Supporting Information

Shimatani et al. 10.1073/pnas.0908805106

SI Text

Cell Lines. The BA-1 (B220$^+$ pro-B cell leukemia) cell line was established from the bone marrow of SPA-1$^{-/-}$ mice by *bcr-abl* (*GFP*) transduction. The Wo-1 (pro-T cell leukemia) cell line was established from the T cell leukemia developed in the recipients of C3G-transfected Spa-1$^{-/-}$ bone marrow cells. These leukemia cells in blood were monitored by flow cytometry for GFP.

Flow Cytometry and Cell Cultures. The following antibodies were used: antibodies to CD3ε, CD4, CD8, CD25, CD44, CD45.1, CD45.2, CD62L, CD69, B220, TCR-Vβs, Foxp3, IL-7Rα, IL-15Rβ, anexin V (eBioscience), CD121b, and TCRβ (BD PharMingen). The cells were cultured in wells (1×10^5 cells/well) coated with anti-CD3 antibody (5 μg/mL) with or without soluble anti-CD28 antibody (2.5 g/mL) or IL-2 (100 U/mL). CD4$^+$ T cells from OT-IITg mice (5×10^4 cells/well) were cultured in the presence of γ-ray–irradiated B6 splenocytes (5×10^5 cells) and OVA (1 μM).

qRT-PCR Analysis. Total RNA was extracted from the purified CD4$^+$ T cell subpopulations with a NucleoSpin RNA kit (Macherey-Nagel), followed by cDNA synthesis with SuperScript III (Invitrogen). The primer pairs used are listed in Table S2. The transcripts of each gene were normalized to those of cyclophilin.

Immunoblot Analysis. Cells were lysed with ice-cold RIPA lysis buffer containing protease inhibitors, and the extracts were subjected to immunoblotting. Antibodies used included those to β-actin, Bcl6, C/EBPα, ERK2, pERK1/2, cFos, IRF8, OcaB, Satb1, VDR (Santa Cruz), c-Jun, c-Myc, and cyclin D1 (Cell Signaling Technology).

SA-β-Gal Staining. The microscopic images of SA-β-Gal staining were stored in Tiff files, and blue color signal per cell was quantified using MetaMorph software (Molecular Devices).

DNA Microarray and Clustering Analysis. PMT levels were adjusted to achieve 0.1%–0.5% pixel saturation, and each Tiff image was analyzed using GenePix Pro 6.0 (Molecular Devices). Data were filtered to remove low-confidence measurements and globally normalized per array such that the median log2 (Cy3/Cy5 fluorescence ratio) was 0 after normalization.

