PNAS
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Supplementary Information for

The histone methyltransferase DOT1L prevents antigen-independent differentiation and safeguards epigenetic identity of CD8$^+$ T cells

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www.pnas.org/cgi/doi/10.1073/pnas.1920372117

Supplementary Materials and Methods

Mice

Lck-Cre;*Dot1L*$^{fl/fl}$ mice have been described elsewhere previously (1) and were based on the Dot1Ltm1a(KOMP)Wtsi line generated by the Wellcome Trust Sanger Institute (WTSI) and obtained from the KOMP Repository (www.komp.org) (2). Mice from this newly created *Lck*-Cre;*Dot1L* strain were breeding in a mendelian ratio and had no welfare issues. *Lck*-Cre$^{+/-}$;*Dot1L*$^{fl/fl}$ (KO) mice were compared to *Lck*-Cre$^{+/-}$;*Dot1L*$^{wt/wt}$ (WT) mice and were indicated with *Lck*-Cre$^{+/-}$;*Dot1L*$^{fl/wt}$ (Het) mice in order to eliminate any Cre-specific effects (3). The *Lck*-Cre;*Dot1L*$^{fl/fl}$ mice were crossed with OT-I (B6J) mice (a kind gift from the Ton Schumacher group, originally from Jackson labs) to generate *Lck*-Cre;*Dot1L*$^{fl/fl}$;*OT-I* mice. Both strains were bred in-house. Mice used for experiments were between 6 weeks and 8 months old and of both genders. For each individual experiment mice were matched for age and gender. Mice were housed under specific pathogen free (SPF) conditions at the animal laboratory facility of the Netherlands Cancer Institute (NKI; Amsterdam, Netherlands). All experiments were approved by the Animal Ethics Committee of the NKI and performed in accordance with institutional, national and European guidelines for animal care and use.

Flow cytometry

Single cell suspensions were made from spleen and thymus. Erylysis was performed on blood and spleen samples. Cells were stained with fluorescently labeled antibodies in a 1:200 dilution unless otherwise indicated (*SI Appendix,* Table S3). Of note, for OT-I tetramer stains the CD8 antibody clone 53-6.7 was used (4). For intracellular staining, cells were fixed and permeabilized using the Transcription Factor Buffer kit (Benton Dickinson). Antibodies for intracellular staining were diluted 1:200 in Perm/Wash buffer (*SI Appendix,* Table S4). For H3K79me2 staining, cells were first stained with surface markers and fixed and permeabilized as described before. After fixation and permeabilization cells were washed with Perm/Wash containing 0.25% sodium dodecyl sulfate (SDS) to expose the epitope. α-H3K79me2 (Millipore) was diluted 1:200 into Perm/Wash + 0.25% SDS and cells were incubated for 30 min. Cells were washed with Perm/Wash and incubated with the secondary antibodies Donkey anti-Rabbit AF555 (Thermo Scientific) or Goat-anti-Rabbit AF488 (Invitrogen) 1:1000 in Perm/Wash. Flow cytometry was performed using the LSR Fortessa (BD Biosciences) and data were analyzed with FlowJo software (Tree Star inc.) Histograms were smoothed.

Immunohistochemistry

Thymus tissues were fixed in EAF (ethanol, acetic acid, formol saline) for 24 hours and subsequently embedded in paraffin. Immunohistochemistry was performed with H3K79me2 antibody (RRID:AB_2631106) (5) as described in (1).

PCR

Sorted CD4$^+$ and CD8$^+$ T cells were lysed in DirectPCR Lysis Reagent (mouse tail) (Viagen Biotech) with 1mg/ml proteinase K (Sigma). *Dot1L*$^{fl/fl}$ and *Dot1L*$^{\Delta/\Delta}$ was detected by PCR using the following primers. Dot1L_FWD: GCAAGCCTACAGCCTTCATC, Dot1L_REV: CACCGGATAGTCTCAATAATCTCA and Dot1L_Δ: GAACCACAGGATGCTTCAG and MyTaq Red Mix (GC Biotech).

In vitro stimulation

To determine cytokine production upon *in vitro* stimulation, splenocytes were stimulated with 20 ng/ml PMA (Sigma), 0.5 µg/ml Ionomcyin (Sigma) and incubated with 1µl/ml Golgi Plug protein transport inhibitor (Benton Dickinson) for 4 hours. Cells were stained as described above. To determine IFNγ production upon *in vitro* TCR-mediated stimulation splenocytes were enriched for T cells using CD19 microbeads depletion (Miltenyi Biotec) or pan-T cell Isolation Kit II (Miltenyi Biotec) on LS columns (Miltenyi Biotec). Cells were plated in a 96-wells plate coated with anti-CD3 (145-2C11) (BD) in complete medium (IMDM supplemented with 8% FCS, 100 mM pen/strep and 100 mM β-mercaptoethanol). Anti-CD28 (37.51) (BD) was added to the medium with recombinant IL-2 (30 U/ml) and cells were incubated at 37 ^oC with 5% CO_2. In order to

determine IFNγ production cells were incubated with Golgi Plug protein transport inhibitor (Benton Dickinson) for 4 hours.

In vivo immunization with *Listeria monocytogenes*

Listeria monocytogenes strain LM-OVA was a gift from Ton Schumacher (NKI). Bacteria were grown overnight in Bacto Brain Heart Infusion, Porcine (BHI) medium (BD) to an absorbance at 600 nm of OD 0.9. A sub-lethal dose (10.000 colony forming units; CFUs) of *Listeria monocytogenes* in Hank's Balanced Salt Solution (HBSS) was injected intravenously into the mice. The number of bacteria injected was confirmed by growth on BHI agar plates (BD) with 25 µg/ml chloramphenicol. The immune response was followed in the blood by taking blood samples via tail vein puncture at day -3 and +4. The CFUs were determined for spleen and liver. Single-cell suspensions were made in PBS and the cells were lysed in 0.1 % IGEPAL. Homogenized suspensions were plated on BHI agar plates in three dilutions (undiluted, 1:10 and 1:100). After 48 hours colony counts were determined.

