Supplementary Information

The thymic cortical epithelium determines the TCR repertoire of IL-17-producing γδT cells

Takeshi Nitta, Ryunosuke Muro, Yukiko Shimizu, Sachiko Nitta, Hiroyo Oda, Yuki Ohte, Motohito Goto, Rieko Yanobu, Tomoya Narita, Hiroshi Takayanagi, Hisataka Yasuda, Tadashi Okamura, Shigeo Murata, and Harumi Suzuki

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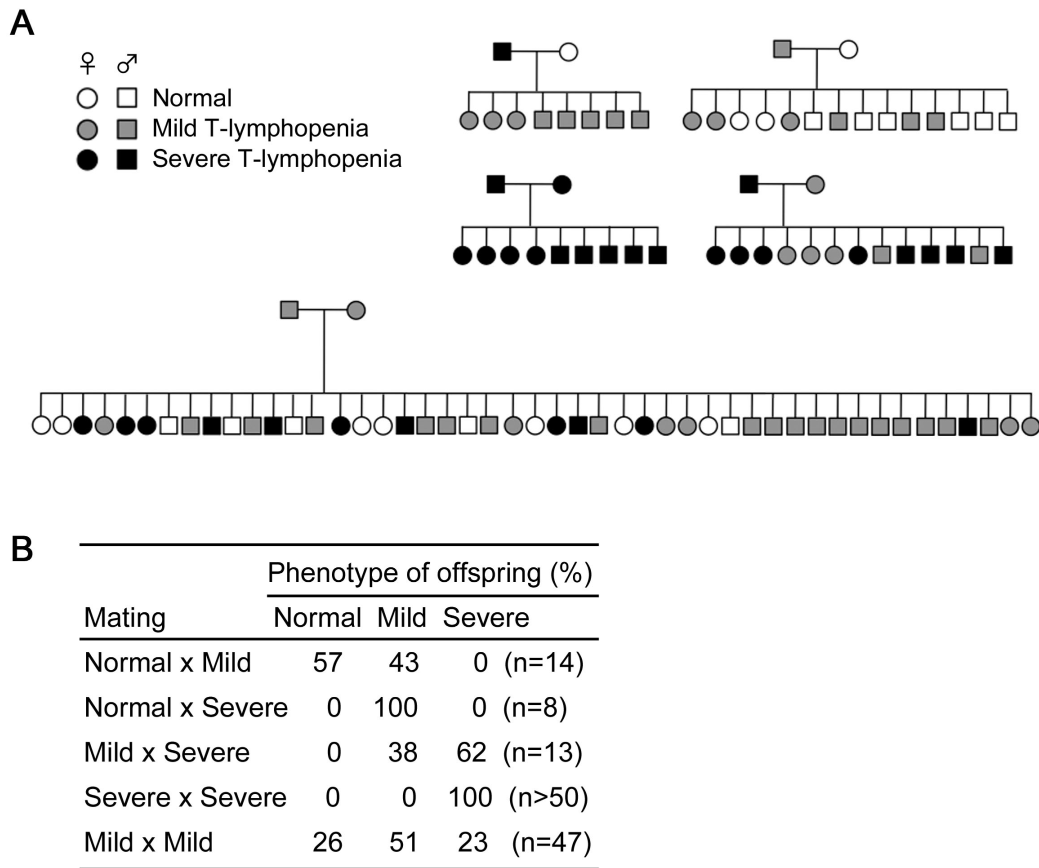
Figure S1



Mating	Phenotype of offspring (%)			
	Normal	Mild	Severe	
Normal x Mild	57	43	0	(n=14)
Normal x Severe	0	100	0	(n=8)
Mild x Severe	0	38	62	(n=13)
Severe x Severe	0	0	100	(n>50)
Mild x Mild	26	51	23	(n=47)

Figure S1. Mendelian inheritance of the *TN* phenotype. (**A**) Peripheral blood leukocytes were analyzed by flow cytometry as described in **Figure 1A**. Mice were categorized according to the frequency of $CD3^{+}CD44^{lo}$ naïve T cells into three groups: normal (>25%), mild T-lymphopenia (6-12%), and severe T-lymphopenia (<3%). Pedigree of the representative families of *TN* mice is shown. (**B**) Summary of the percentage of offspring from indicated breeding pairs.