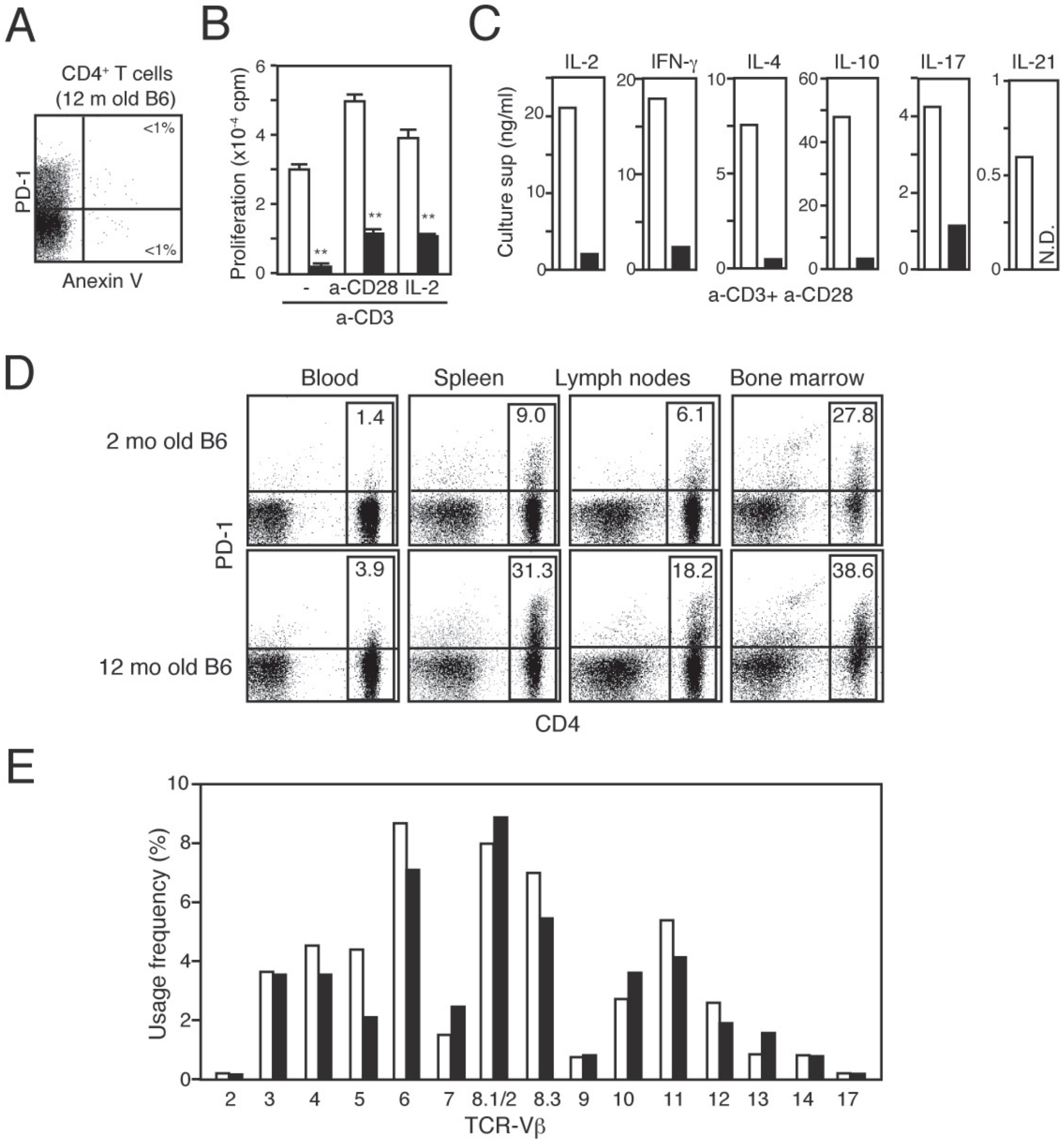

Fig. S1. Proliferation capacity, organ distribution, and TCRβ repertoire of PD-1^{+} MP CD4^{+} T cells. (*A*) Spleen cells from 12-month-old B6 mice were 3-color–analyzed with the indicated antibodies. (*B*) PD-1^{-} (open columns) and PD-1^{+} (closed columns) CD44^{high} CD4^{+} T cells sorted from 12-month-old B6 mice were cultured in the presence of anti-CD3 antibody (5 μg/mL) with or without anti-CD28 antibody (2.5 μg/mL) or IL-2 (100 U/mL) for 3 days and pulsed with ^{3}H-TdR. $**P < .01$. (*C*) PD-1^{-} (open columns) and PD-1^{+} (solid columns) CD44^{high} CD4^{+} T cells sorted from aged mice were cultured in the presence of anti-CD3 plus anti-CD28 antibodies for 3 days, and the indicated lymphokines were assessed by ELISA. ND, not detectable. (*D*) Cells from the blood and indicated lymphoid tissues of 2-month-old and 12-month old B6 mice were 3-color–analyzed with anti-CD3, anti-PD-1, and anti-CD4 antibodies. The profiles in a CD3^{+} gate are indicated. (*E*) PD-1^{-} (open columns) and PD-1^{+} (closed columns) CD44^{high} CD4^{+} T cells sorted from 12-month-old B6 mice were analyzed with a set of anti-TCRβ chain antibodies using FACSCaliber.