In vivo vaccination

The Help-E7SH vaccination was performed as described in (6). The hair on the hind leg was removed using depilating cream (Veet) on day -1. On day 0, mice were anesthetized and 15 µl of 2 mg/ml DNA solution in 10 mM Tris-HCl, 1 mM EDTA pH8.0 was applied to the hairless skin with a Permanent Make Up tattoo machine (Amiea, MT Derm GmgH) using a sterile disposable 9-needle bar with a depth of 1 mm and oscillating at a frequency of 100 Hz for 45 sec.

Mixed bone-marrow chimera

Whole bone marrow cells were isolated from femurs of the indicated mice by flushing out with IMDM medium. Single-cell suspensions from $Ly5.1^+$ and $Ly5.2^+$ were mixed in a 1:1 ratio in HBSS. A total of 10^6 cells were injected intravenously. into $Ly5.1^+$ recipients lethally irradiated with 2x 5.5Gy (Faxitron), separated by a 3-h interval. After 20 weeks mice were sacrificed and spleens isolated for further analysis.

Sort for RNA-Seq, ChIP-Seq and TCR-Seq

For cell sorting, thymus and spleen samples were homogenized. Erylysis was performed on spleen samples. Cells were stained with fluorescently labeled antibodies (1:200) (*SI Appendix,* Table S5) and sorted on FACSAria Ilu (BD Biosciences), FACSAria Fusion (BD Biosciences) or MoFlo Astrios (Beckman Coulter). Cells were collected in tubes coated with FCS.

RNA-Seq sample preparation

Flow cytometrically purified cells from unchallenged mice were resuspended in Trizol (Ambion Life Technologies). Total RNA was extracted according to the manufacturer's protocol. Quality and quantity of the total RNA was assessed by the 2100 Bioanalyzer using a Nano chip (Agilent). Only RNA samples having an RNA Integrity Number (RIN) > 8 were subjected to library generation.

RNA-Seq library preparation

Strand-specific cDNA libraries were generated using the TruSeq Stranded mRNA sample preparation kit (Illumina) according to the manufacturer's protocol. The libraries were analyzed for size and quantity of cDNAs on a 2100 Bioanalyzer using a DNA 7500 chip (Agilent), diluted and pooled in multiplex sequencing pools. The libraries were sequenced as 65 base single reads on a HiSeq2500 (Illumina).

RNA-Seq data preprocessing

RNA-Seq reads were mapped to mm10 (Ensembl GRCm38) using TopHat with the arguments `--prefilter-multihits –no-coverage-search –bowtie1 –library-type fr-firststrand` using a transcriptome index. Counts per gene were obtained using htseq-count with the options `-m union -s no` and Ensembl GRCm38.90 gene models. Subsequent analysis was performed in R version 3.6.3 with Bioconductor packages from release 3.10. Analysis was restricted to genes that have least 20 counts in at least 4 samples, and at least 5 counts in 4 samples included in specific contrasts, to

exclude very low abundance genes. Differential expression analysis was performed on only relevant samples using DESeq2 and default arguments with the design set to either *Dot1L*KO status or cell type. Adaptive effect size shrinkage was performed with the ashr package version 2.2-47 to control for the lower information content in low abundance transcripts. Genes were considered to be differentially expressed when the p-value of the negative binomial Wald test was below 0.01 after the Benjamini-Hochberg multiple testing correction. Sets of differentially expressed genes in indicated conditions were called 'gene signatures'. Exceptions were made for the Ezh2 KO RNA-Seq data from He et al., (7) wherein the dispersion was estimated with a local fit, using `estimateDispersion` function in DESeq2 with the argument `fitType = "local"` and using an adjusted p-value cutoff of 0.05, to increase the number of detected differential genes. Principal component analysis was performed using the `prcomp` function on variance stabilizing transformed data with the `vst` function from the DESeq2 package using default arguments. For analyses where we performed expression matching, we chose genes with an absolute log2 fold changes less than 0.1 and false discovery rate corrected p-values above 0.05 that were closest in mean expression to each of the genes being matched without replacement. GSEAs were performed with the fgsea Bioconductor package using ranked, shrunken fold changes from the differential expression analysis as input.

ChIP-Seq sample preparation

Sorted cells were centrifuged at 500 rcf. The pellet was resuspended in IMDM containing 2% FCS and formaldehyde (Sigma) was added to a final concentration of 1%. After 10 min incubation at RT glycine (final concentration 125 mM) was added and incubated for 5 min. Cells were washed twice with ice-cold PBS containing Complete, EDTA free, protein inhibitor cocktail (PIC) (Roche). Cross-linked cell pellets were stored at -80 ^{o}C. Pellets were resuspended in cold Nuclei lysis buffer (50mM Tris-HCl pH 8.0, 10mM EDTA pH8.0, 1%SDS) + PIC and incubated for at least 10 min. Cells were sonicated with PICO to an average length of 200-500bp using 30s on/ 30s off for 3 min. After centrifugation at high speed debris was removed and 9x volume of ChIP dilution buffer (50mM Tris-HCl pH8, 0.167M NaCl, 1.1% Triton X-100, 0.11% sodium deoxycholate) + PIC and 5x volume of RIPA-150(50mM Tris-HCl pH8, 0.15M NaCl, 1mM EDTA pH8, 0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate) + PIC was added. Shearing efficiency was confirmed by reverse crosslinking the chromatin and checking the size on agarose gel. Chromatin was pre-cleared by adding ProteinG Dynabeads (Life Technologies) and rotation for 1 hour at 4 ^{o}C. After the beads were removed 2µl H3K79me1, 2µl H3K79me2 (NL59, Merck Millipore) and 1µl H3K4me3 (ab8580, Abcam) were added and incubated overnight at 4^{o}C. ProteinG Dynabeads were added to the IP samples and incubated for 3 hours at 4 ^{o}C. Beads with bound immune complexes were subsequently washed with RIPA-150, 2 times RIPA-500 (50mM Tris-HCl pH8, 0.5M NaCl, 1mM EDTA pH8, 0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate), 2 times RIPA-LiCl (50mM Tris-HCl pH8, 1mM EDTA pH8, 1% Nonidet P-40, 0.7% sodium deoxycholate, 0.5M LiCl2) and TE. Beads were resuspended in 150 µl Direct elution buffer (10mM Tris-HCl pH8, 0.3M NaCl, 5mM EDTA pH8, 0.5%SDS) and incubated overnight at 65 ^{o}C and input samples were included. Supernatant was transferred to a new vial and 1µl RNAse A (Sigma) and 3 µl ProtK (Sigma) was added per sample and incubated at 55 ^{o}C for 1 hour. DNA was purified using Qiagen purification columns.

