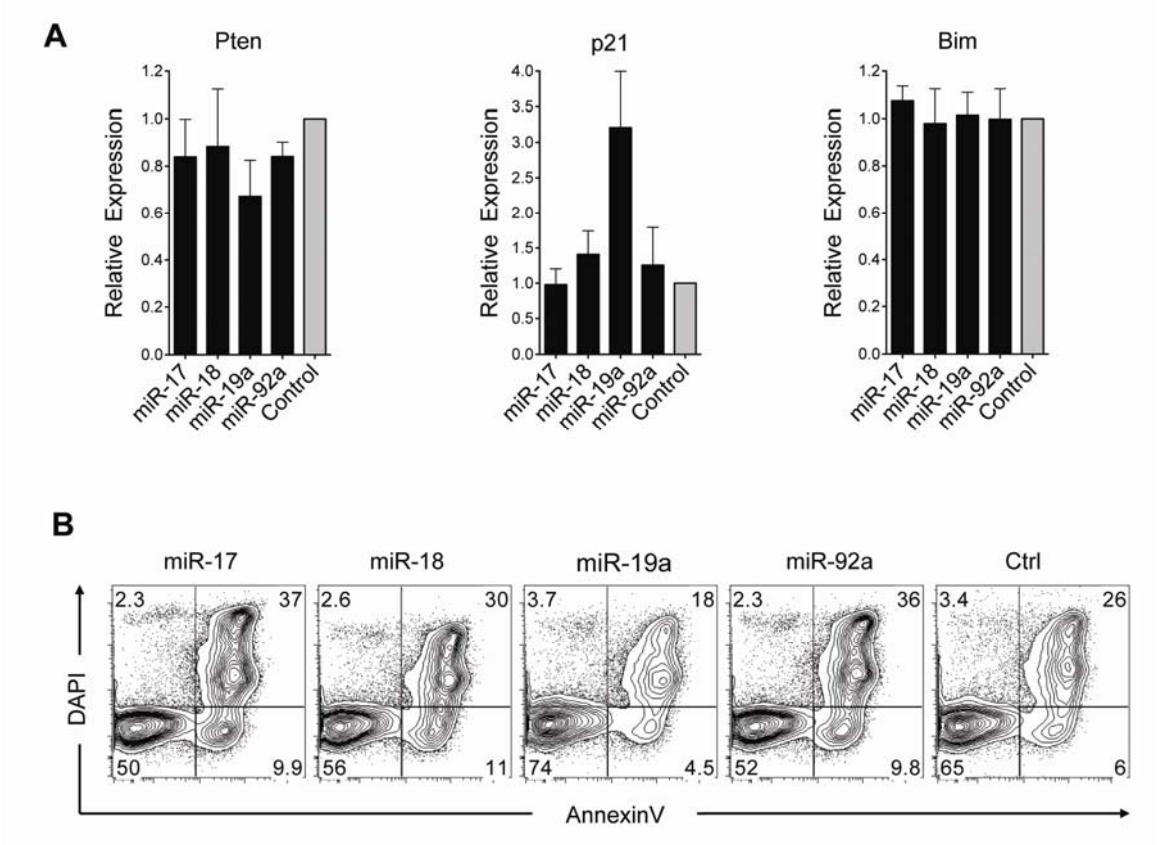

Figure S4 (related to Figure 3). Effects of miR-17~92 in DGCR8-defiecient helper T cells.

(A) Expression of previously validated miR-17~92 target mRNAs. DGCR8-deficient $CD4^+$ T cells were transfected with individual miRNAs of the miR-17~92 cluster. RNA was isolated 24 hours following transfection and subjected to qRT-PCR for the indicated genes. mRNA expression was normalized to Gapdh and is presented relative to expression in cells transfected with control miRNA. Values are means ± SD from 3 biological replicates.