Figure S2

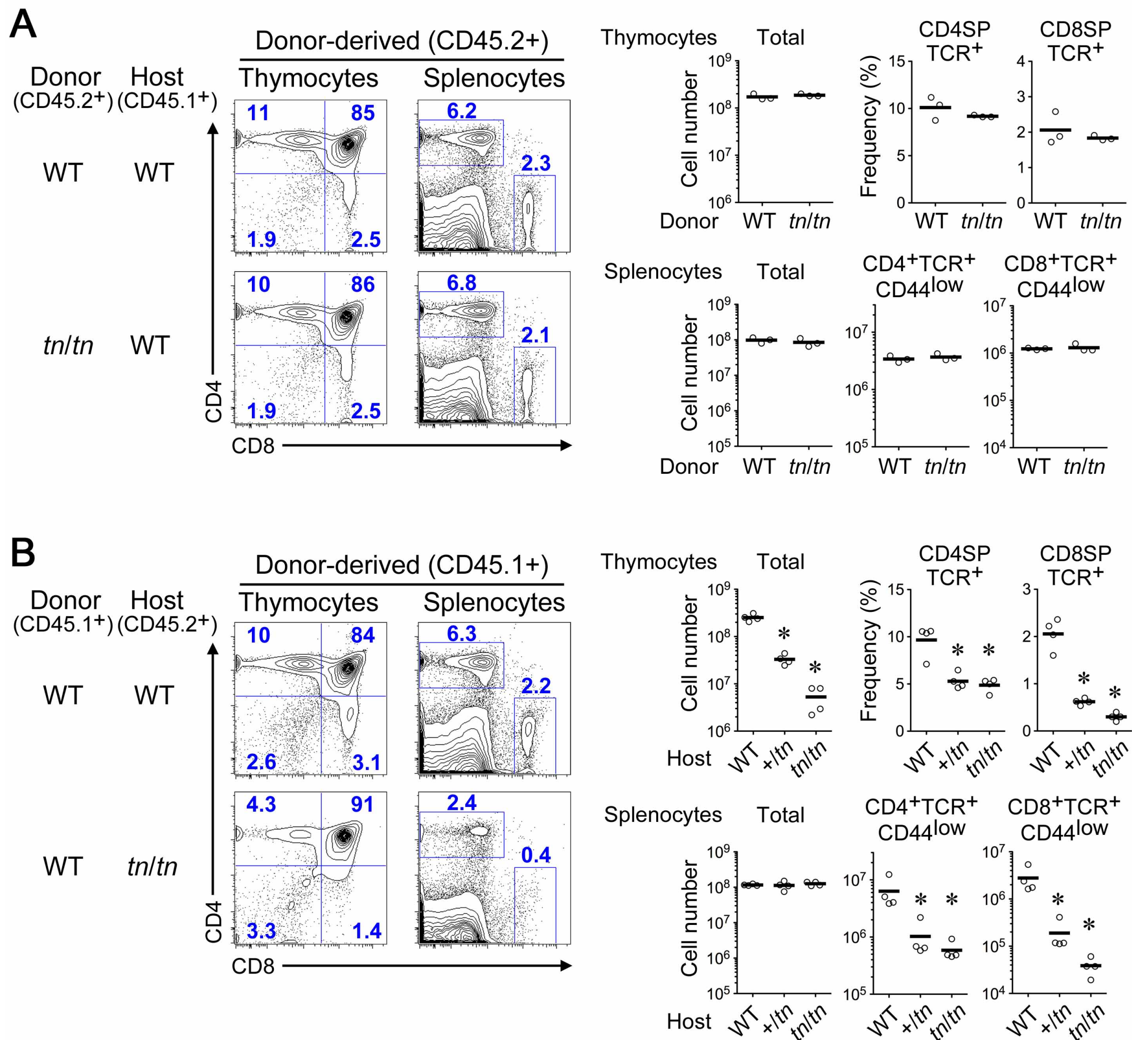


Figure S2. Impaired T cell development in *TN* mice is due to defects in nonhematopoietic thymic stromal cells. (**A**) T cell-depleted bone marrow cells from CD45.2^{+} WT or *tn*/*tn* donor mice were transferred into lethally irradiated CD45.1^{+} WT host mice (three mice per group). (**B**) T cell-depleted bone marrow cells from CD45.1^{+} WT donor mice were transferred into lethally irradiated CD45.2^{+} WT, +/*tn*, or *tn*/*tn* host mice (four mice per group). Thymocytes and splenocytes were analyzed 5 weeks after transplantation. Flow cytometry profiles for CD4 and CD8 were shown

(left). Graphs (right) show cell number or frequency of indicated populations of donor-derived thymocytes or splenocytes. Each circle represents an individual mouse and horizontal bars indicate the mean. Asterisks indicate significant difference ($p<0.01$) from wild-type control.

