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Supplementary Information for

TOX and TOX2 transcription factors cooperate with NR4A transcription factors to impose $CD8^+$ T cell exhaustion

Hyungseok Seo, Joyce Chen, Edahí González-Avalos, Daniela Samaniego-Castruita, Arundhoti Das, Yueqiang H. Wang, Isaac F. López-Moyado, Romain O. Georges, Wade Zhang, Atsushi Onodera, Cheng-Jang Wu, Li-Fan Lu, Patrick G. Hogan, Avinash Bhandoola and Anjana Rao

Anjana Rao
Email: arao@lji.org

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Supplementary Information Text

Materials and Methods

Retroviral vector (MSCV-myc-CAR-2A-Thy1.1) containing chimeric antigen receptor (CAR) has been described previously (1). Briefly, the chimeric antigen receptor was generated using the published part of the clone FMC63 human CD19 single chain variable fragment (2), the published parts of the murine CD3ζ and CD28 sequences. The sequence for the myc tag on the N-terminus was obtained from published work (3). This CAR construct was then cloned into an MSCV-puro murine retroviral vector in place of PGK-puro.

Construction of retroviral vectors containing human CD19 (hCD19). The hCD19 DNA fragment was cloned into an MSCV-puro (Clontech) murine retroviral vector.

Short hairpin RNA (shRNA) retroviral vector. *Tox* or non-targeting (NT) shRNA retroviral vectors were purchased from the Functional Genomics Sequencing core at the La Jolla Institute. The sequences of the shRNAs targeting *Tox* are "TGCTGTTGACAGTGAGCGAACGGGGAGAACATGTATATGATAGTGAAGCCACAGA TGTATCATATACATGTTCTCCCCGTCTGCCTACTGCCTCGGA" , "TGCTGTTGACAGTGAGCGAAAGAACAGAAACAGGTCTATATAGTGAAGCCACAGAT GTATATAGACCTGTTTCTGTTCTTCTGCCTACTGCCTCGGA", "TGCTGTTGACAGTGAGCGAAGCTCCCATCCTCAGATGGCATAGTGAAGCCACAGA TGTATGCCATCTGAGGATGGGAGCTGTGCCTACTGCCTCGGA" and "TGCTGTTGACAGTGAGCGCGCAGCATACAGAGCCAGCCTATAGTGAAGCCACAGA TGTATAGGCTGGCTCTGTATGCTGCTTGCCTACTGCCTCGGA"

Generation and transfer of CAR T cells. Splenic $CD8^+$ T cells from C57BL/6 or *Tox2-/-* mice were isolated and activated with 1 μg/ml anti-CD3 and anti-CD28 for 1 day, then removed from the plates and retrovirally transduced using 15 μg/ml of polybrene at 37℃ for 1 h at 2000 g. After transduction, cells were cultured in T cell media containing 100U of IL-2 per ml. A second transduction was performed the next day using the same protocol. On the day of adoptive transfer, the number of CAR-transduced T cells was obtained using a hemocytometer and the cells were then analyzed by flow cytometry. Cells were washed with PBS twice, resuspended in PBS, and adoptively transferred into recipient mice by retro-orbital intravenous injection. In wild type CAR experiments (in Fig. 1), C57BL/6J mice were used as donors and recipients. In *Tox*-deficient CAR experiments (Fig. 2 and Fig. 4), C57BL/6N mice were used as recipients since the donor Tox2-/- mice were maintained on this background.

Cloning of TOX and TOX2 overexpression vectors. The genes for mouse *Tox*: the mouse *Tox* gene was amplified from thymocytes and cloned in to the XhoI and EcoRI sites of the plasmid MIGR1, and the mouse *Tox2* was synthesized as a gBlock (Integrated DNA Technologies) and cloned into the BglII and EcoRI sites of the plasmid MIGR1 (Addgene plasmid # 27490), kindly provided by Warren Pear.

Mouse tumor models. The B16-hCD19 tumor cell line was previously described (1). $5x10^5$ B16-hCD19 tumor cells were subcutaneously injected into the right flank of recipient mice after shaving the injection site. Once tumors were palpable, CAR T cells were adoptively transferred into the mice and isolated from the tumors at indicated time points. Tumor volumes did not exceed the volumes permitted by the LJI IACUC animal experiments protocol.

Isolation of tumor-infiltrating lymphocytes (TILs). Mice were sacrificed and perfused with PBS before tumor isolation. Tumors were cut to 2 to 5 mm by scissors and then placed into C tubes (Miltenyi Biotec) containing RPMI 1640 with 10% FBS and Collagenase D (1mg/mL; Roche), hyaluronidase (30 unit/mL; Sigma-Aldrich), and DNase I (100ug/mL; Sigma-Aldrich). Tumors were dissociated using the gentleMACS dissociator with Octo Heaters (Miltenyi Biotech), incubated with shaking at 200 rpm for 30 min at 37°C, filtered through a 70 μM filter and spun down. Lymphocytes were separated using lymphocyte separation medium (MP Biomedicals, cat. no.: 0850494)

Eukaryotic cell lines. The B16-hCD19, MC38-hCD19 and EL4-hCD19 cell lines were previously described (1); briefly the cell lines were generated by transducing an amphotropic virus encoding human CD19 (hCD19) into B16-OVA and MC38 tumor cell line, followed by sorting for cells expressing high levels of hCD19. The EL4 mouse thymoma was purchased from the American Type Culture Collection (ATCC); the B16-OVA and MC38 cell lines were kindly provided by S. Schoenberger (4) and A.W. Goldrath respectively. The Platinum-E Retroviral Packaging Ecotropic (PlatE) cell line was purchased from Cell Bio Labs. Every cell line was found to be negative for mycoplasma contamination, and was used at passage 4 after thawing from stock.

Antibodies and staining. Fluorochrome-conjugated antibodies were purchased from BD Bioscience, Thermo Scientific and Biolegend. For surface marker staining, cells were stained with 1:200 dilution of antibodies in 50% of 2.4G2 (Fc Block) and 50% of FACS buffer (PBS+1% FBS, 2 mM EDTA) for 15 min. For cytokine staining, cells were activated with 10 nM PMA, 500 nM ionomycin or with EL4-hCD19 in some instances and 1 μg/ml Brefeldin A at 37°C for 4 hours. After stimulation, cells were stained for surface markers and fixed with 4% paraformaldehyde for 30 min, permeabilized with 1X BD perm/wash (BD Bioscience) for 30 min, and stained for cytokines at a final concentration of 1:200 in 1X BD perm /wash buffer. For detection of transcription factors, cells were stained for surface markers first, after which the Foxp3/transcriptional staining kit was used according to the manufacturer's protocol. All transcription factor antibodies were used at 1:200 dilution.

