Supplemental experimental procedures

Preparation of cell suspensions

Thymus and spleen cell suspensions were obtained by pushing the organs through 70 μm nylon cell strainers (Fisher Scientific, San Diego, CA, USA) and re-suspending in PBS with 5% FBS. Red blood cells were lysed with red blood cell lysing buffer (Sigma Aldrich, St Louis, MO, USA). To prepare intraepithelial T cells, small intestines were cleaned of connective tissue. Peyer's patches and feces were removed before the small intestines were cut open and washed three times in HBSS + 5% FBS. The intestines were next cut into small pieces and transferred into 50ml conical tubes containing 20ml HBSS supplemented with 5% FBS and 1mM DTT (Sigma Aldrich, St Louis, MO, USA). Such prepared samples were shaken for 20 minutes at 37°C and 220 rpm. Remaining pieces of intestines were removed using metal strainers. Obtained cells were further purified on 40/70% discontinuous Percoll gradient (GE Healthcare, Uppsala, Sweden) and centrifuged for 20 minutes at 600g at RT and washed with HBSS plus 5% FBS.

Hybridoma generation

Intraepithelial T cells from the small intestine were prepared from 10-12 week old male B6 mice. The pure population of CD8$\alpha\alpha^{+}$ expressing TCR$\alpha\beta^{+}$ intraepithelial T cells was isolated from the lymphocytes preparation by double FACS sorting (FACS Aria, BD Biosciences, San Jose). Obtained cells were fused in a 1:5 ratio with the BW5147 thymoma cell line (TCR$\alpha\beta^{-/-}$ variant), a kind gift from Drs. Marrack P. and Kappler J. (National Jewish Health, Denver, CO) in the presence of 50% PEG 1500 (Roche Diagnostics, Indianapolis, IN) according to the standard method described by Sydora et al. (Sydora et al., 1993). The cells were plated at the density of 1-10 x 10^5 per well in a final volume of 100uL IMDM medium with 10% FBS, 1.5 x HAT supplement (Sigma Aldrich, St Louis, MO) and 75ug/ml gentamycin (Sigma Aldrich, St Louis, MO) in flat-bottomed 96-well plates containing 1 x 10^4 mouse peritoneal exudate cells per well (50uL volume). 50uL of fresh medium with 1 x HAT was added on day 1, and 150 uL of medium was replaced with identical fresh medium on day 3. TCR positive cells were sub-cloned using a limited dilution method.

Kidney capsule transplantation

Thymus tissue used for the transplantation was collected from retrogenic mice 17 days after receiving the bone marrow injection. Thymus tissue was harvested in DMEM supplemented with 5% FBS, washed and transferred to sterile PBS. The recipient mice were anesthetized with an *i.p.* injection of ketamine/xylazine (100mg/kg ketamine and 10 mg/kg xylazine) and maintained on a heated pad throughout the procedure. Petrolatum ophthalmic ointment (Dechra, Overland Park, KS, USA) was applied to the eyes to prevent drying during the procedure. A trimmer was used to remove fur from the left side of the mouse in a central area over the incision site starting about 2 cm above the tail along the spine, approximately 1 cm wide. Prior to making an incision, the mouse was placed on its right side with the shaved left side facing the surgeon and the area was cleaned using povidone-iodine. All instruments were sterilized prior to surgery using a heat bead sterilizer. A 10-15 mm skin incision was made in a longitudinal direction between the last rib and the hip joint. The connective tissue under the skin was loosened using a fine forceps and a 5-10 mm longitudinal incision was made in the abdominal wall. The kidney was gently squeezed out onto the exterior surface of the abdominal wall and kept moist by application of a small amount of sterile PBS. A 1-2 mm hole was made in the kidney capsule and the thymus was inserted above the kidney parenchyma under the kidney capsule. Once the thymus was under the capsule, the kidney was returned to the body cavity and two sutures were made in the muscle layer using 5-0 braided absorbable sutures (Covidien, Mansfield, MA, USA). The skin layer was stapled together with 3-5 Reflex 7 staples (CellPoint Scientific, Gaithersburg, MD, USA). After surgery, mice received buprenorphine (0.05-0.1mg/kg) *s.c.* and were kept in an incubator to recover. Buprenorphine was administered for an additional 48h, and the staples were removed 8-10 days post surgery.

Adoptive Transfer of Splenocytes

Spleen tissue from BM chimeric mice day 17 post reconstitution, was harvested in PBS supplemented with 5% FBS and single cell suspensions were prepared using a 70 μm nylon cell strainer (Fisher Scientific, San Diego, CA, USA). To enrich for GFP^+ DN

TCRβ$^+$ splenocytes, cells were stained with antibodies for CD4, CD8α, TCRβ, TCRα, Thy1.2 and CD69 before two rounds of purification done by cell sorting using a BD FACS Aria III (BD Biosciences, San Jose, CA, USA). Cell contaminants, if present, were below the detection limit. The recipient mice were injected via the retro-orbital vein with $2x10^4$-$6x10^4$ sorted GFP$^+$ DN TCRβ$^+$ splenocytes suspended in 100 µl PBS. The mice were euthanized and their tissues analysed 5 weeks post reconstitution.