Figure S3

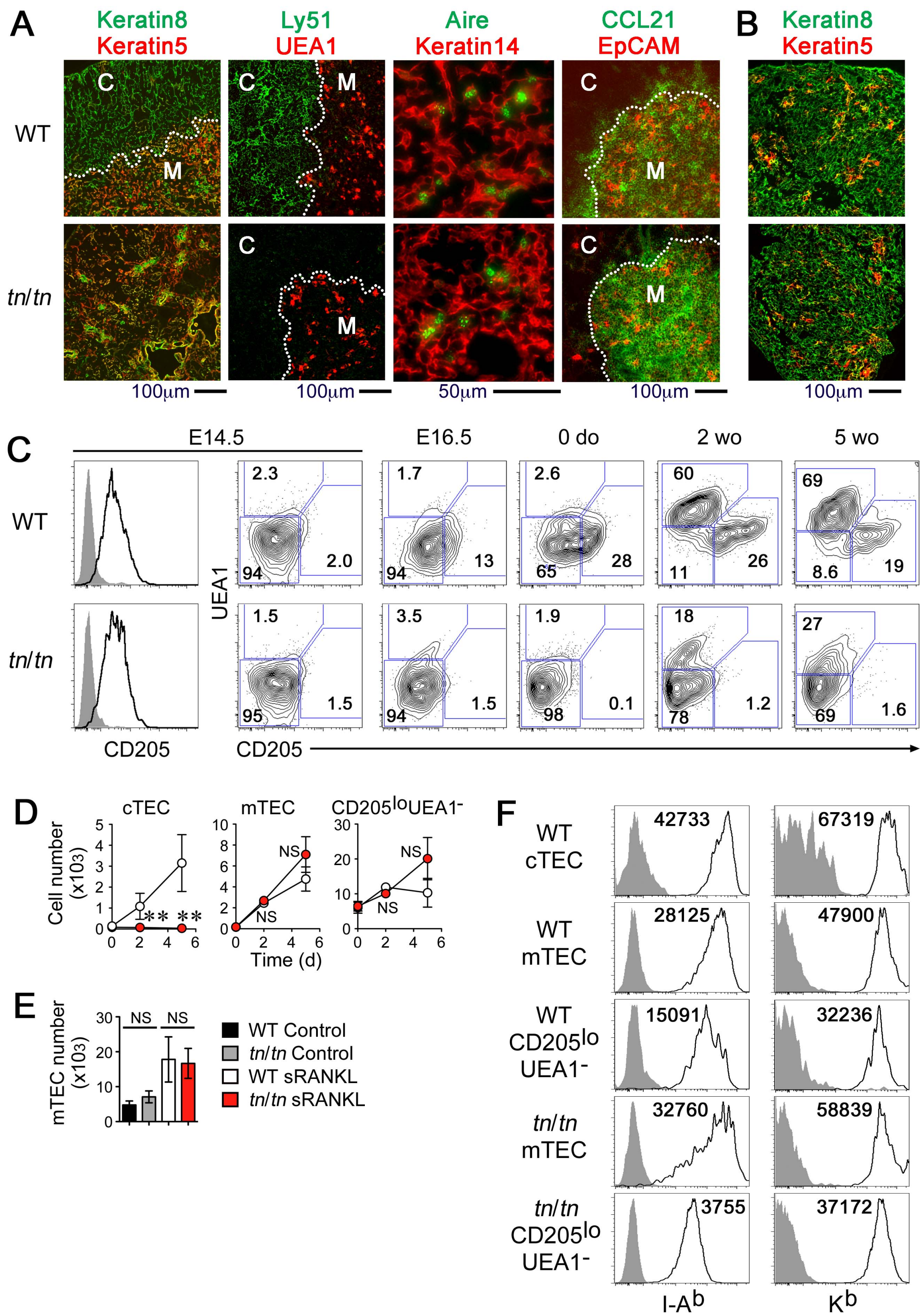


Figure S3. Impaired development of mature cTECs in *TN* mice. (**A**) Thymus

sections from 5-week-old WT or *tn*/*tn* mice were stained for Keratin 8, Keratin 5, Ly51, UEA1, Aire, Keratin 14, CCL21, and EpCAM. Keratin 8^{+}Keratin 5^{-} and $Ly51^{+}$ cTECs were almost undetectable in *tn*/*tn* mice, whereas functional mTECs expressing Aire and CCL21 were readily detected. C denotes cortex and M denotes medulla. Dotted lines indicate cortex/medulla boundary. (**B**) Thymus sections from E14.5 WT or *tn*/*tn* embryos were stained for Keratin 8, Keratin 5. (**C**) Flow cytometry profiles for CD205 and UEA1 of $EpCAM^{+}Keratin^{+}$ TECs prepared from indicated ages of WT or *tn*/*tn* mice. Histograms show CD205 staining profiles (solid lines) and control staining profiles (shaded lines). Numbers indicate percentage of cells within indicated areas. Data are summarized in **Figure 2D**. (**D**) Organ culture of E14.5 fetal thymic lobes from WT (white circles) or *tn*/*tn* mice (red circles). Graphs show numbers of $CD205^{hi}UEA1^{-}$ (cTEC), $CD205^{lo}UEA1^{+}$ (mTEC), or $CD205^{lo}UEA1^{-}$ TECs per lobe (n= 3-5). (**E**) E14.5 fetal thymic lobes were cultured with or without sRANKL (1 μg/ml) for 5 days. Graph shows numbers of $CD205^{lo}UEA1^{+}$ mTECs per lobe (n= 3-5). Mean ± s.e.m. **, $p<0.01$; NS, not significant (unpaired t test). (**F**) Thymic stromal cells prepared from 2-week-old mice were analyzed by flow cytometry as described in **C**. Histograms show expression of I-A^{b} (MHC class II) and K^{b} (MHC class I) in gated $CD205^{hi}UEA1^{-}$ (cTEC), $CD205^{lo}UEA1^{+}$ (mTEC), and $CD205^{lo}UEA1^{-}$ TECs. Shaded lines indicate control staining. Numbers in histograms indicate mean fluorescence intensity (MFI). $CD205^{lo}UEA1^{-}$ TECs in *tn*/*tn* mice show traits of immature cTECs. Data represent three independent experiments.