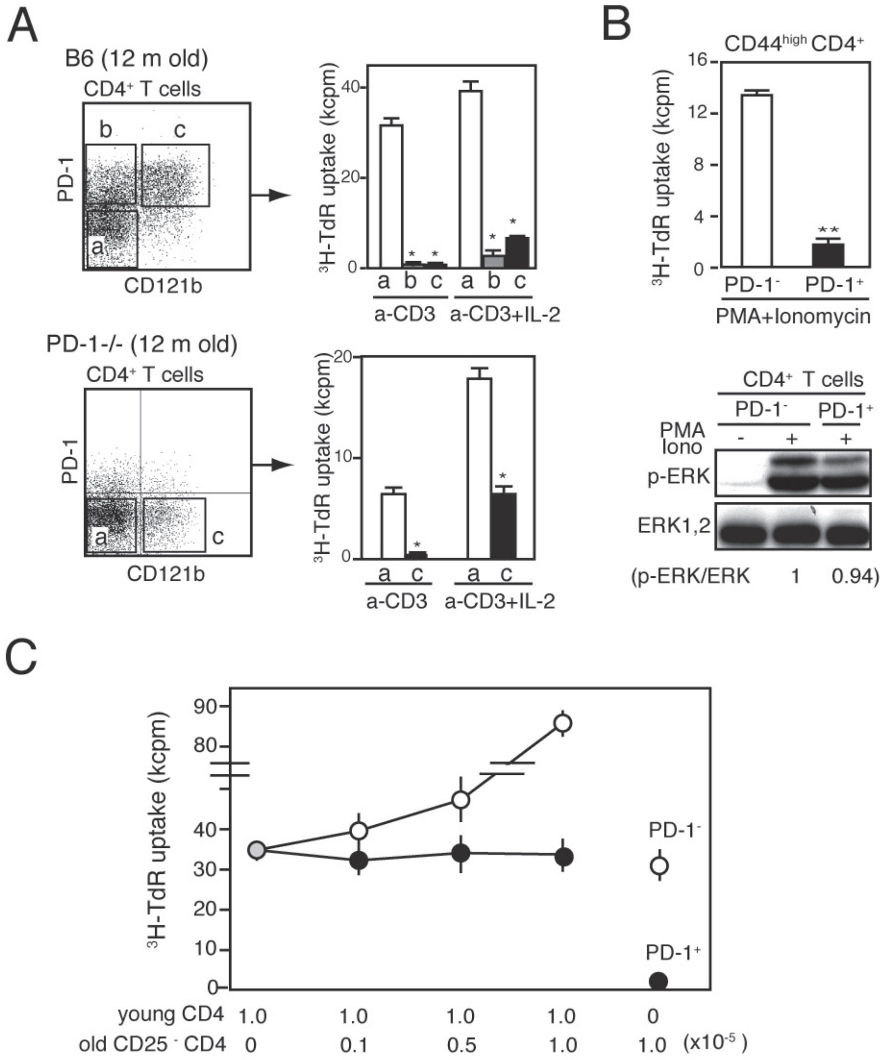

Fig. S2. PD-1$^+$ MP CD4$^+$ T cells are intrinsically defective for TCR-mediated proliferation. (*A*) Spleen cells from B6 and PD-1$^{-/-}$ mice at age 12 months were 3-color–analyzed with the indicated antibodies (*Left*). PD-1$^-$ CD121b$^-$ (a, open column), PD-1$^+$ CD121b$^-$ (b, gray column), and CD121b$^+$ (c, solid column) CD4$^+$ T cells were sorted and cultured in the presence of anti-CD3 antibody with or without IL-2 for 3 days (*Right*). $*P < .01$. (*B*) PD-1$^-$ (open column) and PD-1$^+$ (closed column) CD44$^+$ CD4$^+$ T cells from aged B6 mice were cultured in the presence of PMA plus ionomycin for 10 min to detect ERK activation and for 3 days to assess proliferation. The relative *p*-ERK/ERK ratio was assessed by densitometry. $**P < .01$. (*C*) PD-1$^-$ (open circles) and PD-1$^+$ (closed circles) CD25$^-$ CD4$^+$ T cells sorted from aged mice were co-cultured with CD4$^+$ T cells from young mice at various ratios in the presence of anti-CD3$^+$ and-CD28 antibodies for 3 days.