ChIP-Seq Library preparation

Library preparation was done using KAPA LTP Library preparation kit (Roche) using the manufacturer's protocol with slight modifications. Briefly, after end-repair and A-tailing adaptor were ligated followed by Solid Phase Reversible Immobilization (SPRI) clean-up. Libraries were amplified by PCR and fragments between 250-450 bp were selected using AMPure XP beads (Beckman Coulter). The libraries were analyzed for size and quantity of DNAs on a 2100 Bioanalyzer using a High Sensitivity DNA Kit (Agilent), diluted and pooled in multiplex sequencing pools. The libraries were sequenced as 65 base single reads on a HiSeq2500 (Illumina).

ChIP-Seq data preprocessing

ChIP-Seq samples were mapped to mm10 (Ensembl GRCm38) using BWA-MEM with the option '-M' and filtered for reads with a mapping quality higher than 37. Duplicate reads were removed

using MarkDuplicates from the Picard toolset with `VALIDATION_STRINGENCY=LENIENT` and `REMOVE_DUPLICATES=false` as argument. Low quality reads with any FLAG bit set to 1804 were removed. Bigwig tracks were generated by using bamCoverage from deepTools using the following arguments: `-of bigwig –binsize 25 –normalizeUsing RPGC –ignoreForNormalization chrM –effectiveGenomeSize 2652783500`. For visualization of heatmaps and genomic tracks, bigwig files were loaded into R using the `import.bw()` function from the rtracklayer R package. TSSs for heatmaps and read counting were taken from Ensembl GRCm38.90 gene models by taking the first base pair of the 5' UTR of transcripts. When such annotation was missing, the most 5' position of the first exon was taken.

Defining DOT1L targets involved in transcription regulation

Genes were selected that are down in KO with adjusted p-value < 0.01, with H3K79me2 normalized reads > 20 and the GO annotation "negative regulator of transcription by RNA polymerase II" GO:0000122 from AmiGO (8).

TCRβ-Seq

Between 100,000 and 1,100,000 sorted cells were used for TCRβ-Seq. DNA was extracted using Qiagen DNA isolation kit (Qiagen) and collected in 50 µl TE buffer. The concentration was determined with Nanodrop. Samples were sequenced and processed by Adaptive Biotechnologies using immunoSEQ mm TCRB Service (Survey).

Statistics

Statistical analyses were performed using Excel. Variance was determined using a F-test and an unpaired Student's t-test with two-tailed distribution was used for statistical analyses. Data are presented as means ± SD unless otherwise indicated in the figure legends. For Fig. 1*B* and *SI Appendix*, Fig. S1*D*, *E* and *H* an Student's t-test with Bonferroni correction was performed in R. A p-value <0.05 was considered statistically significant.