Flow cytometry for analysis and sorting. All flow cytometry analysis was performed using a BD LSRFortessa FACS analyzer and BD LSR-II FACS analyzer; cells were sorted using BD FACS Aria-I or Aria-II at the LJI Flow Cytometry Core Facility. For determining MFI, geometric mean fluorescence intensity was used and total population gated for MFI value. All flow data were analyzed with FlowJo (v 10.5.3).

In vitro killing assay. 10,000 B16-hCD19 or MC38-hCD19 cells in 100 μl of T cell media was plated as target cells in E-plate96(ACEA Biosciences Inc, San Diego). 30 min

later, the plates were incubated in the xCELLigence Real-Time Cell Analysis (RTCA) instrument (ACEA Bioscience Inc, San Diego, CA). The next day, the plates were removed from the machine and CAR T cells were added as effector cells in 100 μl T cell media (0.2% Triton X was added to estimate 100% lysis, and media was added to estimate spontaneous lysis). 30 min later, the plates were placed in the machine, and 5 hours later, the Cell Index (CI) was obtained from themachine. Specific lysis percentage was calculated as % of specific lysis =100-(CI^{well}/(CI^{pos}-CI^{neg}))*100.

Quantitative real-time PCR assay. Total RNA was extracted with Rneasy Micro Kit(Qiagen) according to the manufacturer's protocol and reverse transcribed using SuperScript III reverse transcriptase and oligo(dT) primers (Invitrogen). Synthesized cDNA was quantified with Power SYBR® Green PCR Master Mix (Thermo scientific) and Step one real time PCR system (Invitrogen). The expression levels of target genes were normalized to the amount of *Hprt* expression. Primers used in the analyses were as follows:

mouse *Tox* forward 5' -AGTCACCCAGTCGTCTCTT-3'

mouse *Tox* reverse 5'-TTCTCCTCTCTCTCCTTCATCTC-3'

mouse *Tox2* forward 5' ATGAGTGACGGAAATCCAGAG-3'

mouse *Tox2* reverse 5'-GGAGATTGGGAGGCGTTATAG-3'

mouse *Hprt* forward 5'-AGCAGGTCAGCAAAGAACTTA-3'

mouse *Hprt* reverse 5'- CAAACTTTGCTTTCCCTGGTT-3'

RNA-seq and ATAC-seq sample and library preparation. Whole RNA was isolated using the RNeasy plus Micro kit (Qiagen). SMARTseq2 libraries were prepared as described(5). ATAC-seq samples were prepared as described(6) with minor changes. 50,000 cells were sorted and then lysed. The transposition reaction was performed using Nextera enzyme (Illumina) and purified using the MinElute kit (Qiagen) prior to PCR amplification (KAPA Biosystems) with 12 cycles using barcoded primers and 2x50- cycle paired-end sequencing (Illumina).

ATAC-seq analysis. Through the use of Illumina Basespace FASTQ format paired-end sequencing files were generated. Reads were mapped to mouse (mm10) genome using a two-step mapping strategy. To note, bowtie (v1.0.0 (7)) was used to map the untrimmed reads with parameters "-p 4 -m 1 --best --strata -X 2000 --tryhard -S --fr --chunkmbs 2048". The unmapped reads were collected and processed with Trim_galore! using parameters "--paired --nextera --length 37 --stringency 3 --three_prime_clip_R1 1 --three_prime_clip_R2 1". These filtered reads were mapped again with same parameters plus "-X 2500 -v 3 -e 100". Both final bam files were merged and processed to remove duplicated reads (Picard "MarkDuplicates") and reads aligning to either mitochondrial genome (custom perl script) or ENCODE blacklisted regions. To identify peaks of accessibility, first the filtered mapping results were processed to identify nucleosome-free DNA fragments less than 100nt in length with samtools and awk '{if(sqrt($9*$9)<100){print $0}}'. These subnucleosomal fragments were used to call peaks summits for each sequenced sample using MACS2 (8) with parameters " callpeak -g mm -q 0.001 --keep-dup all --nomodel --call-summits". Resulting summits were further expanded to uniform 200 bp sized regions and merged into a global set of peaks

and filtered to remove the Y chromosome. For the differential enrichment of accessibility signal analysis between sample groups we first collected the number of transposase insertions overlapping each peak from the global set using "summarizeOverlaps" function from the GenomicAlignments Package(9). These insertion counts were further normalized between replicates using "voom" from Limma package(10). Finally, pairwise contrast were performed with limma and differentially accessible regions were filtered based on a FDR adjusted p-value of less than 0.05 and an estimated fold change of at least 2. For DeNovo Motifs analysis we used findMotifsGenome.pl from HOMER(11).

RNA-seq analysis. RNA-seq reads were mapped against the mm10 using STAR (STAR_2.5.3) with the following parameters "--genomeLoad LoadAndRemove --outFilterMultimapNmax 1 --outFilterType BySJout --alignSJoverhangMin 8 --alignSJDBoverhangMin 1 --alignIntronMin 20 --alignIntronMax 1000000 --alignMatesGapMax 100000 --outFilterMismatchNmax 0" and the RefSeq gene annotation was obtained from the UCSC genome Bioinformatics database. The number of reads mapping to each gene was counted using featureCounts (subread-1.4.3-p1) with the following parameters "-g gene_name -s 0". The DESeq2 package (1.16.1) was used to normalize raw counts and identify Differentially Expressed Genes. For publically available datasets, genes with less than 10 counts in all conditions were filtered out from the DESeq2 analysis, and an "alpha=0.05" with at least two-fold change were used as cutoff to defined Differentially Expressed Genes. For the data set generated in this study, genes with less than 1 TPM in at least 4 samples were filtered out from the DESeq2 analysis, an "alpha=0.05", and a "lfcThreshold=1", change were used as cutoff to defined Differentially Expressed Genes. Transformed values (rlog) were also calculated with DESeq2 and used to generate the PCA plots.