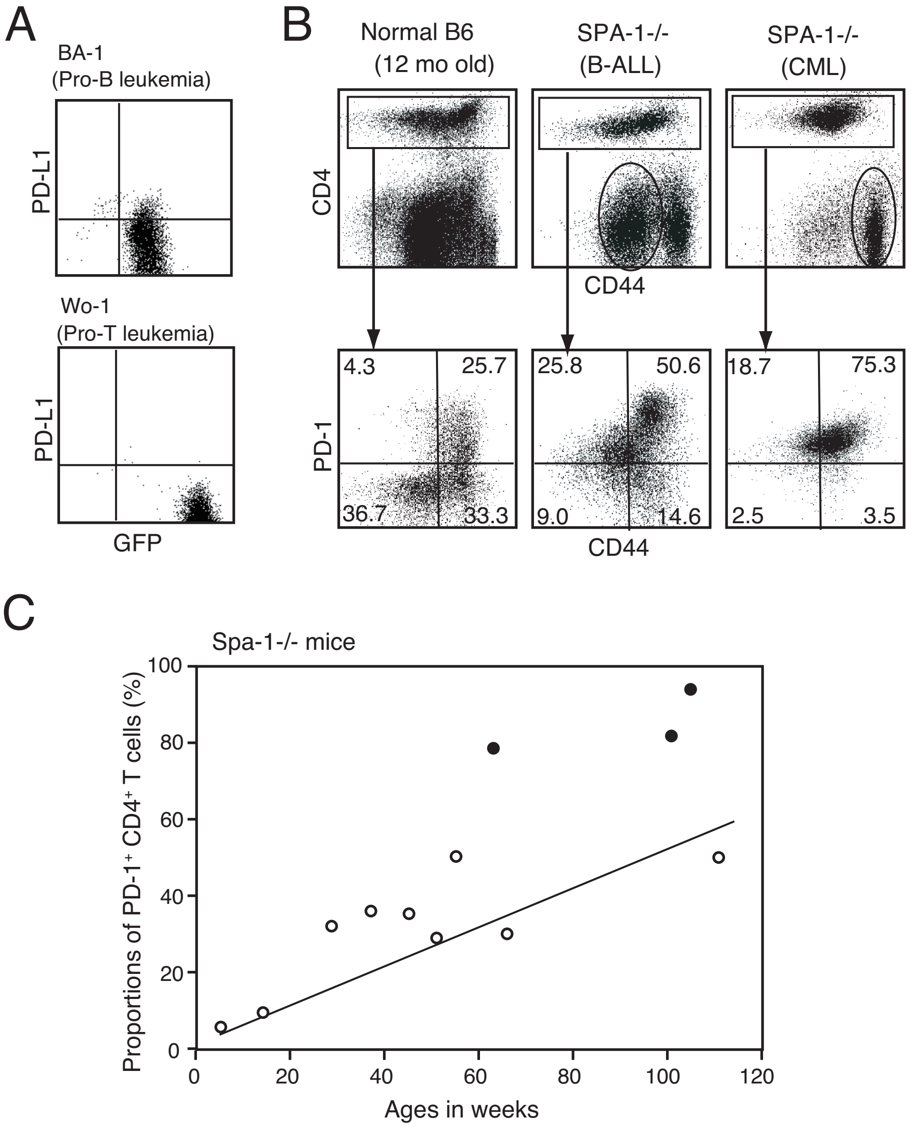

Fig. S3. PD-1$^+$ MP CD4$^+$ T cells increase in number as Spa-1$^{-/-}$ mice spontaneously develop frank leukemia. (*A*) PD-L1 expression was analyzed in the leukemia cell lines BA-1 (pro-B cell leukemia) and Wo-1 (pro-T cell leukemia), both of which are capable of inducing the rapid generation of PD-1$^+$ MP CD4$^+$ T cells in vivo. (*B*) Spleen cells from Spa-1$^{-/-}$ mice with frank leukemia (acute B lymphoblastic leukemia and chronic myelogenous leukemia) and from age-matched (12-month-old) control B6 mice were 3-color–analyzed with the indicated antibodies. Circles indicate the leukemia cells. (*C*) The proportions of PD-1$^+$ MP CD4$^+$ T cells in the spleen cells from Spa-1$^{-/-}$ mice at various ages are shown. Open circles indicate mice with no evidence of leukemia; closed circles, mice with frank leukemia.