(B) Viability analysis on day five of culture for DGCR8-deficient cells transfected with the miR-17~92 cluster miRNAs. Representative of 3 independent experiments.

	miRNA* sequence	No. miRNA* reads	Mature miRNA sequence	No. miRNA Reads
miR-29a	ACTGATTTCTTTTGGTGTTCAG	213	TAGCACCATCTGAAATCGGTTA	20157
miR-29b-1	GCTGGTTTCATATGGTGGTTTAGATTTA	53	TAGCACCATTTGAAATCAGTGTT	23808
miR-29b-2	CTGGTTTCACATGGTGGCTTAGATT	6	TAGCACCATTTGAAATCAGTGTT	23875
miR-29c	TGACCGATTTCTCCTGGTGTTC	8	TAGCACCATTTGAAATCGGTTA	89

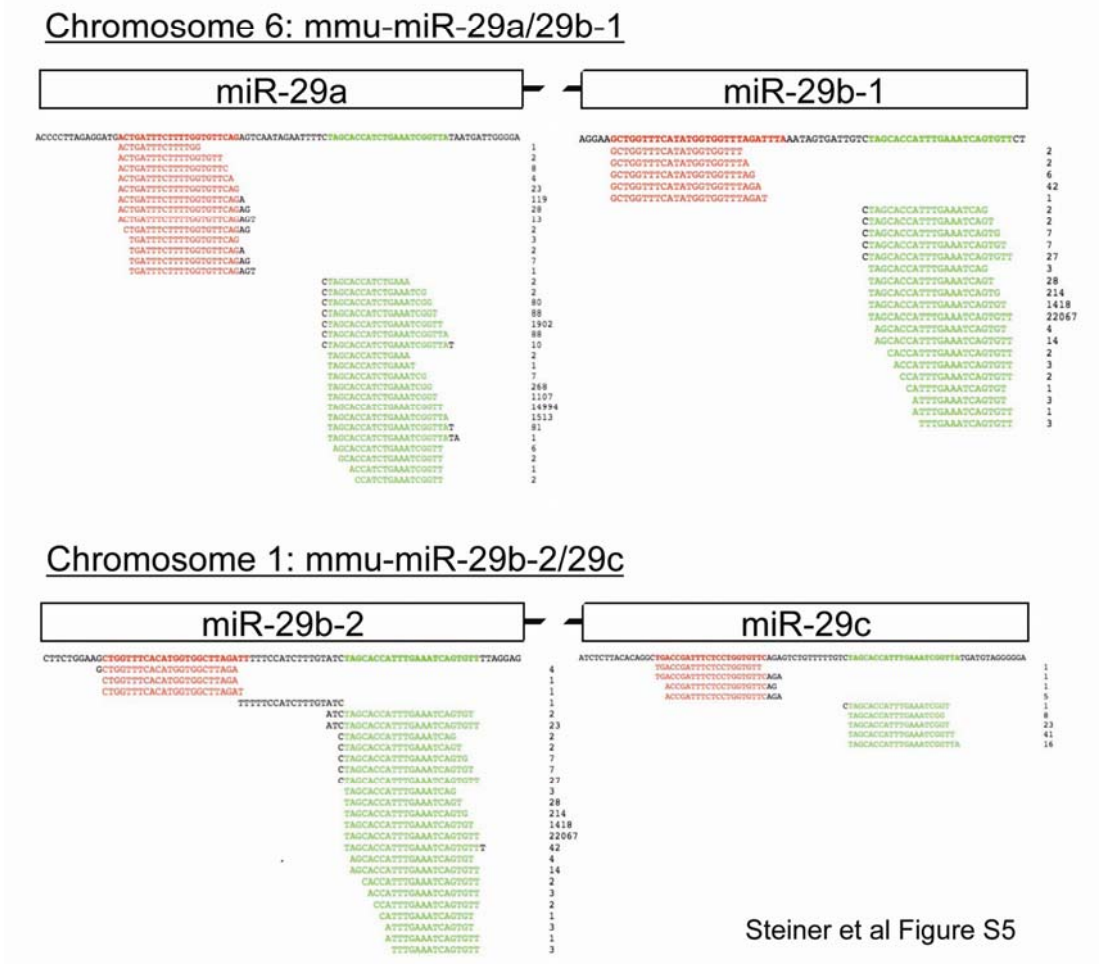

Figure S5 (related to Figure 4). Deep sequencing analysis of miR-29 cluster expression in activated helper T cells.
Small RNA reads mapping to the miR-29 sequence clusters on chromosome 1 and 6. Deep sequencing libraries of small (18-30 bp) RNAs were generated from $CD4^+$ T cells stimulated *in vitro* for 44 hours with anti-CD3 and anti-CD28. Read numbers were obtained from two independent small RNA libraries and represent only perfect matches to the miR-29 loci. Note that miR-29b sequence reads map to both the miR-29b-1 and the miR-29b-2 loci because they share identical mature miRNA sequences. However, the loci are distinguished by different miR-29b* sequences as well as the linked miR-29a or miR-29c sequences.