Chromatin immunoprecipitation and quantitative PCR (ChIP–qPCR). ChIP-qPCR samples were prepared as in a previous study (18) with minor modifications. $CD8^+$ T cells were isolated from C57BL/6J mice as above, activated with plate-bound anti-CD3/CD28 (1μg/ml) antibodies, transduced with either empty (Mock) or retrovirus vector expressing *Tox* or *Tox2* with 3x flag-tag on the N terminus. $CD8^+$ T cells were cultured for a total of 3 days post-stimulation. For fixation, Ultra-pure grade formaldehyde (10%, Polyscience, Inc.) was added directly to the cells (1.5x 10^7 cells) to a final concentration of 1% and incubated at room temperature for 10 min. Glycine (final 125mM) was added to quench the fixation and the cells were washed twice with ice-cold PBS. For nuclei isolation, cell pellets were thawed on ice and lysed with lysis buffer supplemented with 1% Halt protease inhibitor (ThermoFisher) for 10 min at 4 °C with constant rotation. Pellets were washed once with washing buffer and twice with shearing buffer. Nuclei were resuspended in 1 ml shearing buffer, transferred to 1 ml milliTUBE (Covaris, Woburn, MA), and sonicated with Covaris E220 for 1050 seconds (Duty Cycle 5%, intensity 140 Watts, cycles per burst 200). After sonication, insoluble debris was removed by centrifugation at 14,000rpm for 10 min at 4 °C. The concentration of chromatin was quantified using Qubit DNA BR assay (ThermoFisher). Five μg of chromatin was mixed with equal volume of 2× Conversion buffer in a 1.5-ml low-binding tube (Eppendorf) and used for immunoprecipitation. Either 5% of input chromatin was saved as control. Chromatin was pre-cleared using 30 μl washed protein A/G magnetic dynabeads (ThermoFisher) for 1h at 4 °C with constant rotation. Pre-cleared chromatin was transferred to new tube, added with 10 μg anti-flag antibody (M2) or anti-rabbit IgG as a control and 30 μl washed protein A/G magnetic dynabeads, and incubated at 4 °C

overnight with constant rotation. Bead-bound chromatin was washed twice with RIPA buffer, once with high salt washing buffer, once with Lithium washing buffer, and once with TE. All washes were incubated for 10 min at 4 °C with constant rotation. Chromatin was eluted from beads by incubating with elution buffer at room temperature for 30 min in the presence of 0.5 mg/ml of RNaseA (Qiagen) with constant shaking (1,000 rpm) in a ThermoMixer (Eppendorf). To de-crosslink protein and DNA, proteinase K (final 0.5 mg/ml) and NaCl (final 200 mM) were added to the recovered supernatant and incubated at 65 °C overnight with constant shaking (1,200 rpm) in the ThermoMixer. DNA was purified by using Zymo ChIP DNA clean and concentration kit (Zymo Research). Eluted DNA was analyzed by qPCR using SYBR Select Master Mix (ThermoFisher).

The following primers were used for ChIP-qPCR:

chr1:94074907–94075062 156 bp:

Pd1.4A_qF1 (forward) 5′-ACCTTTCCTGTGCCTACGTC-3′,

Pd1.4A_qR1 (reverse) 5′-TAAGAGTGGTGGTGGTTGGG-3′.

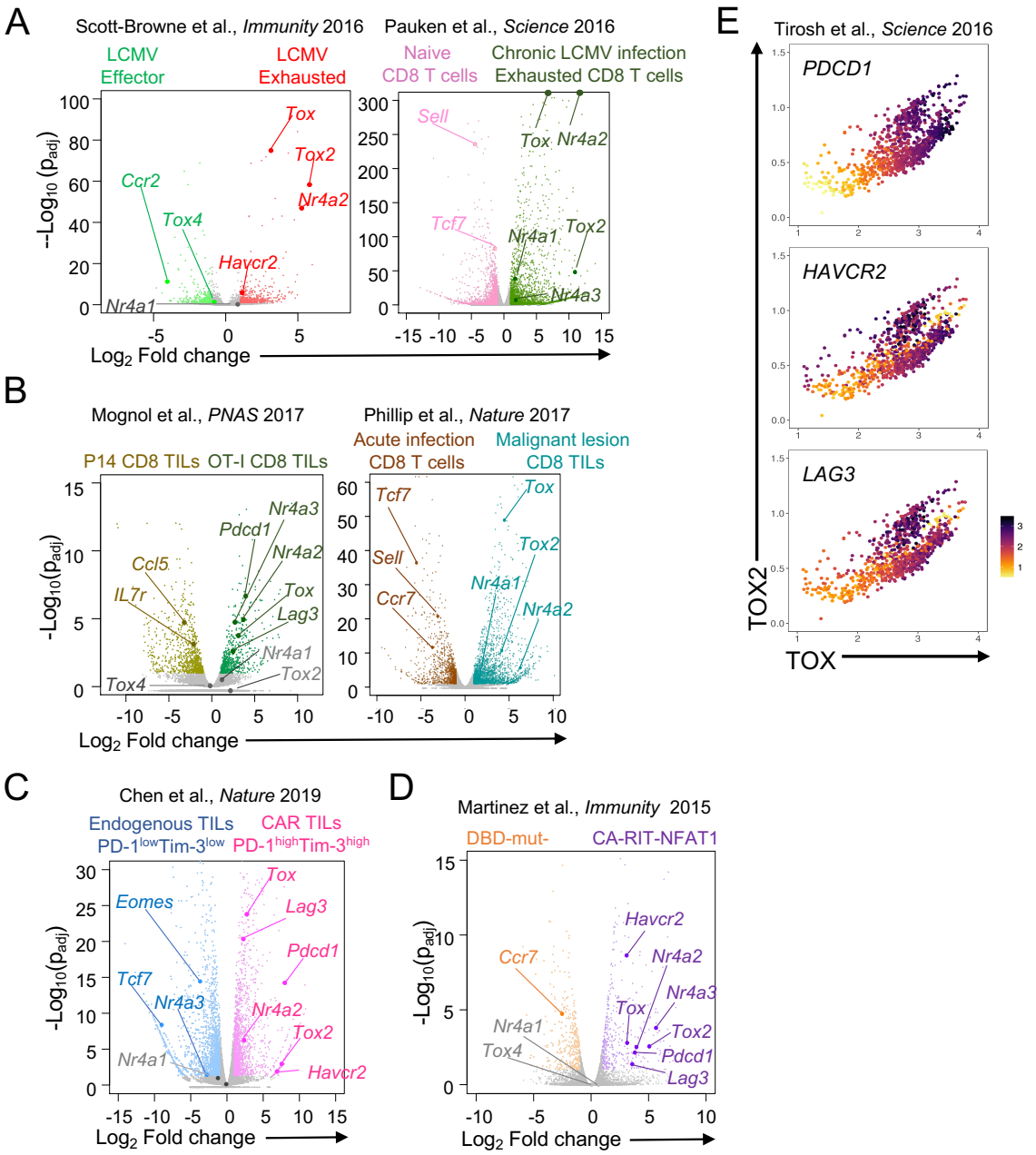

Fig. S1. Expression of mRNAs encoding TOX and NR4A transcription factors in control versus hyporesponsive CD8⁺ T cells. A, Volcano plots of genes differentially expressed in effector compared to exhausted CD8⁺ T cells (*left*) or naïve versus exhausted CD8⁺ T cells (*right*) from mice chronically infected with LCMV Clone 13 (GSE88987, GSE86881)(12, 13). **B,** Volcano plots of genes differentially expressed in P14 CD8⁺ T cells compared to OT-I CD8⁺ T cells infiltrating B16-OVA tumors (*left*) or CD8⁺ T cells from mice acutely infected with Listeria versus CD8⁺ TILS from an autochthonous liver tumor model (*right*) (GSE93014 , GSE89309) (14). **C,** Volcano plot of genes differentially expressed in PD-1^{high} TIM3^{high} CAR TILs compared to endogenous PD-1^{low} TIM3^{low} TILs infiltrating B16-OVA-hCD19 tumors (GSE123739). **D,** Volcano plots of genes differentially expressed in CD8⁺ T cells retrovirally transduced with a DNA binding mutant CA-RIT-NFAT1 (DBD-mut) compared to cells transduced with CA-RIT-NFAT1 (GSE64409)(15). **A-D,** Selected

differentially expressed genes with an adjusted p value ≤0.05 and Log_2 Fold Change >1 or -1 are highlighted. **E,** *PDCD1*, *HAVCR2* and *LAG3* mRNA expression as a color scale, superimposed on a plot showing *TOX* and *TOX2* expression (x and y axis), reanalyzed from single-cell RNA sequencing data performed on human TILs from melanoma patients (16).

A

Group	Mouse strain	Retroviral Transduction
PBS	N/A	n/a
WT CAR T	TOX2+/+	CAR + non-targeting shRNA (shNT)
***Tox* KD CAR T**	TOX2+/+	CAR + shTOX
***Tox2* KO CAR T**	TOX2-/-	CAR + shNT
***Tox* DKO CAR T**	TOX2-/-	CAR + shTOX

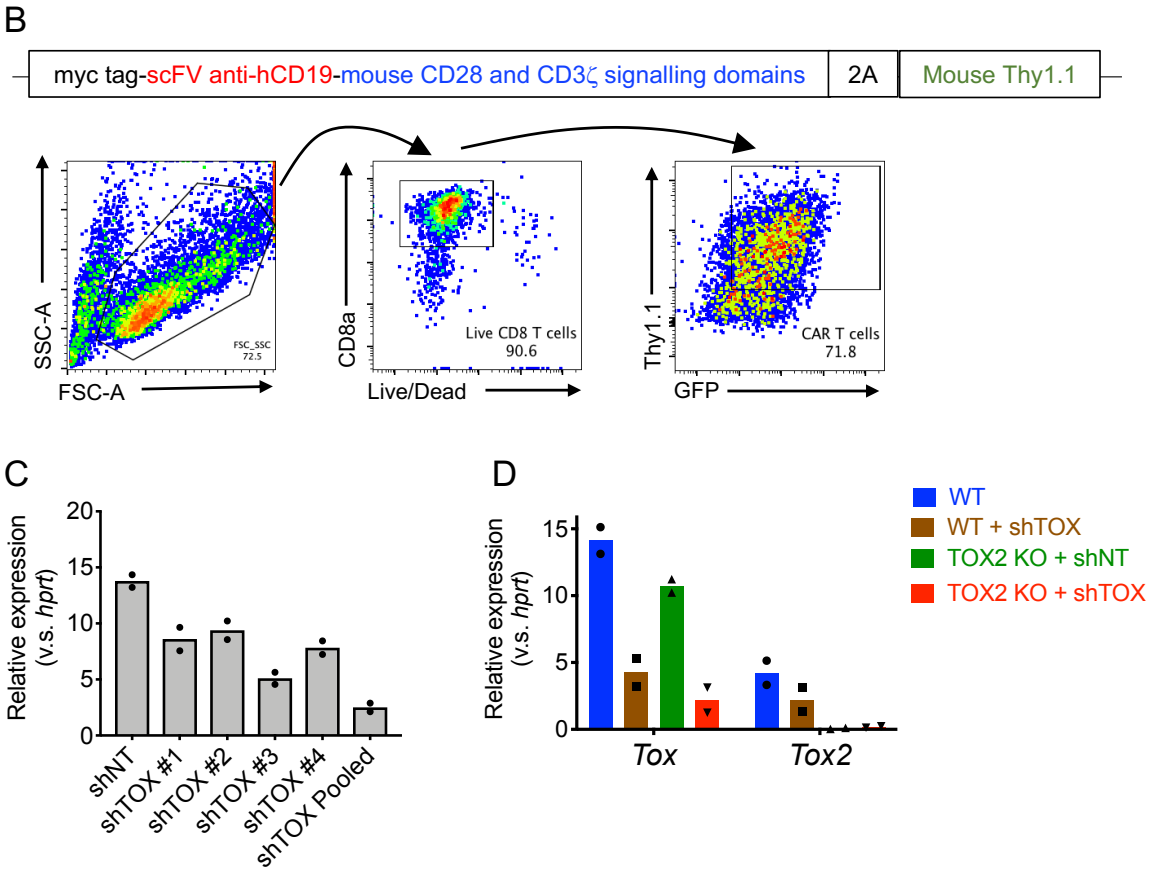

Fig. S2. Generation of CAR T cells deficient in TOX and/or TOX2 and quantification of *Tox* and *Tox2* mRNA expression in the CAR T cells. A, Table showing the strategy used to generate CAR T cells deficient in *Tox*, *Tox2* or both, for in vivo studies. **B,** *Top*, diagram of the CAR retrovirus(1). *Bottom*, Transduction efficiencies of the CAR and short hairpin (sh) RNA retroviruses, assessed by expression of Thy1.1 and GFP respectively. **C,** Efficiency of depletion of *Tox* mRNA by individual and pooled shRNA retroviruses targeting *Tox*. The results of two biologically independent experiments using two different mice were shown. **D**, *Tox* or *Tox2* mRNA expression levels in the indicated CAR T cells. The results of two biologically independent experiments using two different mice were shown.

