Figure S1: TCRαβ chains are expressed in cell lines and efficiently cleaved *in vitro* and *in vivo* (related to Figure 1).

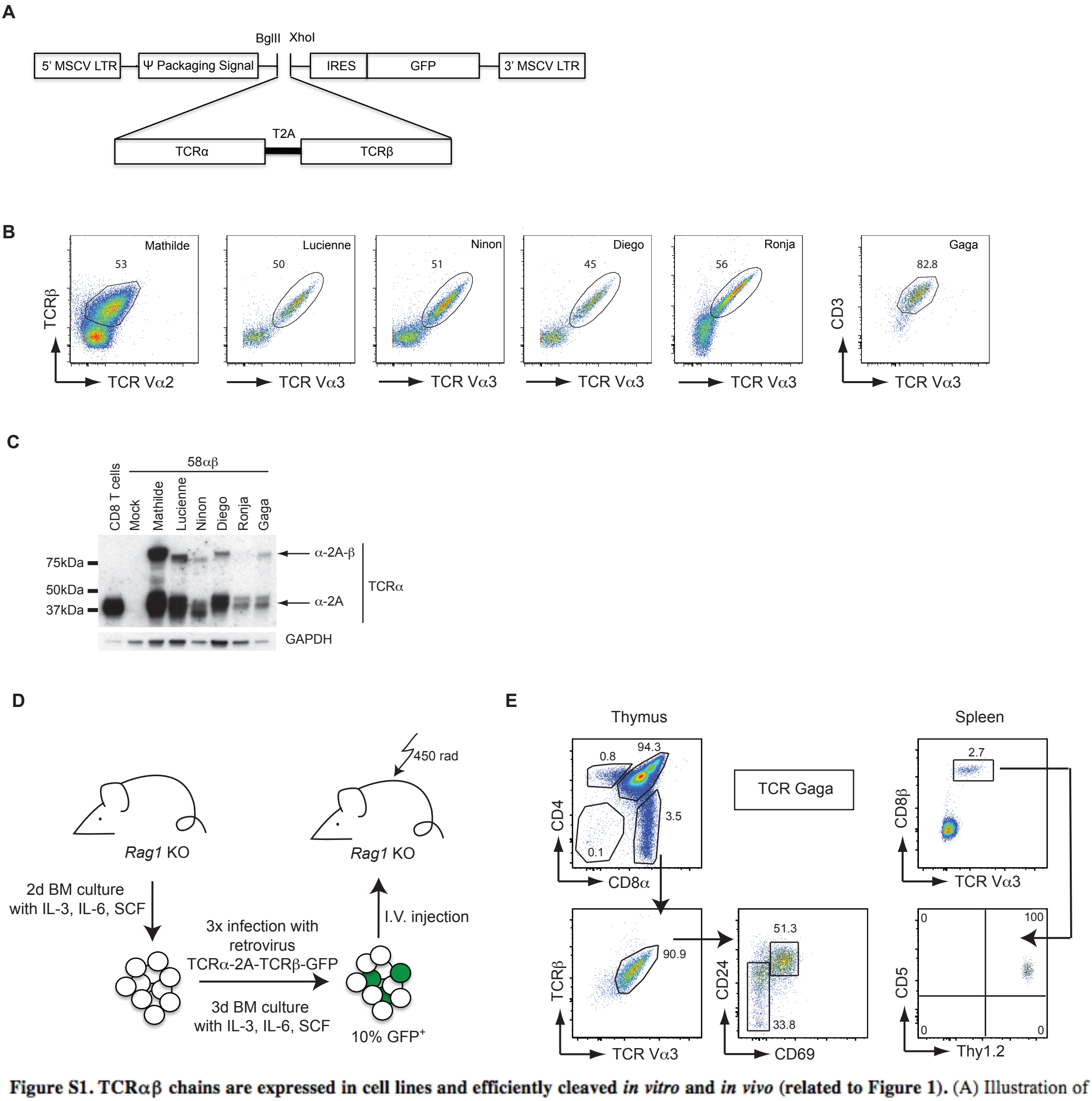

Figure S1. TCRαβ chains are expressed in cell lines and efficiently cleaved *in vitro* and *in vivo* (related to Figure 1). (A) Illustration of the retroviral vector and 2A-linked TCR constructs used. The MSC long terminal repeat promoter was used to express the 2A-linked construct. The IRES directs translation of GFP. Key restriction sites used for cloning are indicated. MSCV, Murine Stem Cell Virus; T2A, 2A regions of the *Thosea Asigna* virus. (B) $58\alpha^{-}\beta^{-}$ cells were transiently transfected with plasmids carrying the 2A-linked TCRαβ chains from each clone, as indicated, stained with anti-TCRβ, anti-TCR Vα2, anti-TCR Vα3 or anti-CD3, and analysed by flow cytometry. (C) $58\alpha^{-}\beta^{-}$ cells transfected as described in (B) were lysed and the lysates were resolved by SDS-PAGE. Membranes were probed with anti-TCRα and anti-GAPDH antibodies. Cells transduced with empty vector and sorted $CD8^{+}$ T cells were used as negative and positive controls, respectively. (D) Schematic representation of the procedure for generation of retroviral bone marrow chimeras. BM, bone marrow; d, days; IL, interleukin; SCF, Stem Cell Factor. (E) FACS analysis of GFP^{hi} thymocytes (left panels) and splenocytes (right panels) from *Rag1*$^{-/-}$ chimeras expressing TCR Gaga isolated from a $CD8\alpha\beta^{+}$ $TCR\alpha\beta^{+}$ intraepithelial T cells and analysed 28 days post reconstitution. Representative plots are shown from two independent experiments (n=4). The genes for the β-chain of TCR Gaga are TRBV13-1, TRBD1, TRBJ1-1 and the sequence for the CDR3 is CASSEGQDTEVFF. The genes for the α-chain of TCR Gaga are TRAV9N-3, TRAJ5 and the sequence for the CDR3 is CAVSRPQVVGQLTF.

Figure S2: DN TCRαβ⁺ T splenocytes harbour the potential to migrate to the small intestine (related to Figure 2).

