Figure S1

A

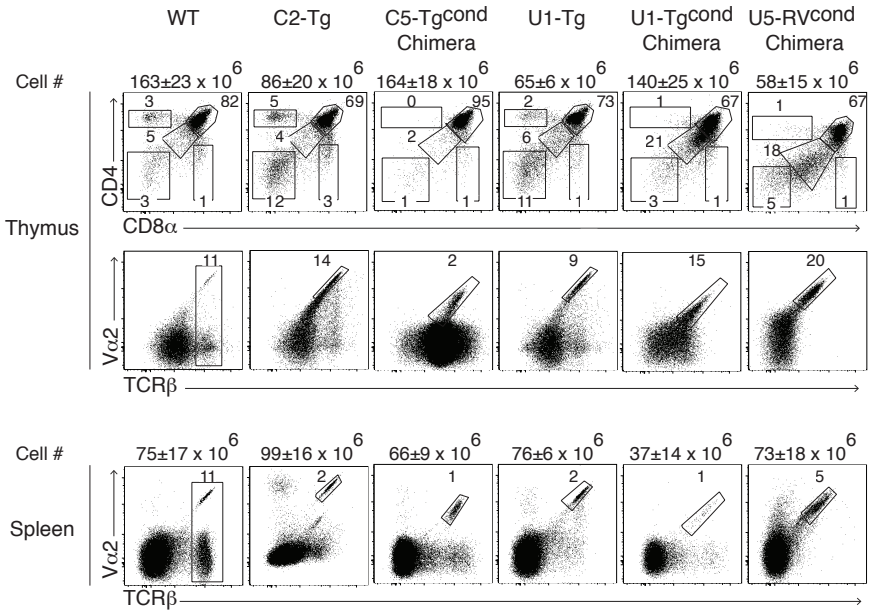
WT
C2-Tg
C5-Tg cond Chimera
U1-Tg
U1-Tg cond Chimera
U5-RV cond Chimera
Cell #
163±23 x 10^6
86±20 x 10^6
164±18 x 10^6
65±6 x 10^6
140±25 x 10^6
58±15 x 10^6
Thymus
CD4
CD8α
Vα2
TCRβ
Cell #
75±17 x 10^6
99±16 x 10^6
66±9 x 10^6
76±6 x 10^6
37±14 x 10^6
73±18 x 10^6
Spleen
Vα2
TCRβ

Figure S1: Distinct TCRs direct unconventional iIEL development, related to Figure 2. A, Flow cytometry of thymus and spleen gated on all lymphocytes from WT littermate and various TCR Tg, TCR Tg^{cond}, and TCR RV^{cond} mice. The absolute number (mean+/-s.e.m.) of lymphocytes is displayed above each plot. Data are pooled from 13 experiments.

Supplemental Experimental Procedures

Mice

C57BL/6, TCR$\alpha^{-/-}$ (B6.129S2-Tcra^{tm1Mom}/J), Bim$^{-/-}$ (B6.129S1-Bcl2l11$^{tm1.1Ast}$/J), Bcl-xL transgenic (B6.Cg-Tg(LCKprBCL2L1)12Sjk/J), β_2m$^{-/-}$ (B6.129P2-B2m^{tm1Unc}), CIITA$^{-/-}$ (B6.129S2-C2ta^{tm1Ccum}/J), MHC II$^{-/-}$ (B6.129S2-*H2*$^{dlAb1\text{-}Ea}$/J), and CD45.1 (B6.SJL-Ptprca Pep3b/BoyJ) mice were from Jackson Laboratories. Cd4-Cre (B6.Cg-Tg(CD4-cre)1Cwi), K^{b}D$^{b-/-}$ (B6.129P2-H2-K^{btm1} H2-D^{btm1}), I-A$^{b-/-}$ (B6.SJL(129)-Ptprca/BoyAiTac H2-Ab1^{tm1Gru}), and MHC I/II$^{-/-}$ (B6.129-H2-Ab1^{tm1Gru}-B2m^{tm1Jae}) mice were from Taconic. All mice were maintained in a specific pathogen-free environment at the University of Chicago and experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

TCR Transgenic Constructs and Transgenic Mice

All transgenic and retroviral TCR used in this study were obtained from and expressed in mice of the C57BL/6 background. The TCR Vβ8 transgenic construct was generated by cloning a Vβ8^{+} TCRβ chain from K^{b}D$^{b-/-}$ unconventional iIEL into pTβCass using XhoI and SacII (Kouskoff et al., 1995). The TCRα transgenic constructs were generated by cloning Vα2^{+} TCRα chains isolated from conventional or unconventional iIEL into pTαCass using XmaI and SacII (Kouskoff et al., 1995). For conditional transgenic expression (Tg^{cond}), the same TCRs were cloned using MfeI and BbvCI into a construct where expression is conditional on Cre-mediated excision of a lox-flanked STOP inserted