Supplemental Experimental Procedures

T cell stimulation and culture

Purified $CD4^+$ T cells were stimulated with hamster anti-mouse CD3 (clone 2C11, 0.25 μg/mL) and anti-mouse CD28 (clone 37.51, 1 μg/mL) on plates coated with goat anti-hamster IgG (0.3 mg/mL in PBS for 2 hours at room temperature; MP Biomedicals) for 60-68 hours at an initial cell density of 0.7-1 x 10^6 cells/mL. Following stimulation, cells were expanded in media with 20 units/mL of recombinant IL-2 (National Cancer Institute). ThN (non-polarizing, no exogenous cytokines or blocking antibodies), Limiting IL-4 (10units/mL IL-4), anti-IFN-γ only (5 μg/mL anti-IFN-γ), Th2 (500 units/mL IL-4, 5 μg/mL anti-IFN-γ clone XMG1.2), or Th1 (10ng/mL IL-12, 10 μg/mL anti-IL-4) conditions were maintained throughout stimulation and expansion. The resulting cultures were free of $CD8^+$ T cells (<2%) when analyzed by flow cytometry 5 d after activation. For experiments involving carboxyfluorescein succinimidy ester (CFSE), cells were labeled for eight minutes with 5 uM CFSE, quenched with an equal volume of fetal bovine serum (FBS), and washed twice in 10% FBS prior to stimulation and culture. T cell culture was in DMEM high glucose media supplemented with 10% FBS, pyruvate, nonessential amino acids, MEM vitamins, L-arginine, L-asparagine, L-glutamine, folic acid, beta mercaptoethanol, penicillin, and streptomycin.

T cell transfection and synthetic miRNA and siRNA oligonucleotides.

Transfections were performed using 2-3 x 10^7 cells/mL in 10 μL “R buffer” (Invitrogen) with a RNA concentration of 500 nM unless otherwise specified. Optimized setting used was 1550 V with three 10 ms pulses. All transfection experiments involving cytokine analysis consisted of one transfection after 24 h of stimulation and a second transfection at 90-100 h. For the initial transfection, cells were removed from plates, transfected, and returned to plate-bound stimulation (anti-CD3/28). Following the second transfection, cells were returned to media with 20 units/mL IL-2.

Mouse Tbx21 siRNA #1; target sequence: ACACACACGUCUUUACUU).

Mouse Eomes siRNA #3); target sequence: AACACTGAAGAGTACAGTAAA).

miR-29a and miR-29b antisense hairpin inhibitors (Dharmacon) were combined and used at 500 nM of each. Negative control miRNA and inhibitor were based on cel-miR-67 (mature sequence UCACAACCUCCUAGAAAGAGUAGA, Dharmacon).

Dgcr8, pri-miR constructs, and miRNA sensors

Dgcr8 cDNA was subcloned into MSCV-IRES-Thy1.1 DEST (www.addgene.org: plasmid 17442). Pri-miR constructs were generated by amplifying and subcloning the endogenous miRNA hairpin with ~150 nucleotides of 5' and 3' flanking sequence into MSCV-IRES-Thy1.1 DEST. Pri-miR-29a parent construct was provided by A.Goga. MiRNA sensor retroviral constructs were generated by subcloning 4 perfectly complementary miRNA binding sites immediately downstream of a GFP coding gene with each miRNA binding site separated by 4 base pairs. The GFP-miRNA binding site (GFP-miR-BS) segment was then inserted into a MSCV-PGK-hCD25 retroviral construct to provide expression of a human CD25 reporter gene that is not affected by miRNA activity. Cells transduced with MSCV-GFP-miR-BS-PGK-hCD25 retroviruses were transfected with synthetic miRNA oligonucleotides on day 4, and analyzed for GFP expression on day 5. To control for possible differences in transduction efficiency between samples, GFP analysis was limited to cells with matched surface expression of the miRNA-independent hCD25 transduction reporter gene. For luciferase reporter assays, the full-length 3'UTR of *Tbx21* and *Eomes* were amplified from murine activated T cell cDNA with the following primers and cloned into psiCHECK-2 (Promega):
T-bet fwd, 5'-CTCGAGGAAAATGCCGCTGAATTG-3'
T-bet rev, 5'-GCGGCCGCTTTACCAGGTCCATGTTTATTTC-3';
Eomes fwd, 5'-CTCGAGAGCATTATTTTAACCTTTAACC-3';
Eomes rev, 5'-GCGGCCGCTACAGAAGACAGAGCTATACC-3'.