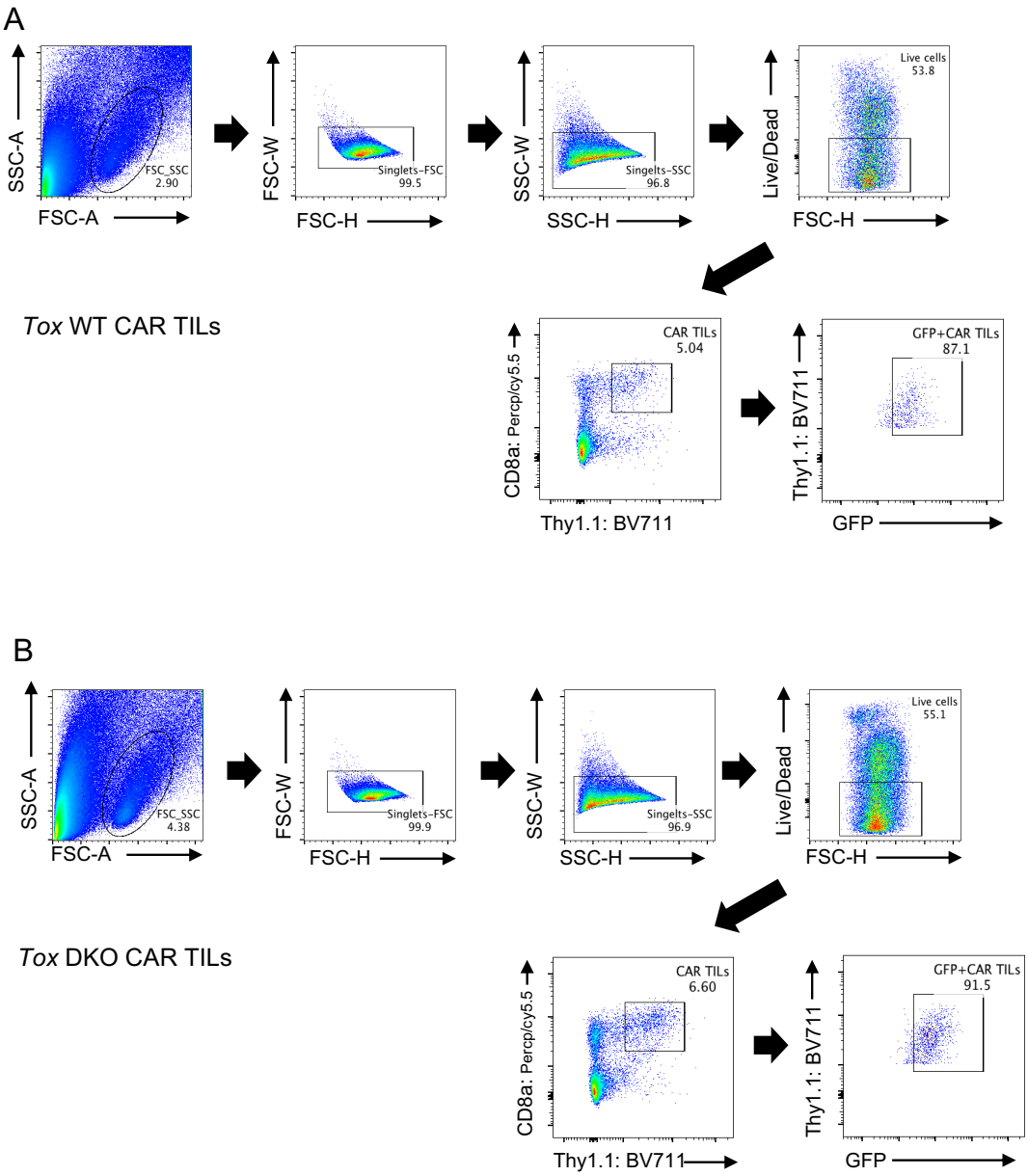

Fig. S3. Flow cytometry gating strategy for CAR TIL analysis. Flow cytometry gating scheme for surface markers, cytokines, transcription factors expressed by WT CAR TILs (**A**) and *Tox* DKO CAR TILs (**B**).