Table S1. DNA microarray analysis

		Normal aged B6		
		MP CD4$^+$ T cells		
		PD-1$^-$	PD-1$^+$	Ratio
Overexpressed genes				
Transcription factors				
Batf	Basic leucine zipper transcription factor, ATF-like	883	2,824	3.2
Nfil3	Nuclear factor, interleukin-3, regulated	237	740	3.1
Zdhhc2	Zinc finger, DHHC domain–containing 2	145	388	2.7
Hif1a	Hypoxia-inducible factor 1, alpha subunit	292	1,003	3.4
Plagl1	Pleiomorphic adenoma gene-like 1	143	826	5.8
Tox	Thymocyte selection-associated HMG box gene	684	2,017	3.0
Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha	183.4	970.5	4.8
Pou2af1	POU domain, class 2, associating factor 1	265.8	948.4	3.2
Bcl6	B-cell leukemia/lymphoma 6	1,147.3	3,914.7	3.1
Egr2	Early growth response 2	765.1	1,622.8	1.9
Vdr	Vitamin D receptor, mRNA	183.8	893.1	4.4
Membrane proteins				
Pdcd1	Programmed cell death 1	107	646	6.0
Cacna1d	Calcium channel, voltage-dependent, L type, alpha 1D	40	161	4.0
Cd121b	Interleukin 1 receptor, type II	79	584	7.4
Ptger2	Prostaglandin E receptor 2 (subtype EP2)	63	241	3.8
Tnfsf8	Tumor necrosis factor (ligand) superfamily, member 8	333	959	2.9
Cxcr4	Chemokine (C-X-C motif) receptor 4	168	874	5.2
Cd200	Cd200 antigen	204	678	3.3
Cd83	CD83 antigen	310	1,491	4.8
Blr1	Burkitt lymphoma receptor 1	529	1,757	3.3
Cd81	CD 81 antigen (Cd81)	3426.2	11,450.9	3.0
Cytokines/secreted proteins				
Sostdc1	Sclerostin domain–containing 1	397	5,375	13.5
Ccl3	Chemokine (C-C motif) ligand 3 [Source: MarkerSymbol;Acc:MGI:98260]	107	646	6.0
Ccl4	Chemokine (C-C motif) ligand 4	203	926	4.6
Il4	Interleukin-4	176	421	2.4
Il21	Interleukin-21	173	1,017	5.9
Stfa1	Stefin A3	19	229	12.3
Spp1	Pogo transposable element with ZNF domain	96	1,418	14.7
Esm1	Endothelial cell–specific molecule 1	179	530	3.0
Cytoplasmic proteins				
Cdkn2b	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	75	294	3.9
Sccpdh	Saccharopine dehydrogenase (putative)	92	461	5.0
Rgs16	Regulator of G-protein signaling 16	97	492	5.1
Srxn1	Sulfiredoxin 1 homolog (*Saccharomyces cerevisiae*)	135	361	2.7
Pfn2	Profilin 2	66	241	3.7
Stk39	Serine/threonine kinase 39, STE20/SPS1 homolog (yeast)	502	1,154	2.3
Cxxc5	Ring finger protein 128	103	266	2.6
Stx11	Syntaxin 11	325	1,241	3.8
Tbc1d4	TBC1 domain family, member 4	551	1,696	3.1
Underexpresssed genes				
Transcription factors				
Cbfa2t3 h	Core-binding factor, runt domain, alpha subunit 2	385	54	0.1
Jun	Jun oncogene	466	114	0.2
Irf8	Interferon regulatory factor 8	607	73	0.1
Satb1	Special AT-rich sequence binding protein 1	912	83	0.1
Cd74	*Mus musculus* CD74 antigen	8,101.8	2,714.8	0.3
Membrane proteins				
Cd226	CD226 antigen	309.4	18.5	0.1
Cd74	*Mus musculus* CD74 antigen	8,101.8	2,714.8	0.3
Cd8b1	CD8 antigen, beta chain 1	451	164	0.4
H2-Ob	Histocompatibility 2, O region beta locus	686	332	0.5
Sell	Selectin, lymphocyte	695	163	0.2
Il7r	Interleukin 7 receptor	1,039	470	0.5
Ccr7	Chemokine (C-C motif) receptor 7	238	95	0.4
Trat1	T cell receptor–associated transmembrane adaptor 1	1,144	309	0.3
Cd72	CD72 antigen	477	130	0.3