Retroviral Transduction

$CD4^+$ cells were stimulated as described for 48 hours and transduced with retrovirus produced by Phoenix-E packaging cells transfected with retroviral plasmids. Following 6 hours of incubation with virus and 8 μg/mL polybrene, media was replaced and cells were cultured and expanded for analysis.

Intracellular staining and antibodies

After 5 days of culture, cells were restimulated for 4 hours with 10 nM phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin in the presence of 5 μg/mL brefeldin A, fixed with 4% formaldahyde and permeabilized and stained in PBS containing 0.5% saponin, 1% bovine serum albumin (BSA), and 0.1% sodium azide. For phosphorylated (Y701) STAT1, cells were fixed and permeabilized in 100% methanol and stained in

PBS containing 1% BSA. Fluorophore-conjugated antibodies for Eomes, T-bet, IFN-γ, and IL-4 were from eBioscience. APC-pSTAT1 and biotin-IFNGR1 were from BD Biosciences. Data was collected using a LSRII with FACSDiva software (BD Biosciences), and analyzed with Flowjo software.

RNA isolation and Quantitative PCR

Total RNA was isolated with Trizol LS reagent (Invitrogen). For mRNA expression analysis cells were harvested on day 2 or day 5, 24 hours after miRNA transfection. cDNA was synthesized using the Superscript III Kit for mRNA (Invitrogen). For miRNA analysis, total RNA from d5 cells was subject to polyA addition and cDNA synthesis (Ncode kit, Invitrogen). Real-time PCR analyses for miRNAs were performed with FastStart Universal SYBR green (Roche) using universal reverse qPCR primer (Invitrogen) and forward primers with sequence identical to that of the mature miRNA sequence. Solaris mRNA assays (Dharmacon) were used for *Pten, CDKN1a, Bim,* and *Eomes* and 5'-FAM labeled probes were used for *Tbx21* and *Gapdh*, All qPCR was done using a realplex2 (Eppendorf).

Tbx21 sense 5′-CAACAACCCCTTTGCCAAAG-3′;
Tbx21 anti-sense 5′-TCCCCCAAGCAGTTGACAGT-3′;
Tbx21 probe FAM-5′- CCGGGAGAACTTTGAGTCCATGTACGC-3′-Tamra.
Gapdh sense 5'-CTCGTCCCGTAGACAAAATGG-3';
Gapdh antisense 5'-AATCTCCACTTTGCCACTGCA-3';
Gapdh probe FAM-5'-CGGATTTGGCCGTATTGGGCG-3'-Tamra.

Solexa Sequencing and Bioinformatics Analysis

Purified $CD4^+$ T cells were activated for 44 h under Th1 (10 ng/mL recombinant IL-12 (Peprotech)) or Th2 conditions. Total RNA was isolated using miRNeasy kit (Qiagen). Small (18-30 bp) RNA libraries were constructed as described previously (Thomas and Ansel, 2010) and samples were sequenced on an Illumina 1G Genome Analyzer. Adaptor sequences were trimmed from sequence reads as described previously (Babiarz et al., 2008). All adaptor-extracted reads 15-30 nt in length were mapped to the the mouse genome (UCSC mm8 assembly) and only sequences with perfect matches to the genome were used for further analysis. Mouse scRNA, snRNA, srpRNA and rRNA sequences annotations were compiled from the UCSC genome browser RepeatMasker track (Karolchik et al., 2004), and miRNA sequences were annotated using the

miRanalzyer online database (http://web.bioinformatics.cicbiogune.es/microRNA/miRanalyser.php) (Hackenberg et al., 2009). Mouse tRNA annotations were compiled from the Lowe lab tRNA database website (http://lowelab.ucsc.edu/GtRNAdb/credits-citation.html) (Chan and Lowe, 2009) and mouse snoRNA annotations were compiled as described previously (Taft et al., 2009).

Supplemental References

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