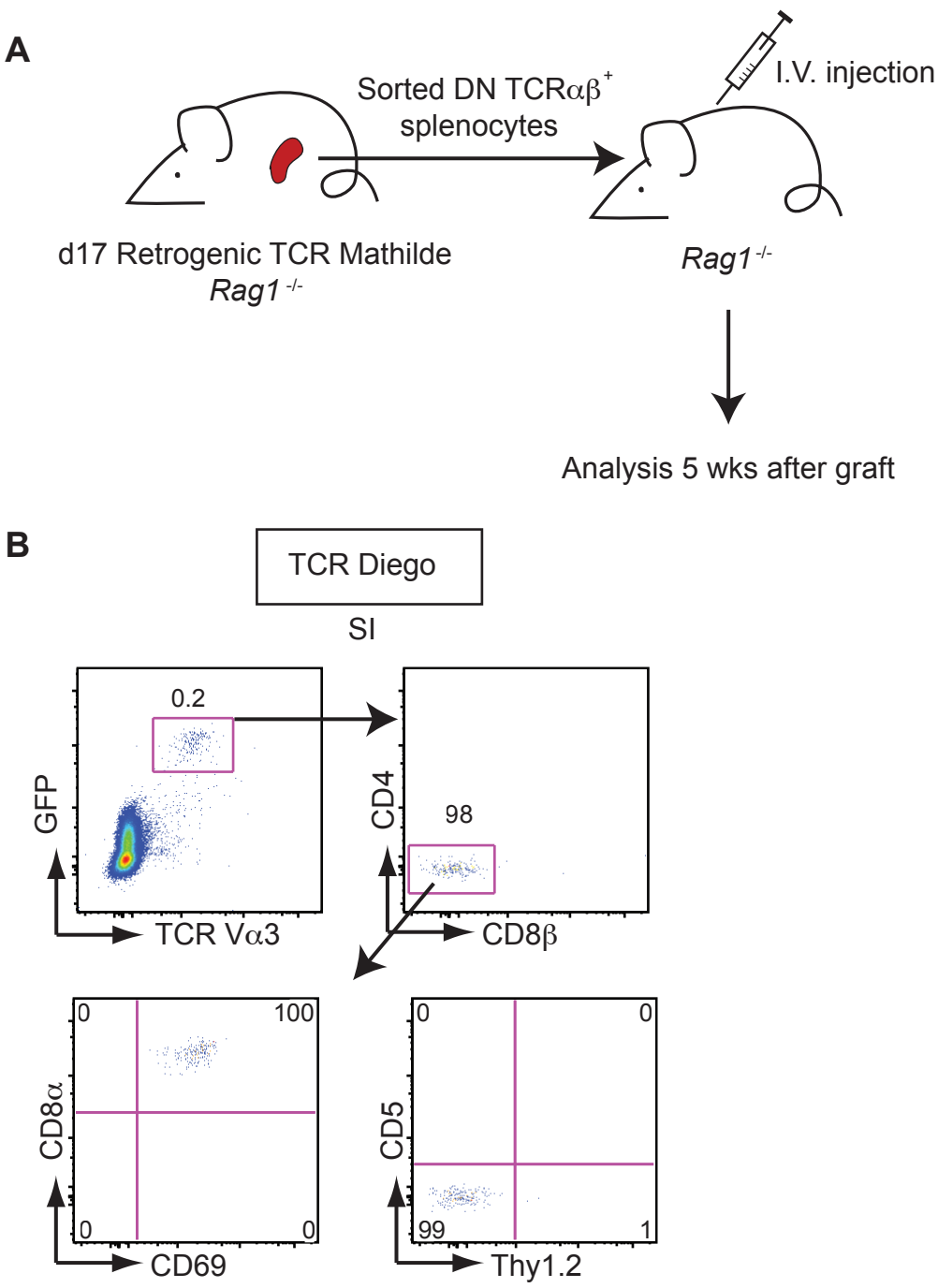

Figure S2. DN TCRαβ⁺ T splenocytes harbour the potential to migrate to the small intestine (related to Figure 2). (A) Schematic representation of the experimental set-up. (B) Sorted GFP⁺ DN TCRαβ splenocytes isolated from retrogenic donor mice were adoptively transferred to *Rag1*$^{-/-}$ recipients. Representative flow cytometry plots of GFP⁺ intraepithelial T cells from chimeras injected with GFP⁺ DN TCRαβ Diego splenocytes and analysed five weeks post-transfer. Two independent experiments were performed for each TCR, TCR Mathilde (n=2) and TCR Diego (n=2).

Figure S3: DN TCRαβ⁺ T cells develop in a competitive mix BM chimera set-up (related to Figure 2).