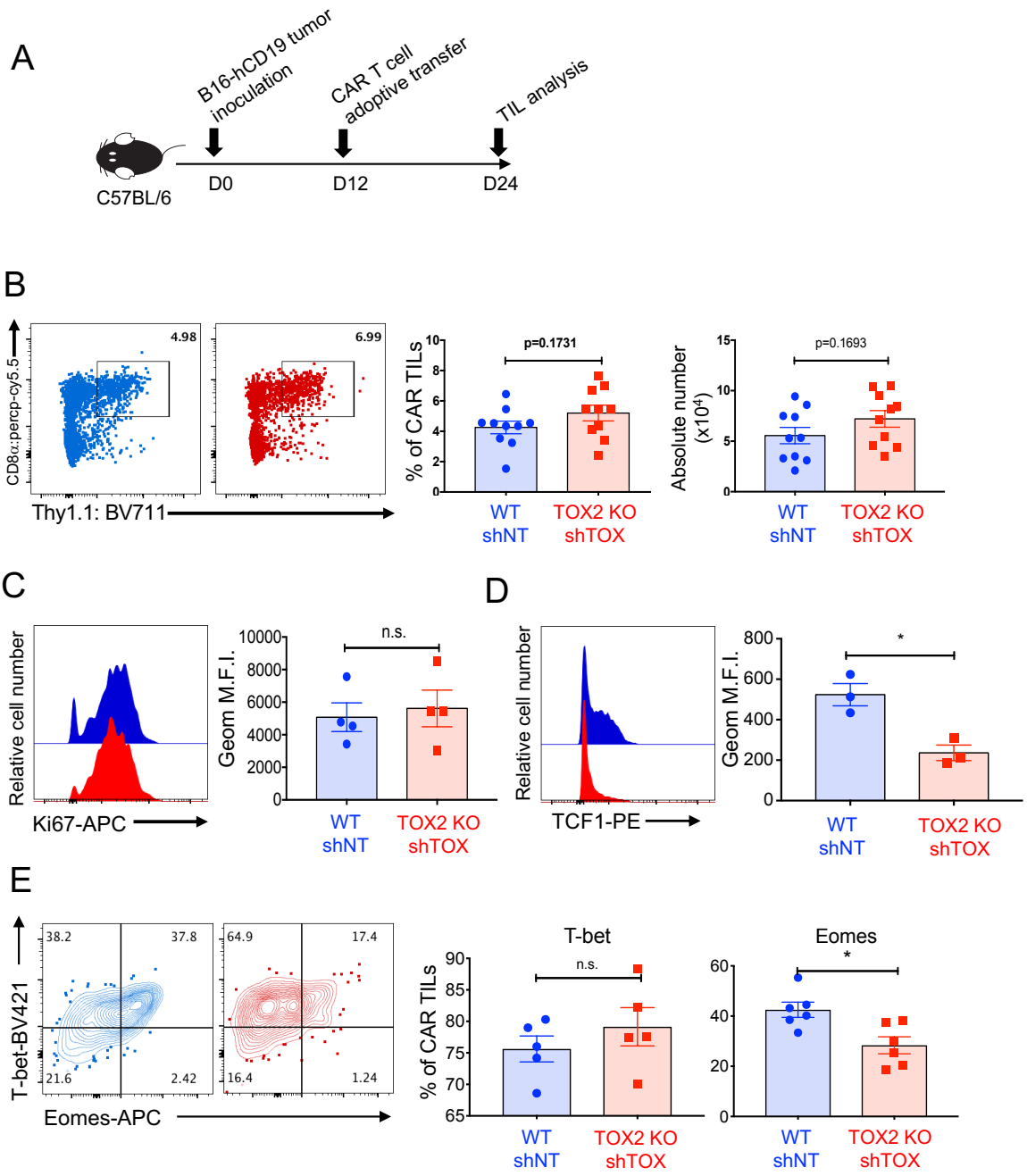

Fig. S4. Number of CAR T cells and expression of transcription factors in WT and *Tox* DKO CAR TILs. A, Experimental scheme for phenotypic analysis. $5x10^5$ tumor cells were inoculated into C57BL/6 mice, the mice were adoptively transferred with WT or *Tox* DKO CAR T cells ($1.5x10^6$) 12 days later, and CAR TILs were isolated on day 24. **B,** Flow cytometry plot for measuring the percentages and numbers of CAR TILs. The absolute number of CAR TILs was calculated as total TIL number multiplied by the percentage of $Thy1.1^+CD8^+$ cells in the total TIL population. **C,** Ki67 expression was analyzed by intracellular staining and flow cytometry. **D,** T-bet and Eomes expression on CAR TILs analyzed by flow cytometry. In all bar graphs, each dot represents CAR TILs from a single recipient mouse. The data from **B**, **C** and **D** were analyzed by Student's t test. *P≤0.05; **P≤0.01; ***P≤0.001; ****P≤0.0001; n.s.=not significant.

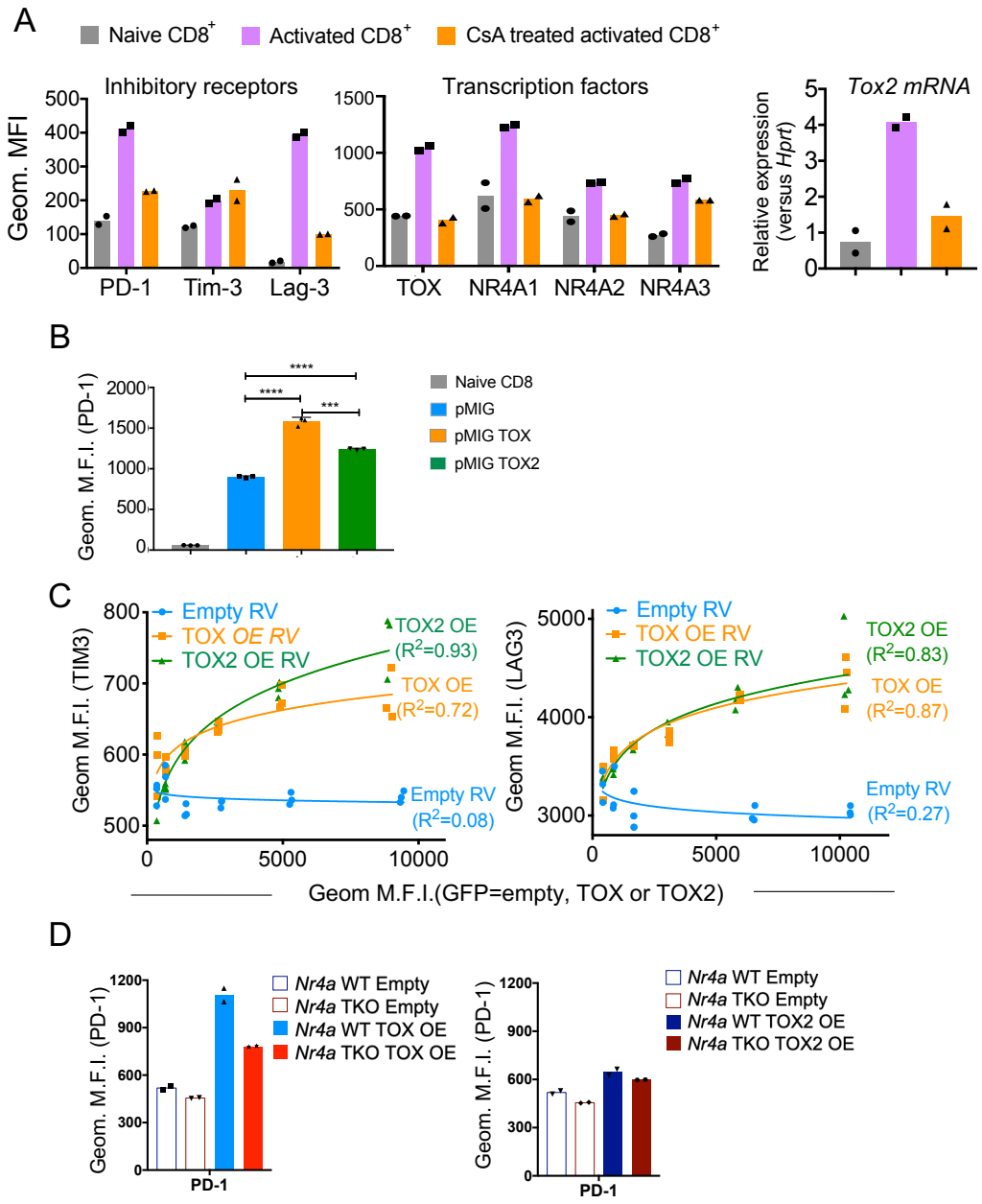

Fig. S5. TOX and NR4A transcription factors induce inhibitory receptors on CD8⁺ T cells. A. Splenic CD8⁺ T cells from C57BL/6 mice were incubated with or without cyclosporin A (CsA) for 30 min and then stimulated with anti-CD3 and anti-CD28 for 16 hours. *Left*, The expression of inhibitory receptors PD-1, TIM3 and LAG3, and transcription factors TOX, NR4A1, NR4A2 and NR4A3 was analyzed by flow cytometry. *Right*, The expression of *Tox2* mRNA was measured by qPCR and normalized to the level of *Hprt* mRNA expression. Naïve CD8⁺ cell from splenic CD8⁺ cells were used as a control. The data were representative of two biologically independent experiments. **B,** PD-1 expression was analyzed by flow cytometry in CD8⁺ T cells from C57BL/6 mice, transduced with empty retrovirus (pMIG), TOX OE RV or TOX2 OE RV with an IRES-GFP cassette (see **Fig. 3C** for details). **C,** TIM3 and LAG3 expression levels were analyzed by flow cytometry in splenic CD8⁺ T cells from C57BL/6 mice, transduced with empty RV, TOX OE RV and TOX2 OE RV. Mean fluorescence intensities (MFI) of TIM3 and LAG3 were determined

for each increment of GFP (i.e. TOX or TOX2) expression (see **Fig. 3C** for details). *Blue*, Empty RV; *orange*, TOX OE RV; *green*, TOX2 OE RV. **D,** PD-1 expression was analyzed by flow cytometry in *Nr4a* WT or *Nr4a* TKO $CD8^+$ T cells from C57BL/6 mice, transduced with empty retrovirus (pMIG), TOX OE RV (*top*) or TOX*2* OE RV (*bottom*).