Figure S1

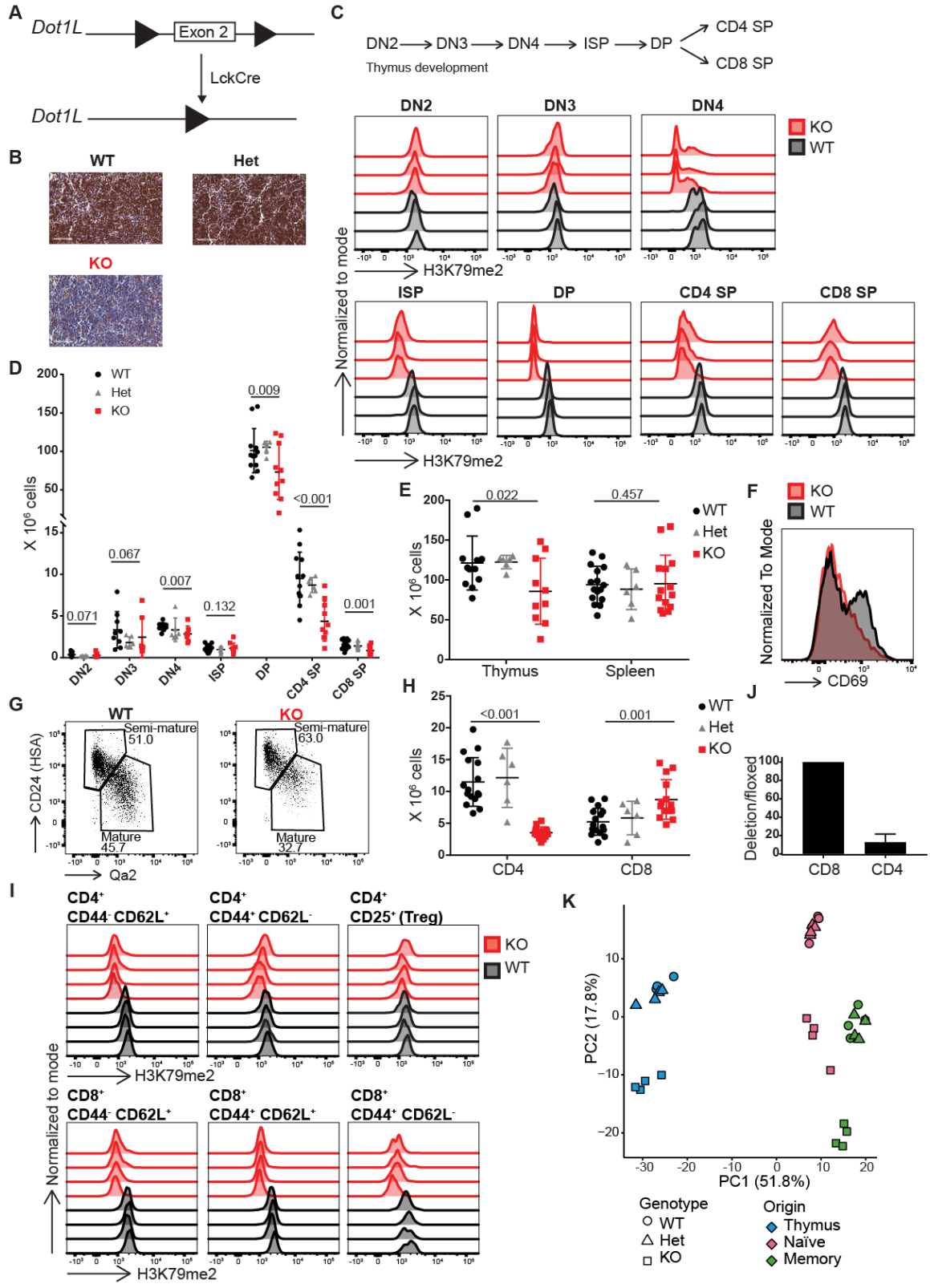
A
Dot1L
Exon 2
LckCre
Dot1L
B
WT
Het
KO
C
DN2 → DN3 → DN4 → ISP → DP
CD4 SP
CD8 SP
Thymus development
DN2
DN3
DN4
KO
WT
Normalized to mode
H3K79me2
ISP
DP
CD4 SP
CD8 SP
D
WT
Het
KO
X 10^6 cells
0.071
0.067
0.007
0.132
0.009
<0.001
0.001
DN2
DN3
DN4
ISP
DP
CD4 SP
CD8 SP
E
0.022
0.457
X 10^6 cells
Thymus
Spleen
F
KO
WT
Normalized To Mode
CD69
G
WT
KO
Semi-mature
51.0
Mature
45.7
Semi-mature
63.0
Mature
32.7
CD24 (HSA)
Qa2
H
<0.001
0.001
X 10^6 cells
CD4
CD8
J
Deletion/floxed
CD8
CD4
I
CD4+ CD44- CD62L+
CD4+ CD44+ CD62L-
CD4+ CD25+ (Treg)
CD8+ CD44- CD62L+
CD8+ CD44+ CD62L+
CD8+ CD44+ CD62L-
KO
WT
Normalized to mode
H3K79me2
K
PC2 (17.8%)
PC1 (51.8%)
Genotype
WT
Het
KO
Origin
Thymus
Naïve
Memory

Figure S1: Validation of the Lck-Cre;Dot1L$^{fl/fl}$ mouse model.
(*A*) Schematic overview of the *Lck*-Cre*;Dot1L*$^{fl/fl}$ mouse model. (*B*) Immunohistochemistry with α-H3K79me2 (brown) on fixed thymus tissue of WT, and heterozygous and homozygous *Dot1L*-KO. (*C*) Flow-cytometry analysis of H3K79me2 in thymocyte subsets in WT and homozygous *Dot1L*-KO (KO). Data from one experiment with three mice per genotype. (*D*) Absolute number of thymocyte subsets defined by flow-cytometry. Subsets are defined as follows: DN2 ($CD4^-CD8^-CD25^+CD44^{high}$), DN3 ($CD4^-CD8^-CD25^+CD44^{low}$), DN4 ($CD4^-CD8^-CD25^-CD44^-$), ISP ($CD4^-CD8^+CD3^-$), DP ($CD4^+CD8^+$), CD4 SP ($CD4^+CD8^-$) and CD8 SP ($CD4^-CD8^+CD3^+$). Data from 2-4 individual experiment with 2-3 mice per genotype per experiment. Data is represented as mean ±-SD. (*E*) Absolute number of cells in thymus and spleen. Data from 2-5 individual experiments with 2-4 mice per genotype per experiment, represented as mean ± SD. (*F*) Representative plot of CD69 expression on WT and *Dot1L*-KO CD8 SP thymocytes. (*G*) Representative plots of Qa-2 and CD24 (HSA) expression on CD8 SP thymocytes. (*H*) Absolute number of $CD4^+$ and $CD8^+$ T cells in the spleen. Data from 2-5 individual experiments with 2-4 mice per genotype per experiment, represented as mean ± SD. (*I*) Flow-cytometry analysis of H3K79me2 on indicated $CD4^+$ and $CD8^+$ T cells subsets in spleen. Data from one experiment with three mice per genotype. (*J*) PCR analysis of Dot1L deletion on sorted $CD4^+$ and $CD8^+$ splenocytes, intensity of Dot1L$^{\Delta}$ band over Dot1L^{fl} band. Data from three mice, normalized per mouse with CD8 set to 100. (*K*) Principal component analysis of RNA-Seq data from sorted $CD4^-CD8^+CD3^+$ thymocytes (thymus), $CD44^-CD62L^+$ (T_N) $CD8^+$ T cells and $CD44^+CD62L^+$ (T_{CM}) $CD8^+$ T cells, four mice per genotype except for WT thymus where there are three mice. Percentages indicate variance explained by the principal component.