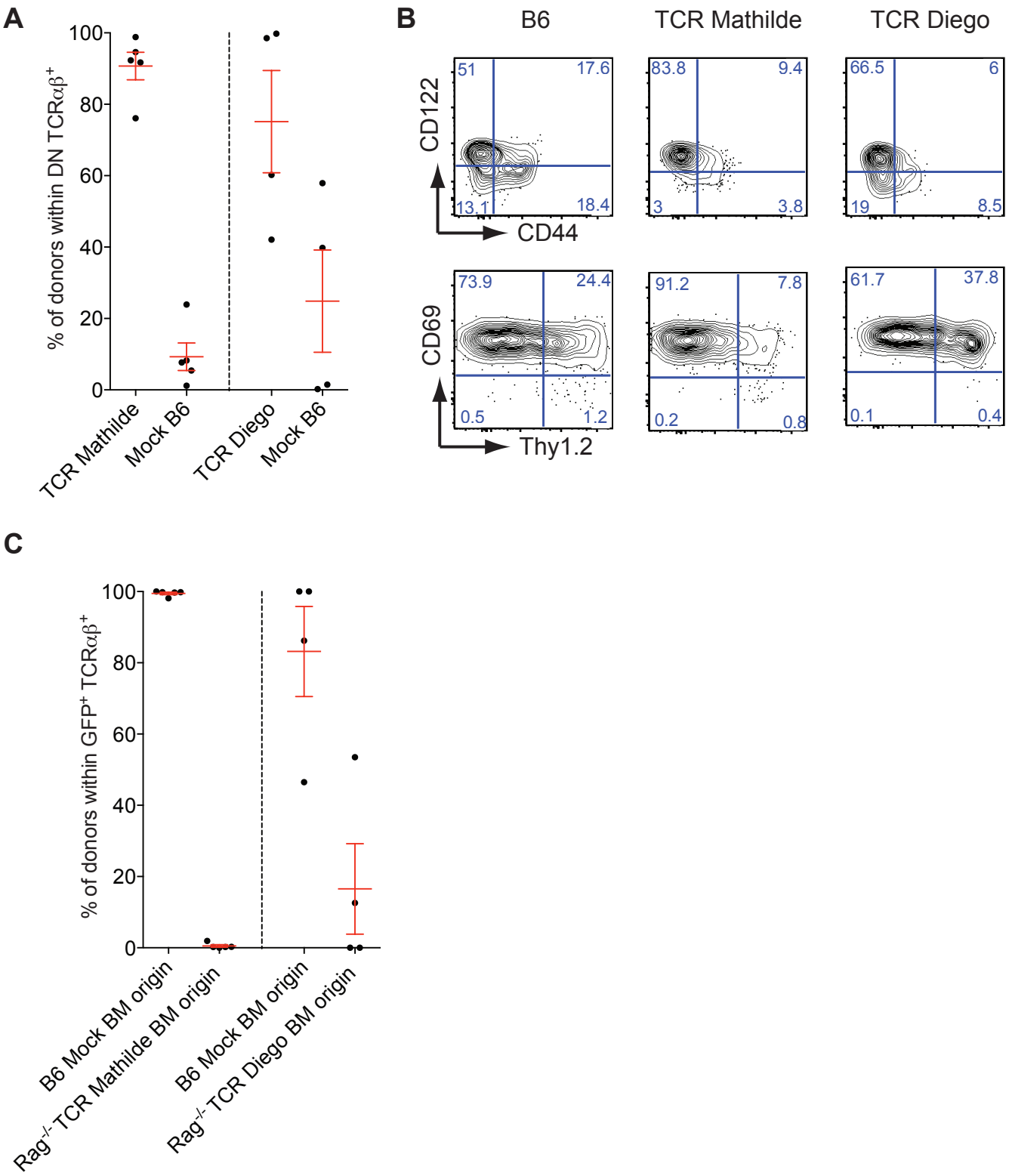

Figure S3. DN TCRαβ⁺ T cells develop in a competitive mix BM chimera set-up (related to Figure 2). An equal number of B6 Ly5.1⁺ BM cells transduced with Mock vector and *Rag1*$^{-/-}$ Ly5.2⁺ BM cells transduced with either TCR-Mathilde or TCR-Diego were injected in irradiated *Rag1*$^{-/-}$ recipient and analysed five weeks post-transfer. (A) Graph represents the percentage of Ly5.1⁻ DN TCRαβ⁺ cell expressing TCR-Mathilde or TCR-Diego and Ly5.1⁺ DN TCRαβ⁺ cell from B6 in gated GFP⁺ DN TCRαβ⁺ intraepithelial T cells from the competitive mix BM chimera. (B) Representative dot plots showing CD122 versus CD44 and CD69 versus Thy1.2 expression on DN TCRαβ⁺ intraepithelial T cells from B6 