		Normal aged B6		
		MP CD4$^+$ T cells		
		PD-1$^-$	PD-1$^+$	Ratio
Ccr2	Chemokine (C-C motif) receptor 2	660	188	0.3
Klrd1	Killer cell lectin-like receptor, subfamily D, member 1	235	43	0.2
H2-DMb2	Histocompatibility 2, class II, locus Mb1	560	90	0.2
Cd7	CD7 antigen	334	133	0.4
Ifitm1	Interferon-induced transmembrane protein 1	303	44	0.1
Cd8a	CD8 antigen, alpha chain	167	52	0.3
Klrc1	Killer cell lectin-like receptor subfamily C, member 1	281	33	0.1
Lair1	Leukocyte-associated Ig-like receptor 1	142		
Cd74	CD74 antigen	13,810	2,083	0.2
Fcer1 g	Fc receptor, IgE, high-affinity I, gamma polypeptide	968	70	0.1
Amica1	Adhesion molecule, interacts with CXADR antigen 1	474	36	0.1
Ly6d	Lymphocyte antigen 6 complex, locus D	412	54	0.1
Ly6c1	Lymphocyte antigen 6 complex, locus C1	1,105	24	0.0
H2-Eb1	Histocompatibility 2, class II antigen E beta [Source: MarkerSymbol;Acc:MGI:95901]	8,877	453	0.1
Siglech	Sialic acid–binding Ig-like lectin H [Source: MarkerSymbol;Acc:MGI:2443256]	633	17	0.0
Mgl1	Macrophage galactose N-acetyl-galactosamine specific	154		
H2-Ab1	Histocompatibility 2, class II antigen A, beta 1	6,391	268	0.0
Igl-V1	Immunoglobulin lambda chain, variable 1	258		
Ly86	Lymphocyte antigen 86	495	16	0.0
H2-Aa	Histocompatibility 2, class II antigen E alpha	14,275	391	0.0
Cytokines/secreted proteins				
Cfp	Complement factor properdin	428	107	0.2
Il1b	Interleukin-1 beta	467	27	0.1
Cytoplasmic proteins				
Hck	Hemopoietic cell kinase	742	174	0.2
Txk	TXK tyrosine kinase	1,112	226	0.2
Prkcn	Protein kinase C, nu	169	17	0.1
Ncf1	Neutrophil cytosolic factor 1	366	38	0.1
Ctsh	Cathepsin H	583	35	0.1
Pld4	Phospholipase D family, member 4	667		
Unc93b1	Unc-93 homolog B1 (*Caenorhabditis elegans*)	425	163	0.4
Nedd4	Neural precursor cell expressed	395	111	0.3
Rnf130	Ring finger protein 130	316	69	0.2
Plac8	Placenta-specific 8	1,943	58	0.0
Tyrobp	TYRO protein tyrosine kinase–binding protein	2,125	65	0.0
Pld4	Phospholipase D family, member 4	667		
Cell cycle–related proteins				
Ccnd1	Cyclin D1 (Ccnd1)	1,818.8	99.0	0.1