Figure S4

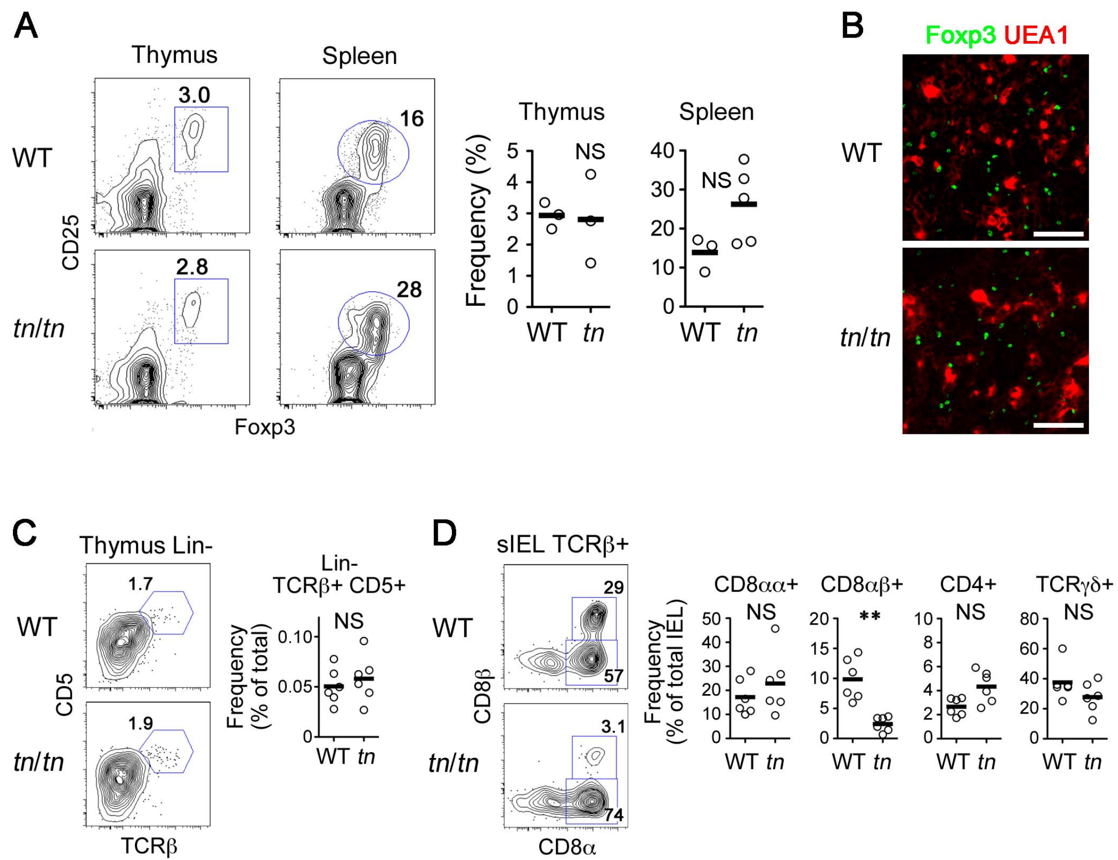


Figure S4. Development of Tregs and IELs in *TN* mice. (**A**) Flow cytometry profiles for Foxp3 and CD25 of CD4SP TCRβ$^+$ thymocytes or splenocytes from 5-week-old mice. Graphs indicate frequency of Foxp3$^+$CD25$^+$ cells (n = 3-4). (**B**) Thymus sections from 5-week-old WT or *tn*/*tn* mice were stained for Foxp3 and mTEC marker UEA1. Foxp3$^+$ cells were detectable in the medulla both in WT and in *tn*/*tn* mice. Scale bars, 50 μm. Data represent two independent experiments. (**C**) Flow cytometry profiles for TCRβ and CD5 of lineage-negative (Lin$^-$, CD4$^-$CD8$^-$CD19$^-$CD11b$^-$CD11c$^-$NK1.1$^-$TCRγδ$^-$) thymocytes (left). Numbers indicate frequency of TCRβ$^+$CD5$^+$ IEL precursor cells. Graph indicates the

frequency of TCRβ^+CD5$^+$ cells in Lin$^-$ cells (right) (n = 6). (**D**) Flow cytometry profiles for CD8α and CD8β of TCRβ^+CD45$^+$ cells prepared from small intestine (left). Graphs indicate the frequency of TCRβ^+CD8$\alpha\alpha^+$, TCRβ^+CD8$\alpha\beta^+$, TCRβ^+CD4$^+$, or TCR$\gamma\delta^+$ cells in total IELs (right) (n=6). Each circle represents an individual mouse and horizontal bars indicate the mean (**A**, **C**, **D**). **, $p<0.01$; NS, not significant (unpaired t test). Data represent three (**A**), two (**B**), or five (**C**, **D**) independent experiments.