WT or mix BM chimera. **C**. Graph represents the percentage of B6 Ly5.1⁺ versus *Rag1*$^{-/-}$ Ly5.2⁺ BM cells within GFP⁺ Thy1.2⁺ CD5⁺ gated splenocytes isolated from the competitive mix BM chimera. Two independent experiments were performed for each TCR, TCR Mathilde (n=5) and TCR Diego (n=4).

Figure S4: CD69 expression on thymocyte subsets (related to Figure 3).

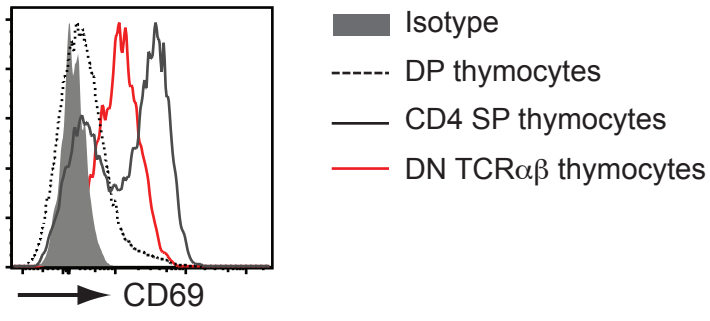

Figure S4. CD69 expression on thymocyte subsets (related to Figure 3). Expression of CD69 on B6 CD4 SP, B6 DP and DN TCRαβ$^+$ Ninon thymocytes. Representative graphs from 2 mice. Similar results were obtained in chimera

Figure S5: DN TCRαβ⁺ T cells are not Qa1-, TL- or CD1d-restricted T cells (related to Figure 5).