Table S2. Primer sequences for quantitative PCR

Gene	Sense	Antisense
Angptl2	CTGGACAGGGACCATGATGT	GGAGTGAGCACAGGCGTTAT
Cbfa2t3 h	CTGACTGTCATCAACCAGCAA	TTACAGCCACTGCACGTCTC
Ccnd1	GAACAAGCTCAAGTGGAACC	CTTCAATCTGTTCCTGGCAG
Ccr8	AGAAGAAAGGCTCGCTCAGA	GGCTCCATCGTGTAATCCAT
Cebpa	TGAGAAAAATGAAGGGTGCAG	CGGGATCTCAGCTTCCTGT
c-myc	CGAAACTCTGGTGCATAAACTG	GAACCGTTCTCCTTAGCTCTCA
Cxcr6	AAGCTACTGGGCTTCTCTTCTG	CCCATCGTACAGAGCTGACTC
Cyclophilin	GACGAAGGTAGCCAGTCACAAG	AATCAGGCCTGTGGAATGTGAG
Ifng	ATCTGGAGGAACTGGCAAAA	TTCAAGACTTCAAAGAGTCTGAGG
Il1r2	CCCATCCCTGTGATCATTTC	GCACGGGACTATCAGTCTTGA
Il2	GCTGTTGATGGACCTACAGGA	TTCAATTCTGTGGCCTGCTT
Il21	TCATCATTGACCTCGTGGCCC	ATCGTACTTCTCCACTTGCAATCCC
Il4	GAGAGATCATCGGCATTTTGA	TCTGTGGTGTTCTTCGTTGC
Irf8	CCAACCAGTTCATCCGAGA	GAATGAGTTTGGAGCGCAAG
Klf2	CTAAAGGCGCATCTGCGTA	TAGTGGCGGGTAAGCTCGT
Ly6c	TCTTGTGGCCCTACTGTGTG	GCAATGCAGAATCCATCAGA
Pdcd1	CTACCTCTGTGGGGCCATC	GAGGTCTCCAGGATTCTCTCTGT
Pou2af1	CCTCCTCGGTGTTGACCTAT	CGGGTGTAGCAGTGCTTCTT
Satb1	ACTGAAACGAGCCGGAATC	CGGAGGATTTCAGAAAGCAA
Slc24a3	GCCTCATTGTAGCCAGACAAG	ACGTTGCTCCCAATGGAAT
Soscdc1	AACAGCACCCTGAATCAAGC	CAGCCCACTTGAACTCGAC
Spp1	CCCGGTGAAAGTGACTGATT	TTCTTCAGAGGACACAGCATTC
Vdr	CACCTGGCTGATCTTGTCAGT	CTGGTCATCAGAGGTGAGGTC
Bace2	CCTGAGAGATGAGAATGCCAGT	ATCATGGGCTGAATGTAGAGC
Bhlhb2	TGAAGCACGTGAAAGCATTG	TTTCTTCCCGACAAATCACC
Npas4	AGGGTTTGCTGATGAGTTGC	CCCCTCCACTTCCATCTTC
Grail	GTAACCCGCACACCAATTTC	GTGAGACATGGGGATGACCT
Apoe	GACCCTGGAGGCTAAGGACT	AGAGCCTTCATCTTCGCAAT
Tnfsf8	GAGGATCTCTTCTGTACCCTGAAA	TTGGTATTGTTGAGATGCTTTGA
Cd121b	CCCATCCCTGTGATCATTTC	GCACGGGACTATCAGTCTTGA
Ecel1	GCCCAACAAGAATCAAATGG	CCCCCGTAGTTCAGAGACTG
Bir1	GGAGGGTACCACTCACATGG	TTGCCTGCTAACTTCCCCTA
Nrbp2	CTGAGTGACCCCAACATGC	CGGTGGAAGAGGAGGTTGT
Bcl6	TTCCGCTACAAGGGCAAC	CAGCGATAGGGTTTCTCACC
Cd83	TGGTTCTGAAGGTGACAGGA	CAACCAGAGAGAAGAGCAACAC
Egr2	GTGCCAGCTGCTATCCAGAAG	GGCTGTGGTTGAAGCTGGAG
Nfil3	AGGCCGATGAGGGTGTAGT	GCCCTTAGGGACCTGTTGTT
Batf	AGCTTCAGCCGCTCTCCT	GCAGCGATGCGATTCTTC
Scin	CACAGTTCTCCGCAGATGG	CACTGTTCTCTACACGCCAGA
Rgs16	GGCCAGTAAGCATAACAAAGAGA	TCAGCAGCAAATCGAAAGAC
Cd200	CTCCACCTACAGCCTGATTTG	CCTGGGTCACCACTTCCA
Hif1a	GCACTAGACAAAGTTCACCTGAGA	CGCTATCCACATCAAAGCAA
Nr4a2	TCAGAGCCCACGTCGATT	TAGTCAGGGTTTGCCTGGAA
Tnfrsf9	TGTGACTCCAGAGGGAGGAC	AGCAGCAAAGCCGATGTC
Cd81	CCTGGAACTGGGAAACAAAC	GCTCCCACAGCAATGAGAAT
Ly6d	TCTGCTCGTCCTCCTTGTCT	GTGCACACGTGACATCGAA
Trat	AGGAAGTGGCTGCACCTG	CCAGTGAGGCATAGCACATC
Ly86	ATTCTGAACTACTCCTATCCCCTTT	GGCCGGCATAGTATATCTGTTCT