Figure S5

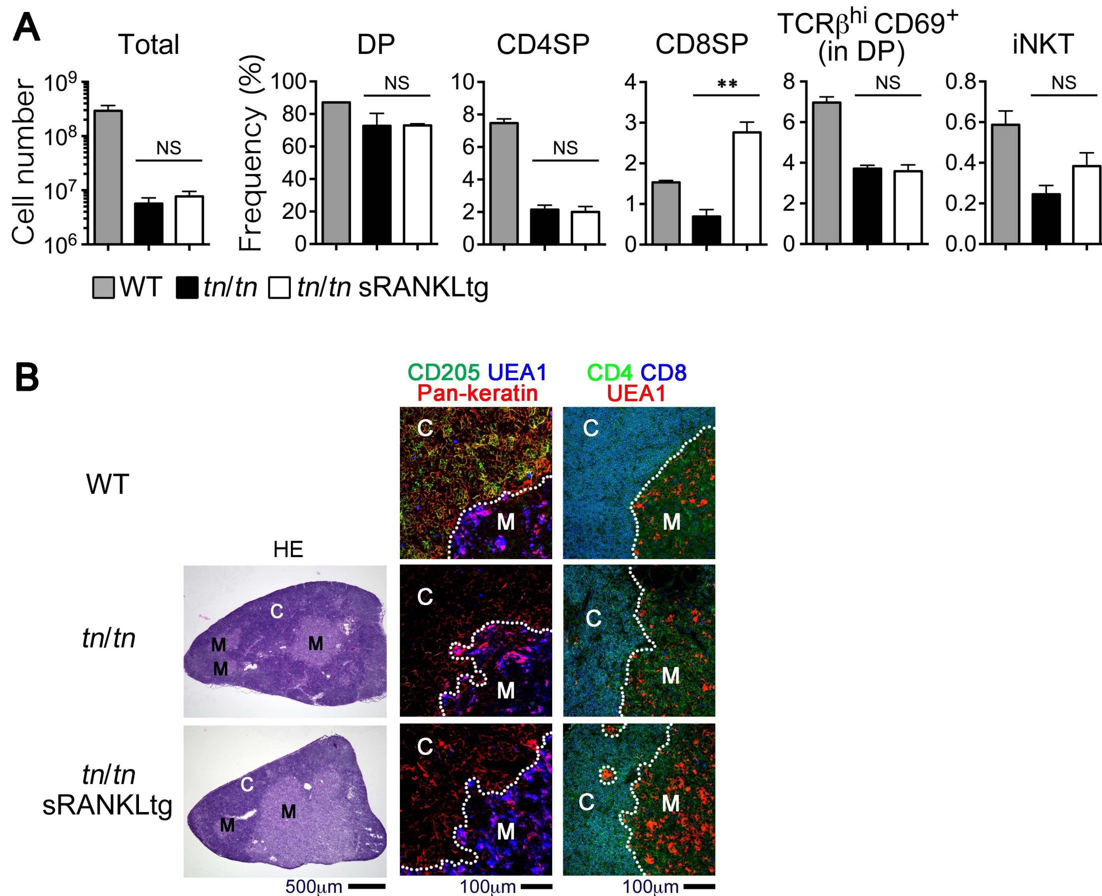


Figure S5. Thymus from *tn*/*tn* sRANKLtg mice. (**A**) Total thymocyte number and frequency of indicated thymic cells (n = 3-5). The reduced frequency of CD4SP cells, TCRβ^{hi}CD69^{+} post-selected DP thymocytes, and TCRβ^{+} αGal-Cer/CD1d tetramer^{+} iNKT cells in *tn*/*tn* thymus was not restored by sRANKL transgene. *tn*/*tn* sRANKLtg mice showed increased frequency of CD8SP thymocytes, which was also observed in wild-type sRANKLtg mice (3.32 ± 0.23 %), indicating that sRANKL causes expansion of CD8SP thymocytes irrespective of the β5t mutation, although the mechanism is unclear. **, $p<0.01$; NS, not significant (unpaired t test). (**B**) Thymus sections from 3-week-old were stained with hematoxylin and eosin (HE), or for CD205, UEA1,

pan-Keratin, CD4, and CD8. C denotes cortex and M denotes medulla. Dotted lines indicate cortex/medulla boundary. Introduction of sRANKL transgene caused enlarged medulla formation but had no effect on impaired development of mature cTECs and cortex/medulla compartmentalization in *tn*/*tn* thymus.

Table S1. CDR3 sequences from Vγ6^{+} and Vγ4^{+} γδT cells.

Cell subset	Sequence			Occurrence	
	Vγ	N	Jγ1	WT	*tn/tn*
Vγ6+	CWD		SSGF	8	7
Total				8	7
Vγ4+	YCSY	GLY	SSGF	5	3
	YCSY	GY	SSGF	3	5
	YCSY	GSY	SSGF	3	4
	YCSY	GRY	SSGF	3	1
	YCSY	GH	SSGF	2	4
	YCSY	G*		2	3
	YCSY	GEY	SSGF	2	1
	YCSY	GN	SSGF	2	0
	YCSY	GPY	SSGF	2	0
	YCSY	GQY	SSGF	2	0
	YCSY	GGY	SSGF	1	2
	YCSY	GVY	SSGF	1	1
	YCSY	GD	SSGF	0	4
Others				14	25
Total				42	53