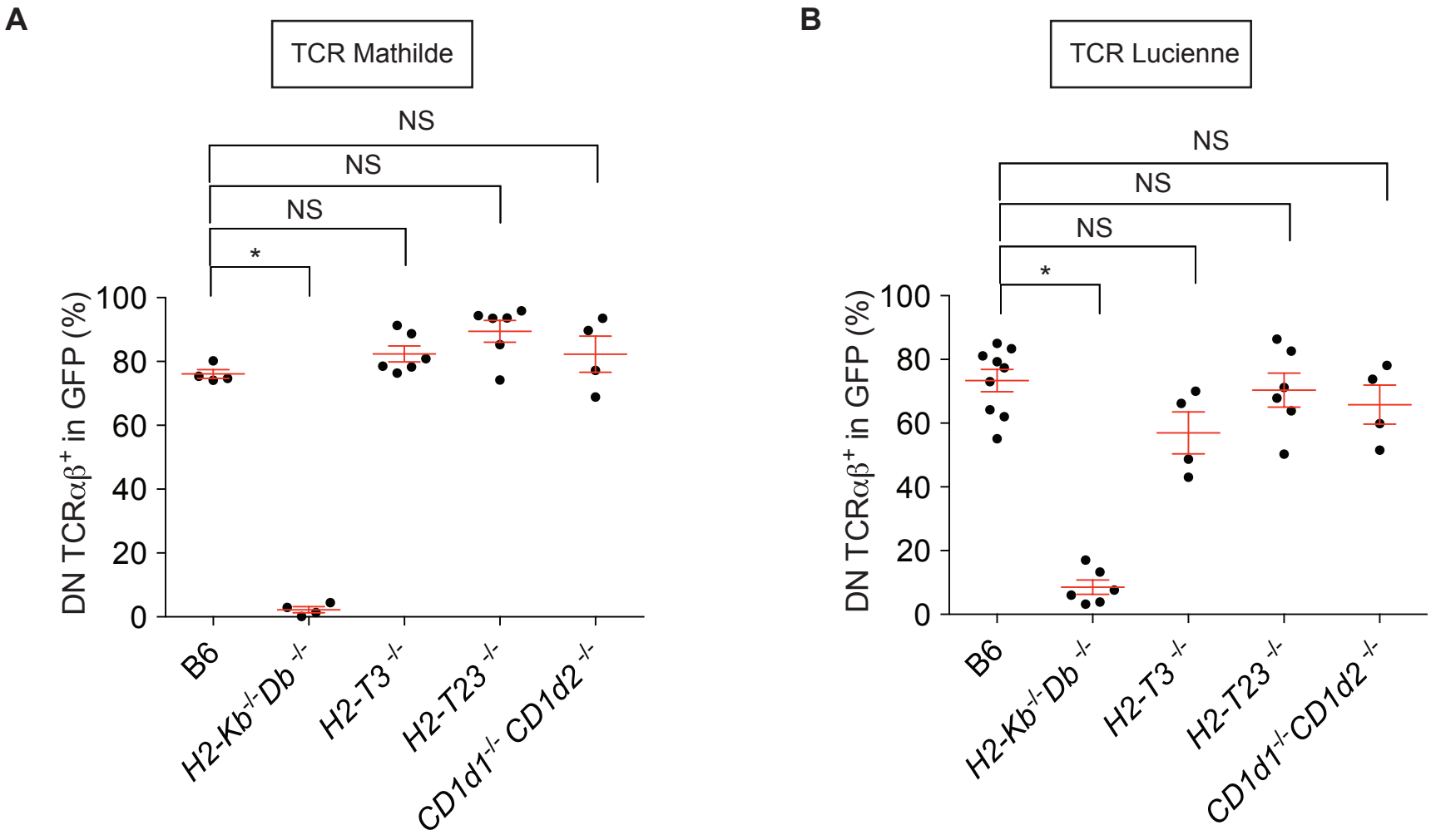

Figure S5. DN TCRαβ⁺ T cells are not Qa1-, TL- or CD1d-restricted T cells (related to Figure 5). Frequency of DN TCRαβ⁺ T cells in GFP⁺ gated intraepithelial T cells isolated from chimeric recipients deficient for the indicated MHC class I molecules. Recipients were analysed five weeks after reconstitution. (A) Analysis of chimera expressing TCR Mathilde from at least two independent experiments (B6 (n=4), *H2-Kb*$^{-/-}$*Db*$^{-/-}$ (n=5), *H2-T3*$^{-/-}$ (n=6), *H2-T23*$^{-/-}$ (n=6) and *CD1d1*$^{-/-}$ *CD1d2*$^{-/-}$ (n=4). (B) Analysis of chimera expressing TCR Lucienne from at least two independent experiments (B6 (n=9), *H2-Kb*$^{-/-}$*Db*$^{-/-}$ (n=6), *H2-T3*$^{-/-}$ (n=4), *H2-T23*$^{-/-}$ (n=6) and *CD1d1*$^{-/-}$ *CD1d2*$^{-/-}$ (n=4). Data points are shown for each mouse, with mean plus s.e.m. A two-tailed Mann-Whitney test was performed. * P <0.05 and NS, not significant.

Figure S6: Table summarizing the TCR specificity for each clone analysed (related to Figure 5 and Figure 6).

Mouse Line \ Clone	Mathilde	Lucienne	Diego	Ronja	Ninon
B6	Yes	Yes	Yes	Yes	Yes
H2-Kb$^{-/-}$ *Db* $^{-/-}$	No	No	Yes	Yes	Yes
H2-T3 $^{-/-}$	Yes	Yes	Yes	Yes	Yes
CD1d1$^{-/-}$ *CD1d2*$^{-/-}$ (Chr. 3)	Yes	Yes	Yes	Yes	Yes
H2-T23 $^{-/-}$	Yes	Yes	Yes	Yes	Yes
Mr1 $^{-/-}$ (Chr. 1)	N.D.	N.D.	Yes	Yes	Yes
Fcgrt $^{-/-}$ (Chr. 7)	N.D.	N.D.	N.D.	N.D.	Yes
Hfe $^{-/-}$ (Chr. 13)	N.D.	N.D.	N.D.	N.D.	Yes
BALB/cJ (*H2-Qa2*$^{+}$)	N.D.	N.D.	No	No	Yes