Figure S2

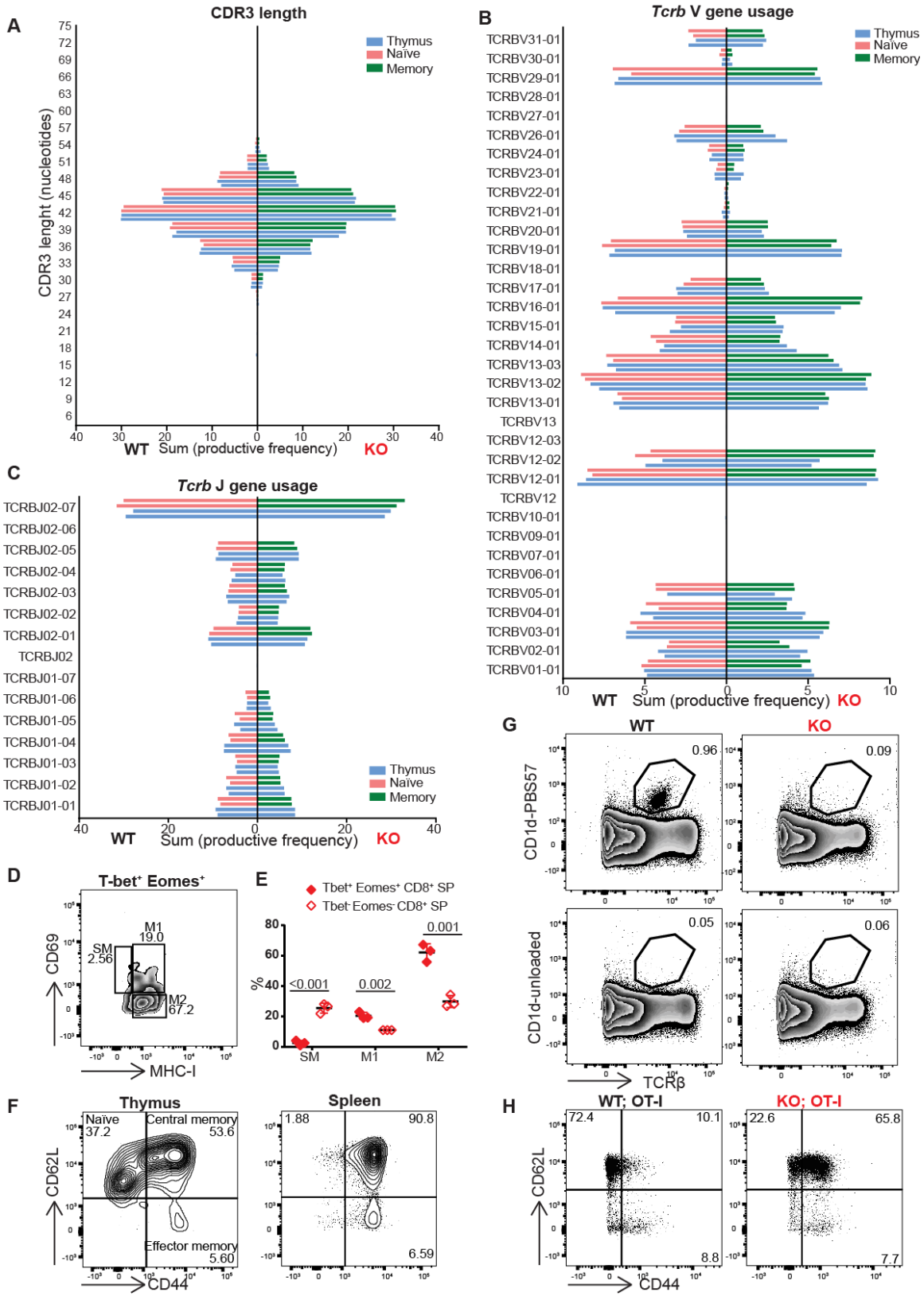
A
CDR3 length
Thymus
Naïve
Memory
CDR3 lenght (nucleotides)
WT
Sum (productive frequency)
KO
B
Tcrb V gene usage
TCRBV31-01
TCRBV30-01
TCRBV29-01
TCRBV28-01
TCRBV27-01
TCRBV26-01
TCRBV24-01
TCRBV23-01
TCRBV22-01
TCRBV21-01
TCRBV20-01
TCRBV19-01
TCRBV18-01
TCRBV17-01
TCRBV16-01
TCRBV15-01
TCRBV14-01
TCRBV13-03
TCRBV13-02
TCRBV13-01
TCRBV13
TCRBV12-03
TCRBV12-02
TCRBV12-01
TCRBV12
TCRBV10-01
TCRBV09-01
TCRBV07-01
TCRBV06-01
TCRBV05-01
TCRBV04-01
TCRBV03-01
TCRBV02-01
TCRBV01-01
C
Tcrb J gene usage
TCRBJ02-07
TCRBJ02-06
TCRBJ02-05
TCRBJ02-04
TCRBJ02-03
TCRBJ02-02
TCRBJ02-01
TCRBJ02
TCRBJ01-07
TCRBJ01-06
TCRBJ01-05
TCRBJ01-04
TCRBJ01-03
TCRBJ01-02
TCRBJ01-01
D
T-bet+ Eomes+
CD69
MHC-I
SM
2.56
M1
19.0
M2
67.2
E
Tbet+ Eomes+ CD8+ SP
Tbet- Eomes- CD8+ SP
%
<0.001
0.002
0.001
SM
M1
M2
F
Thymus
Spleen
CD62L
CD44
Naïve
37.2
Central memory
53.6
Effector memory
5.60
1.88
90.8
6.59
G
WT
KO
CD1d-PBS57
CD1d-unloaded
TCRβ
0.96
0.09
0.05
0.06
H
WT; OT-I
KO; OT-I
CD62L
CD44
72.4
10.1
8.8
22.6
65.8
7.7