Vγ4-Cγ1 and Vγ6-Cγ1 cDNA fragments were PCR-amplified and cloned in the plasmid vector. DNA sequences of randomly picked clones were determined. Listed are amino acid sequences of Vγ6-Jγ1 and Vγ4-Jγ1 joints and the number of times each

sequence occurred. No diversity was detected in Vγ6-Jγ1 and Jγ1-Cγ1 joints. An asterisk indicates the termination of translation.

Table S2. CRISPR/Cas9-mediated gene targeting in +/*tn* mice.

Host embryos	Dose of Cas9/sgRNA (ng/µl)	Survived embryos / Injected embryos	Newborns	Mutant alleles per mouse / Total mice tested		
				2	1	0
BDF1 x TN	100/50	140/213	50(35.7%)	0/47	38/47	9/47
B6 x TN	100/50	145/175	14(9.7%)	2/12	9/12	1/12

hCas9 mRNA and G220R-specific sgRNA were microinjected into the cytoplasm of the fertilized eggs from (BDF1 x *TN*) or (C57BL/6 x *TN*) mating. The number of total alleles mutated in each mouse is listed from 0 to 2.

Table S3. Potential off-target effects of CRISPR/Cas9-mediated gene targeting in +/*tn* mice.

Site name	Sequence	Indel mutation frequency (Mutant/Total)	Coordinate (mm9)
Psmb11 (G220R)	TGCTTATTCAAGGGGCTCAG**TGG**		
Off target 1	gatTTATTCAAGGGGCTCAG**gGG**	14/21[a]	chr11: +97399031
Off target 2	ccCTcATTCtAGGGGCTCAG**GaG**	0/20	chr5: -48885216
Off target 3	TGCTTggaCAAGGGGCTCAG**caG**	0/19	chr18: +86446410

Potential off-targets were predicted by CRISPR Design Tool (http://crispr.mit.edu) searching the mouse genome (mm9) for matches to the 20 nt guide sequence followed by PAM sequence. Mismatches from the on-target sequence are lower-case and underlined. PAM sequences are boldface.

[a] PCR products were sequenced to confirm off-target mutations.