BALB/c ByJ (*H2-Qa2*^{null})	N.D.	N.D.	No	No	Yes
B6.C-H2^{d}/bByJ (*H2-Qa2*^{null})	N.D.	N.D.	No	No	Yes
Tap1 $^{-/-}$	Yes	Yes	No	No	Yes
Conclusions	K^b D^b restricted TAP independent		Restricted to an MHC-Ib molecule from the H2-Q or H2-T or H2-M regions (other than the one tested) and that differ between B6 and Balb/c backgrounds and is localized on chromosome 17. TAP dependent		Restricted to an MHC-Ib molecule other than the one tested. TAP independent

Figure S6. Table summarizing the TCR specificity for each clone analysed (related to Figure 5 and Figure 6). "Yes" indicates the presence of DN TCRαβ^{+} intraepithelial T cells and "No" the absence of DN TCRαβ^{+} intraepithelial T cells. In mice, the MHC locus is localised on chromosome (Chr.) 17 with a few MHC molecules localised on other chromosomes as indicated.

Table S1. TCRβ and TCRα sequences of five individual DN TCRαβ$^+$ intraepithelial T cells isolated from C57BL/6 mice (related to Figure 1).

TCR Name	TCRβ chain				TCRα chain		
	Vβ segment	Dβ segment	Jβ segment	CDR3 amino-acid sequences	Vα segment	Jα segment	CDR3 amino-acid sequences
Mathilde hybridoma	TRBV5	TRBD2	TRBJ2-3	CASSQEDWGPSAETLYF	TRAV14-2	TRAJ48	CAAQANYGNEKITF
Lucienne	TRBV16	TRBD1	TRBJ1-2	CASSPGQANSDYTF	TRAV9N-3	TRAJ23	CAVRNYNQGKLIF
Diego	TRBV16	TRBD1	TRBJ2-4	CASSSPGQGASQNTLYF	TRAV9N-3	TRAJ58	CAVKGTGSKLSF
Ronja	TRBV16	TRBD1	TRBJ2-7	CASSLDRISYEQYF	TRAV9N-3	TRAJ35	CAVRTGFASALTF
Ninon	TRBV16	TRBD2	TRBJ2-7	CASSSKRLGAYEQYF	TRAV9N-3	TRAJ12	CAVSMGTGGYKVVF