Figure S2: *Dot1L* ablation does not affect clonality of the TCR repertoire.
TCRβ repertoire analysis of sorted $CD8^+$ T cell subsets of WT and *Dot1L*-KO. (*A*) Distribution of CDR3 length. (*B*) Usage of the *Tcrb*-V and c) *Tcrb*-J elements. (*D* and *E*) Representative plots and quantification of CD69 and MHC-I expression on *Dot1L*-KO CD8 SP thymocytes that were T-bet^+Eomes^+ or $Tbet^-Eomes^-$, data of one experiment with three mice, represented as mean ± SD. (*F*) Representative plots of CD44 and CD62L expression on T-bet^+Eomes^+ $CD8^+$ thymocytes and splenocytes from *Dot1L* KO. (*G*) Representative plots of iNKT (CD1d-$PBS57^+TCR\beta^+$) cells in total thymus. (*H*) Representative plot of CD44 and CD62L expression on $CD8^+$ T cells in *Lck*-Cre;*Dot1L;OT-I* mice.

Figure S3

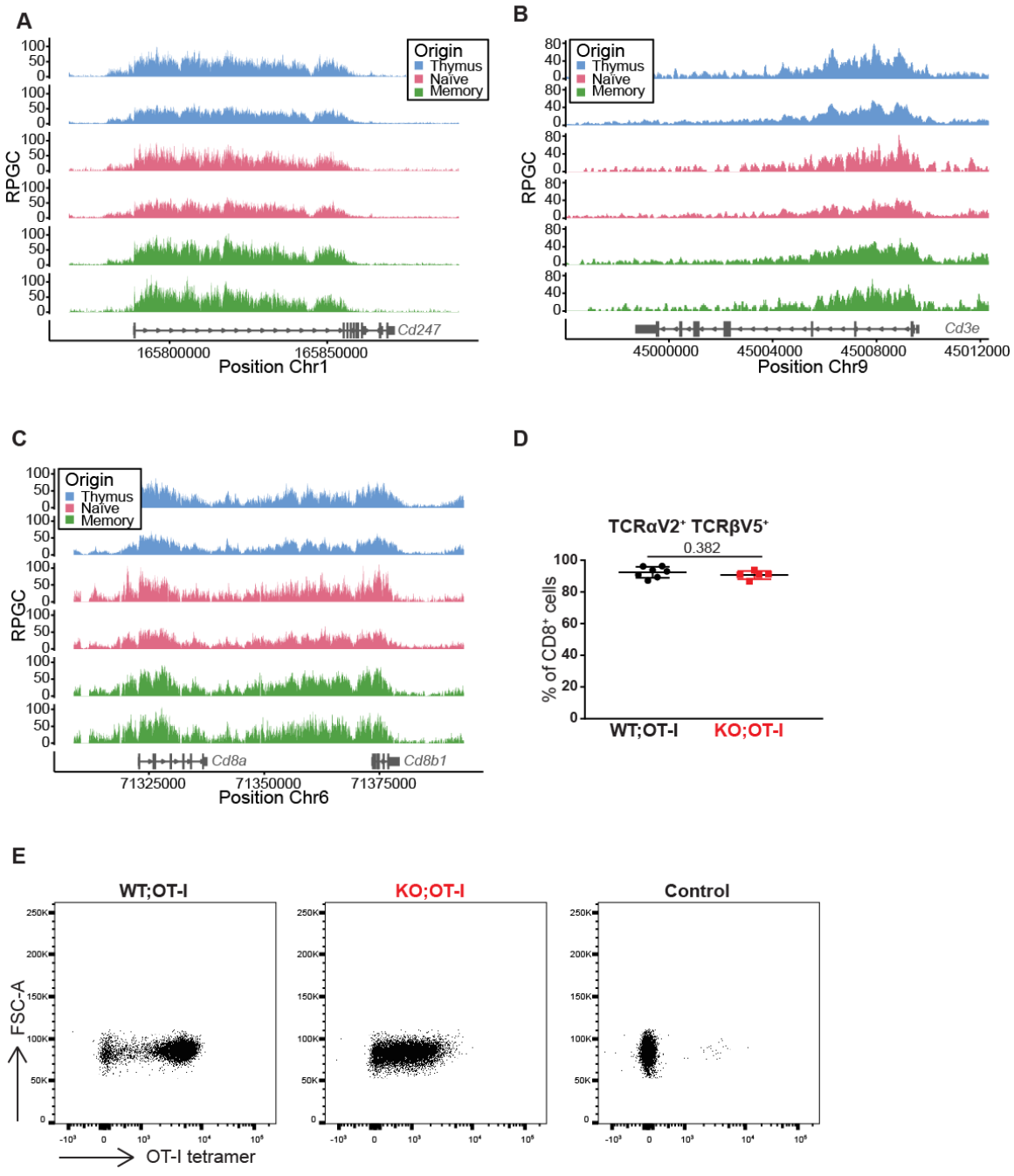

Figure S3: H3K79 methylation of selected TCR component genes in WT, and OT-I staining in WT and *Dot1L* KO.

(*A-C*) H3K79me2 ChIP-Seq data tracks for *Cd247*, *Cd3e*, *Cd8a* and *Cd8b1* in CD8$^+$ SP thymocytes (thymus), and CD8$^+$CD44$^-$CD62L$^+$ (naïve) and CD8$^+$CD44$^+$CD62L$^+$ (memory) splenocytes. Y-axis indicates reads per genomic content. (*D*) Quantification of TCRα V2 and TCRβ V5 expression on CD8$^+$ T cells from spleen. Data from two experiments with 2-4 mice per genotype, represented as mean ± SD. (*E*) Representative plot of SIINFEKL/H-2K^b tetramer staining in *Lck*-Cre$^{+/-}$;*Dot1L*$^{wt/wt}$;*OT-I*, *Lck*-Cre$^{+/-}$;*Dot1L*$^{fl/fl}$;*OT-I* mice and *Lck*-Cre$^{-/-}$;*Dot1L*$^{fl/fl}$ as control. (*E*) Quantification of TCRα V2 and TCRβ V5 expression on CD8$^+$ T cells from spleen. Data from two experiments with 2-4 mice per genotype, represented as mean ± SD.