Generation of Retroviral Mix Bone Marrow Chimeras

B6 Ly5.1$^+$ BM cells transduced with Mock vector and *Rag1*$^{-/-}$ Ly5.2$^+$ BM cells transduced with either TCR-Mathilde or TCR-Diego were prepared as described in the Material and Methods section. At the day of injection, BM cells were harvested, washed in PBS and suspended in PBS. An equal number of BM cells transduced with Mock vector were mixed with BM cells transduced with either TCR-Mathilde or TCR-Diego. BM cells were assessed for retroviral reporter expression (GFP) by flow cytometry. For all experiments, GFP expression on total BM cells after four days of *in vitro* culture ranged from 5% to 15%. *Rag1*$^{-/-}$ recipient mice were irradiated the day before injection at 450 rad. $7x10^6$-$9x10^6$ total mixed BM cells in 100µl PBS were injected into anesthetised mice through the retro-orbital vein. Mice were euthanized and their tissues analysed 4 to 5 weeks post reconstitution.

Antibodies, cell staining and Flow Cytomery

Dead cell discrimination was performed using the LIVE/DEAD Fixable Yellow Dead Stain Kit (Life Technologies, Eugene, Oregon, USA). The following antibodies were purchased from BD Biosciences (San Diego, CA, USA): CD3-PerCP-Cy5.5 (145-2C11), CD4-PE-CF594 (RM4-5), CD8α-PE-CF594 (53-6.7), TCRβ-PE (H57-597), TCRβ-APC-eF780 (H57-597), CD69-PE-CF594 (H1.2F3), CD69-PerCP-Cy5.5 (H1.2F3) and CD8β-PE (H35-17.2). The following antibodies were purchased from eBioscience (San Diego, CA, USA): CD8α-APC-eF780 (53-6.7), Vα3.2-APC (RR3-16), Vα2-APC (B20.1), CD69-eF450 (H1.2F3), CD5-eF450 (53-7.3), CD5-PerCP-Cy5.5 (53-7.3), CD122-eF450 (TM-b1) and CD8β-eF450 (eBioH35-17.2). The following antibodies were purchased from BioLegend (San Diego, CA, USA): CD4-AF700 (GK1.5), CD8α-

AF700 (53-6.7), Thy1.2-PE-Cy7 (30-H12), PD1-PE-Cy7 (RPM1-30) and CD8β-PerCP-Cy5.5 (YTS156.7.7). R-phycoerythrin-conjugated streptavidin-CD1d tetramers loaded with αGalCer were produced as previously described (Sidobre et al., 2002).

Western blot analysis

Cells were lysed in cold RIPA buffer (Pierce) supplemented with protease and phosphatase inhibitor mixture (Roche) for 20 min on ice, and centrifuged at 15,000 rpm for 15 min at 4 °C. Protein content was quantified, and equal amounts of protein were analysed by SDS–polyacrylamide gel electrophoresis (Bio-Rad) for each sample and transferred to a PVDF membrane (Invitrogen). The membrane was blotted with anti-TCRα (H28-710) or anti-GAPDH (FL-335) (Santa Cruz) antibodies. Immunoreactive bands were identified by chemiluminescence (ECL Solution, Santa Cruz).

Primer Sequences

List of primers used for RACE-PCR, RT-PCR and sequencing

Oligonucleotide name	**Sequence 5'-3'**	**Used for**
TCRα RT	GTA GGT GGC GTT GGT CTC TT	RT primer
TCRβ RT	TCC TTG CCA TTC ACC CAC CA	RT primer
Oligo-dC Adaptor	ACA GCA GGT CAG TCA AGC AGT AGC AGC AGT TCG ATA AGC GGC CGC CAT GGA CCC CCC CCC CCC DN	Second-strand cDNA synthesis
Adaptor P1	AGC AGG TCA GTC AAG CAG TA	1st PCR primer
TCRα 1st	GAT ATC TTG GCA GGT GAA GC	1st PCR primer
TCRβ 1st	TCC ACG TGG TCA GGG AAG AA	1st PCR primer
Adaptor P2	GCA GTA GCA GCA GTT CGA TA	2nd PCR primer
TCRα Nested	AAA GTC GGT GAA CAG GCA GA	2nd PCR primer
TCRβ Nested	GAT GGC TCA AAC AAG GAG AC	2nd PCR Primer
TRBV16 (Vβ11)	GCT TCT TGA GAG CAG AAC CA	1st and 2nd PCR
TRBV13-1 (Vβ8.3)	ATG GGC TCC AGG CTC TTT CT	1st and 2nd PCR
TRAV9N-3 (Vα3)	CTG GGG ATA CAC TTT CTC CT	1st and 2nd PCR

Supplemental reference

Sidobre, S., Naidenko, O.V., Sim, B.C., Gascoigne, N.R., Garcia, K.C., and Kronenberg, M. (2002). The V alpha 14 NKT cell TCR exhibits high-affinity binding to a glycolipid/CD1d complex. J Immunol *169*, 1340-1348.

Sydora, B.C., Mixter, P.F., Houlden, B., Hershberg, R., Levy, R., Comay, M., Bluestone, J., and Kronenberg, M. (1993). T-cell receptor gamma delta diversity and specificity of intestinal intraepithelial lymphocytes: analysis of IEL-derived hybridomas. Cell Immunol *152*, 305-322.