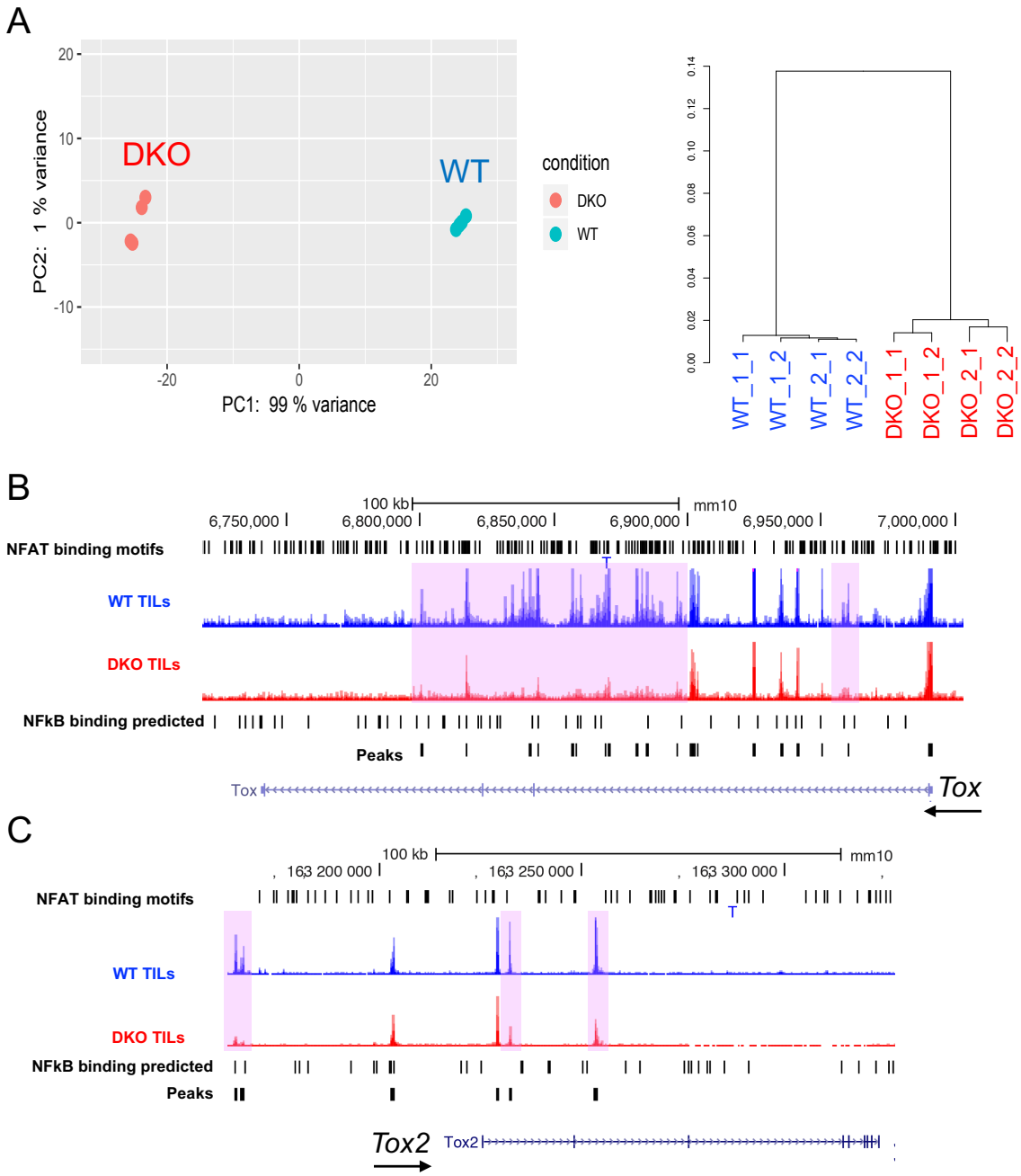

Fig. S6. A, Principal component analysis (PCA) (*left*) and hierarchical clustering (*right*) of RNA-seq data from *Tox* WT and *Tox* DKO CAR TILs. **B-C**, Genome browser views of the **B,** *Tox* and **C,** *Tox2* loci incorporating ATAC-seq data from *Tox* WT and *Tox* DKO CAR TILs. Regions of differential accessibility are highlighted in pink. Consensus binding motifs for NFAT (*top*) and NFκB (*bottom*) are indicated.

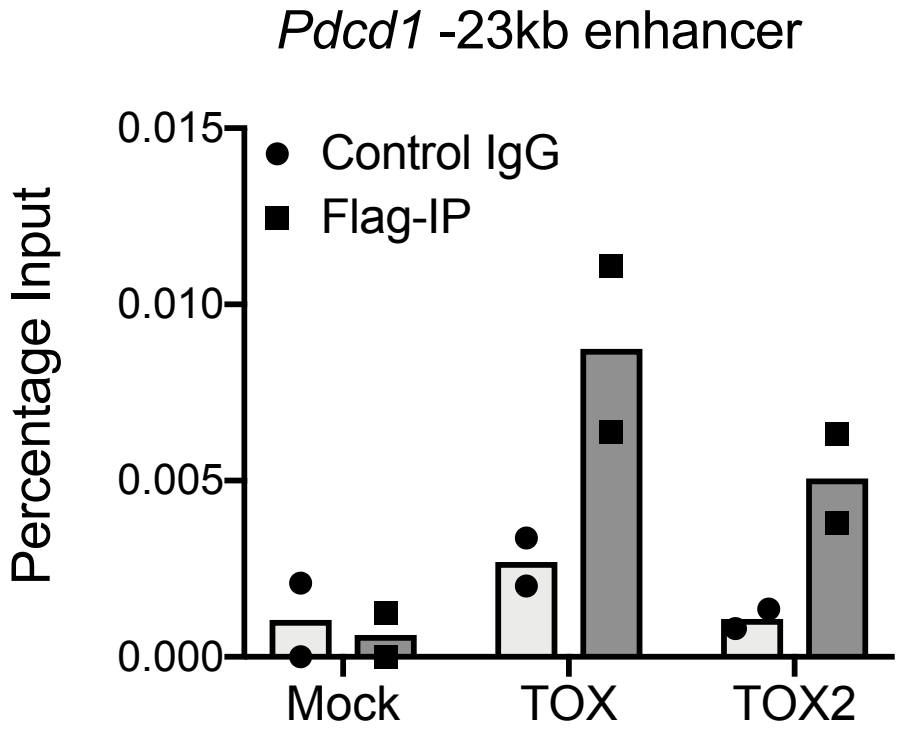

Fig. S7. ChIP–qPCR showing enrichment of 3x flag-tagged TOX or TOX2 over background at the *Pdcd1* enhancer. The data were from two independent biological experiments.