Figure S4

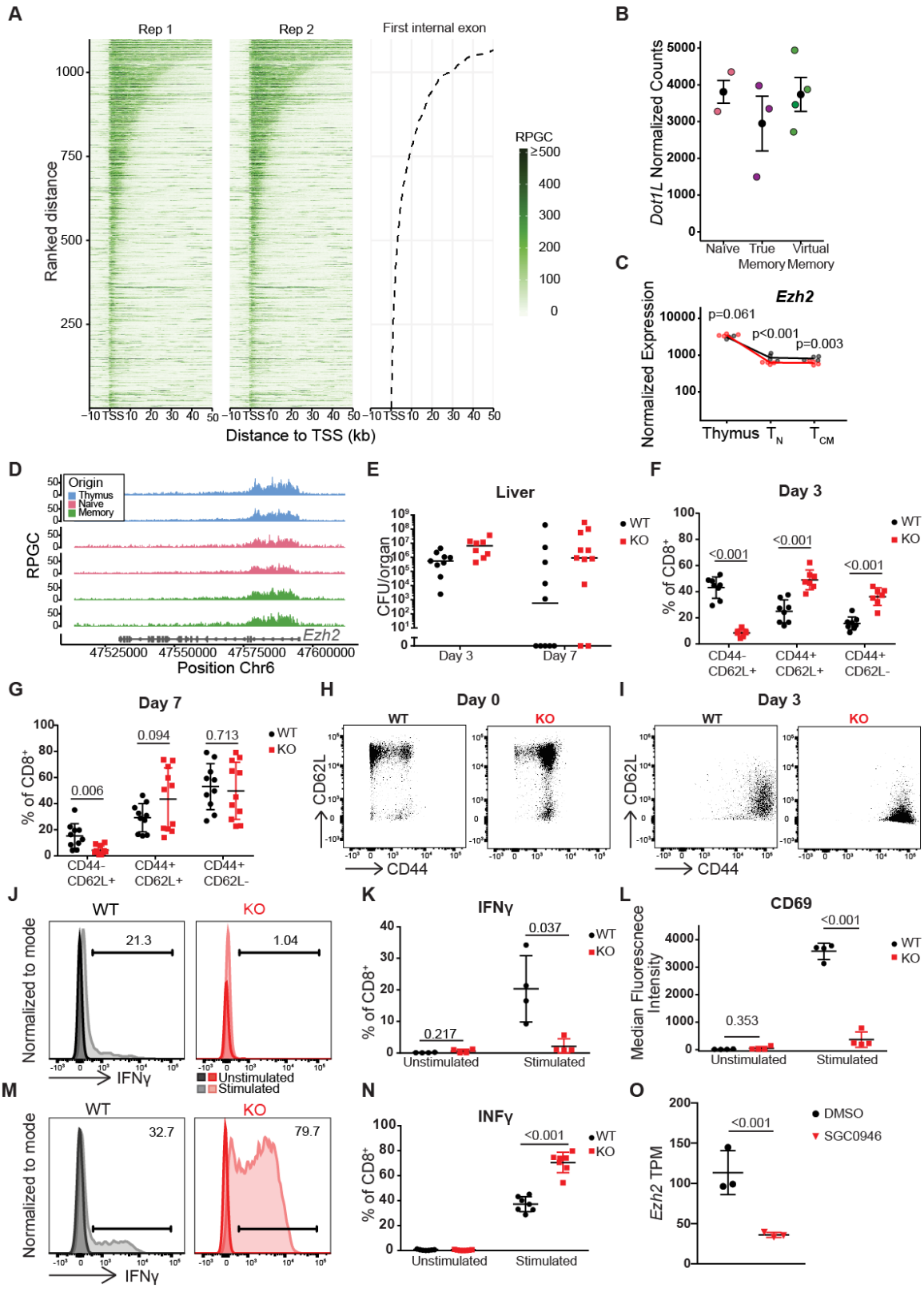
A
Rep 1
Rep 2
First internal exon
Ranked distance
RPGC
Distance to TSS (kb)
B
Dot1L Normalized Counts
Naive
True Memory
Virtual Memory
C
Ezh2
Normalized Expression
p=0.061
p<0.001
p=0.003
Thymus
T_N
T_CM
D
Origin
Thymus
Naive
Memory
RPGC
Ezh2
Position Chr6
E
Liver
CFU/organ
Day 3
Day 7
WT
KO
F
Day 3
% of CD8+
CD44- CD62L+
CD44+ CD62L+
CD44+ CD62L-
G
Day 7
H
Day 0
I
Day 3
CD62L
CD44
J
Normalized to mode
IFNγ
Unstimulated
Stimulated
K
IFNγ
L
CD69
Median Fluorescnece Intensity
M
N
INFγ
O
Ezh2 TPM
DMSO
SGC0946

Figure S4: DOT1L is required for maintenance of the epigenetic identity of CD8$^+$ T cells and for mounting an immune response.
(*A*) Heatmap of H3K79me2 in CD44$^+$CD62L$^+$ (memory) CD8$^+$ T cells on genes ranked by distance from transcription start site to first internal exon. Top 50% of highest normalized expression genes are shown for which the distance to the first internal exon could be calculated. RPGC notes reads per genomic content. (*B*) Normalized counts for *Dot1L* in naïve, true memory and virtual memory CD8$^+$ T cells from(9). (*C*) *Ezh2* expression in indicated CD8$^+$ T cell populations. FDR corrected p-values are based on negative binomial Wald test. (*D*) H3K79me2 on the *Ezh2* gene by ChIP-Seq. (*E*) Clearance of *L. monocytogenes* in liver as defined by the number of CFU per organ. Bars indicate median. Data from two or three individual experiments with 1-5 mice per genotype per experiment. (*F*) Percentage of CD44 CD62L subsets in CD8$^+$ T cells from spleen at day 3 and (*G)* day 7. (*H* and *I*) Representative plots of CD44 and CD62L expression on CD8$^+$ T cells before stimulation (H) and after 3 days of stimulation with α-CD3/ α-CD28 *in vitro (I)*. (*J and K*) IFNγ production by CD8$^+$ cells after stimulation with α-CD3/α-CD28 for 3 days and Golgi-plug incubation for four hours. Data from one experiment with four mice per genotype, represented as mean ± SD. (*L*) Median fluorescence intensity of CD69 on CD8$^+$ T cells. Data from one experiment with four mice per genotype, represented as mean ± SD. (*M* and *N*) IFNγ production of CD8$^+$ cells after PMA/Ionomycin stimulation for four hours. Data from two experiments with 3-4 mice per genotype per experiment, represented as mean ± SD. (*O*) Transcripts per million for *EZH2* in human CD8$^+$ T cells treated with DMSO or the DOT1L inhibitor SGC0946 from (10). Data is represented as mean ± SD. Significance is shown as false discovery rate calculated after edgeR normalization.

Table S1: Productive clonality scores of TCRb sequencing

Sample	Productive Clonality
WT Thymus CD8 MSP rep1	0.0112
WT Thymus CD8 MSP rep2	0.0116
KO Thymus CD8 MSP rep1	0.0144
KO Thymus CD8 MSP rep2	0.0076
WT Spleen CD8 naïve rep1	0.0080
WT Spleen CD8 naïve rep2	0.0084
KO Spleen CD8 memory rep1	0.0071
KO Spleen CD8 memory rep2	0.0090

Table S2: List of candidate negative regulators affected by DOT1L in T cells

Gene	Transcriptional regulator
Hif1a	yes
Srebf2	yes
Lef1	yes
Notch1	yes
Mbd2	yes
Ezr	no
Zeb1	yes
Usp3	yes
Satb1	yes
Fnip1	no
Phb	no
Ezh2	yes
Zbtb20	yes
Rps14	no
Ybx1	yes
Smad7	no
Rpl10	no
Smad4	no
Gatad2a	yes
Ski	no
Etv3	yes
Foxk1	yes

Table S3: Antibodies for surface stains

Antibody	Fluorescent label	Clone	Company
CD3e	APC	145-2C11	BD
CD3e	AF488	145-2C11	eBioscience
CD3e	PerCpCy5.5	145-2C11	BD Pharmingen
CD3	PE	500A2	BD-Pharmingen
TCRβ	PB	H57-597	BioLegend
TCRβ	PE	H57-597	BD
CD4	APC	RM4-5	BioLegend
CD4	PB	RM4-5	eBiosciences/BD Biosciences
CD4	BV785	GK1.5	BioLegend
CD5	Pe-Cy7	53-7.3	BioLegend
CD8a	BV510	53-6.7	BioLegend
CD8a	AF700	53-6.7	BioLegend
CD8a	PB	53-6.7	eBioscience/BD Biosciences
CD8b	PerCpCy5.5	YTS1567.7.7	BioLegend
CD19	APC-H7	1D3	BD
CD19	PE	1D3	BD
CD24	PE	M1/69	BD Pharmingen
CD25	APC	PC61	BioLegend
CD49d	AF488	R-12	BioLegend
CD44	APC-Cy7	IM7	BioLegend
CD44	BV650	IM7	BioLegend
CD44	FITC	IM7	eBioscience
CD45	PerCpCy5.5	30-F11	BioLegend
CD62L	PE	MEL-14	BD
CD62L	PE-Cy7	MEL-14	BioLegend
CD62L	FITC	MEL-14	BD Pharmingen/eBioscience
CD62L	AF700	MEL-14	eBioscience
CD69	PE	H1.2F3	BD
CD69	BB700	H1.2F3	BD
CD90.2	FITC	53-2.1	BD
KLRG1	BV421	2F1/KLRG1	BioLegend
KLRG1	PE-Cy7	2F1/KLRG1	BioLegend
γδTCR	BV605	GL3	BioLegend
TCRαV2	PE	B20.1	BD
TCRβV5	APC	MR9-4	eBioscience
H2Kb	FITC	AF6-88.5	BD Pharmingen
Qa-2	APC	695H1-9-9	BioLegend
Ly5.1	eF450	40-0453	eBioscience
Ly5.2	BV605	104	BioLegend
OT-I tetramer	PE	NA	Schumacher group
E7-tetramer	PE	NA	Borst group(6)
CD1d tetramer (1:100)	AF488	NA	NIH Tetramer Core Facility
LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit	NA	NA	Thermo Fisher
Zombie NIR	NA	NA	BioLegend
Zombie Violet	NA	NA	BioLegend
7-AAD	NA	NA	BioLegend

Table S4: Antibodies for intracellular stains

Antibody	Fluorescent label	Clone	Company
IFNγ	PE	XMG1.2	BD
T-bet	PE-Cy7	4B10	eBioscience
EOMES	AF488	Dan11mag	eBioscience
EOMES	eF450	Dan11mag	eBioscience
IFNγ	PE-Cy7	XMG1.2	eBioscience
GZMB	PE	CLB-GB11	Enzo Life Sciences

Table S5: Antibodies for cell sorting

Antibody	Fluorescent label	Clone	Company
CD3	AF488	145-2C11	eBioscience
CD4	APC	RM4-5	BioLegend
CD8	PerCp-Cy5.5	YTS1567.7.7	BioLegend
CD44	APC-Cy7	IM7	BioLegend
CD62L	PE	MEL-14	BD biosciences
TCRβ	PE	H57-597	BD

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