genenamee	WT1_1	WT1_2	WT2_1	WT2_2	WT average	SD	DKO1_1	DKO1_2	DKO2_1	DKO2_2	DKO average	SD
Pdcd1'	1708.17	1873.70	1877.27	1948.13	1851.82	88.09	1123.75	1105.79	797.83	830.84	964.55	150.80
Havcr2'	170.08	159.81	161.09	154.85	161.46	5.50	15.03	14.06	13.95	13.29	14.08	0.62
Lag3'	67.37	76.02	75.38	85.20	75.99	6.32	7.30	7.21	7.20	6.10	6.95	0.49
Cd244'	13.09	12.38	11.44	10.16	11.77	1.10	1.90	2.22	2.54	2.56	2.30	0.27
Cd38'	36.88	32.50	33.89	29.97	33.31	2.50	0.43	0.43	1.15	0.66	0.67	0.29
Tigit'	1072.95	1058.20	1049.50	1037.20	1054.46	13.03	220.86	219.21	160.73	167.46	192.06	28.08
Nr4a1'	210.42	229.97	235.13	237.04	228.14	10.55	93.74	93.25	81.02	83.13	87.79	5.76
Nr4a2'	287.52	291.90	293.32	298.18	292.73	3.80	47.96	44.36	51.61	47.79	47.93	2.56
Crem'	323.13	293.75	279.41	263.38	289.92	21.98	70.12	68.42	74.56	72.92	71.51	2.39
Nfkbia'	823.80	889.51	906.06	927.21	886.64	38.67	2000.95	1903.36	1676.06	1680.23	1815.15	141.29
Tcf7'	83.63	86.51	84.65	85.87	85.16	1.11	5.59	6.85	8.75	9.61	7.70	1.57

Table S1. Transcript per million (TPM) values of Figure 4B

DKO>WT

Motif	P-value	log P-pvalue	% of Targets	% of Background	STD(Bg STD)	Best Match/Details
	1e-228	-5.263e+02	21.83%	4.32%	78.3bp (71.4bp)	NFkB-p65(RHD)/GM12787-p65-ChIP-Seq(GSE19485)/Homer(0.945) More Information | Similar Motifs Found
	1e-132	-3.052e+02	14.00%	2.98%	79.6bp (78.7bp)	Atf3(bZIP)/GBM-ATF3-ChIP-Seq(GSE33912)/Homer(0.979) More Information | Similar Motifs Found
	1e-126	-2.909e+02	23.96%	8.50%	86.2bp (72.8bp)	ERG(ETS)/VCaP-ERG-ChIP-Seq(GSE14097)/Homer(0.960) More Information | Similar Motifs Found
	1e-48	-1.108e+02	21.98%	11.88%	95.8bp (72.9bp)	Egr2(Zf)/Thymocytes-Egr2-ChIP-Seq(GSE34254)/Homer(0.890) More Information | Similar Motifs Found
	1e-47	-1.097e+02	3.71%	0.55%	46.9bp (73.0bp)	BORIS(Zf)/K562-CTCFL-ChIP-Seq(GSE32465)/Homer(0.919) More Information | Similar Motifs Found
	1e-46	-1.080e+02	9.21%	3.20%	103.4bp (77.2bp)	RUNX1(Runt)/Jurkat-RUNX1-ChIP-Seq(GSE29180)/Homer(0.979) More Information | Similar Motifs Found
	1e-43	-9.964e+01	16.32%	8.09%	83.3bp (76.9bp)	MITF(bHLH)/MastCells-MITF-ChIP-Seq(GSE48085)/Homer(0.916) More Information | Similar Motifs Found
	1e-37	-8.622e+01	19.24%	10.78%	92.9bp (73.3bp)	GATA3/MA0037.3/Jaspar(0.944) More Information | Similar Motifs Found
	1e-30	-6.994e+01	8.27%	3.47%	90.5bp (76.4bp)	Atf2(bZIP)/3T3L1-Atf2-ChIP-Seq(GSE56872)/Homer(0.945) More Information | Similar Motifs Found
	1e-28	-6.524e+01	39.50%	29.40%	97.8bp (78.2bp)	Tbet(T-box)/CD8-Tbet-ChIP-Seq(GSE33802)/Homer(0.869) More Information | Similar Motifs Found

WT>DKO

Motif	P-value	log P-pvalue	% of Targets	% of Background	STD(Bg STD)	Best Match/Details
	1e-983	-2.265e+03	37.19%	8.21%	72.1bp (84.8bp)	Fra1(bZIP)/BT549-Fra1-ChIP-Seq(GSE46166)/Homer(0.993) More Information | Similar Motifs Found
	1e-813	-1.874e+03	49.68%	17.60%	81.9bp (84.3bp)	RUNX1/MA0002.2/Jaspar(0.982) More Information | Similar Motifs Found
	1e-690	-1.589e+03	33.81%	9.34%	76.9bp (77.9bp)	ERG(ETS)/VCaP-ERG-ChIP-Seq(GSE14097)/Homer(0.967) More Information | Similar Motifs Found
	1e-297	-6.854e+02	14.27%	3.55%	80.7bp (82.1bp)	Nur77(NR)/K562-NR4A1-ChIP-Seq(GSE31363)/Homer(0.971) More Information | Similar Motifs Found
	1e-236	-5.447e+02	20.69%	8.09%	85.9bp (82.3bp)	IRF3(IRF)/BMDM-Irf3-ChIP-Seq(GSE67343)/Homer(0.908) More Information | Similar Motifs Found
	1e-153	-3.539e+02	47.95%	32.66%	90.2bp (86.5bp)	Tbx5(T-box)/HL1-Tbx5.biotin-ChIP-Seq(GSE21529)/Homer(0.941) More Information | Similar Motifs Found
	1e-118	-2.732e+02	21.42%	11.63%	89.5bp (84.5bp)	Atf7(bZIP)/3T3L1-Atf7-ChIP-Seq(GSE56872)/Homer(0.923) More Information | Similar Motifs Found
	1e-50	-1.157e+02	4.52%	1.72%	82.3bp (84.1bp)	PB0113.1_E2F3_2/Jaspar(0.619) More Information | Similar Motifs Found
	1e-47	-1.092e+02	5.45%	2.37%	89.2bp (70.3bp)	Sp5(Zf)/mES-Sp5.Flag-ChIP-Seq(GSE72989)/Homer(0.898) More Information | Similar Motifs Found
	1e-45	-1.037e+02	2.23%	0.56%	79.0bp (79.1bp)	MAFG::NFE2L1/MA0089.1/Jaspar(0.675) More Information | Similar Motifs Found

Table S2. Results of de novo motif analysis of regions differentially accessible in *Tox* DKO versus WT CAR TILs.

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