5' of the TCRα coding segment (obtained from Hogquist *et al*.) (Baldwin et al., 2005). Linearized constructs were injected into fertilized C57BL/6 oocytes, and the injected oocytes were implanted into pseudopregnant CD-1 mice. The TCRα Tg mice were crossed to the TCR Vβ7 tg strain and to $TCR\alpha^{-/-}$ mice to generate Vα2Vβ7 tg in a $TCR\alpha^{-/-}$ background. In one case (U1 Tg^{cond}) the TCRα and TCR Vβ7 constructs were co-injected. Tg^{cond} mice were crossed to Cd4-Cre mice to ensure proper TCR expression timing. Mice from one or two founder lines were analyzed for each TCR.

Antibodies and Flow Cytometry

Purified cell populations were incubated with Fc Block (Biolegend) prior to FACS staining. Fluorochrome or biotin conjugated monoclonal antibodies (clone in parentheses) against mouse α4β7 (DATK32), CD3ε (145-2C11), CD4 (GK1.5 or RM4-5), CD5 (53-7.3), CD8α (53-6.7), CD8β (53-5.8), CD45.1 (A20), CD45.2 (104), CD69 (H1.2F3), CD122 (5H4), CD160 (7H1), Ly49CFIH (14B11), PD-1 (29F.1A12), TCRb (H57-597), Thy1.1 (OX-7), Thy1.2 (53-2.1), Vα2 (B20.1), Vβ7 (TR310), Vβ8 (KJ16-133.18), 2B4 (m2B4 (B6)458.1), Rat IgG2A (r2a-21B2), and human CD4 (OKT4) were purchased from Biolegend, eBioscience, or BD Biosciences unless otherwise noted. Annexin V^+ cells were detected using a kit (eBioscience) after culture in RPMI (Corning) supplemented with 10% FCS. Intranuclear staining of Egr2 (erongr2) was performed using the Foxp3 Staining Buffer Set (eBioscience). Intranuclear staining of Bim (Cell Signaling Technology) granzyme B (GB11), Nur77 (12.14; Novus Biologicals), and Tbet (4B10) was performed by fixation in 4% paraformaldehyde (Electron Microscopy Sciences) followed by permeabilization using the permeabilization buffer (eBioscience).

Active Caspase 3 (Asp175; Cell Signaling Technology) was detected following fixation and permeabilization using a kit (BD Biosciences). Fluorescent second step reagents included streptavidin and donkey anti-rabbit antibody (Poly4064). Doublet exclusion and DAPI staining to remove dead cells was used when possible.

Cell Isolation

Small intestines were excised from mice and fat, peyer's patches, and luminal contents removed. The intestine was opened longitudinally and cut into 1 cm pieces. For experiments involving IEL isolation without LPL isolation, pieces were stirred with a magnetic stirrer for 2 hours in RPMI-10% FCS and then purified by running over a glass-wool column (Fisher Science France) followed by centrifugation on 40% percoll (Sigma) to further remove epithelial cells and debris. When both IEL and LPL were analyzed from the same mouse, the intestine was cut into pieces as above, but was shaken twice for 15 minutes in RPMI-1% FCS with 5mM EDTA to remove IEL and the remaining pieces were subjected to two 30-minute digestions with RPMI-20% FCS supplemented with 0.5 mg/mL of collagenase A (Roche) and 0.2 mg/mL of DNase I (Roche). The LPL fraction was subsequently purified by centrifugation over 40% percoll.

Retrovirus Production, Infection, and Chimera Generation

iIEL TCR Vα2 chains were cloned from conventional ($CD8\alpha\beta^+$ $TCR\alpha\beta^+$) iIEL or unconventional ($CD4^-$ $CD8\beta^-$ $TCR\alpha\beta^+$) iIEL isolated from TCRβ transgenic mice. For "negatively selected" thymic TCR, TCR Vα2 chains were cloned from $TCR\alpha\beta^+$ DP^{dull} $PD\text{-}1^{high}$ thymocytes FACS-sorted from TCRβ transgenic mice in a Bcl-xL transgenic

background. The TCRα chain genes were inserted into a conditional retroviral vector kindly provided by Brink *et al*., using EcoRI and AgeI (Turner et al., 2010). In some cases, the conditional retroviral vector was modified to express mouse Thy1.1 (RV^{cond2}) instead of human tail-less CD4 (RV^{cond1}) downstream of the IRES (the IRES-Thy1.1 construct was a gift from Richard Locksley). Constructs were transfected into Plat-E packaging cells using lipofectamine (Life Technologies) (Morita et al., 2000). Harvested retroviral supernatant was filtered through a .45 um filter and frozen in a dry ice/ethanol bath. TCR Vβ7 or Vβ8 transgenic Cd4-cre mice (usually also $TCR\alpha^{-/-}$) were injected with 150 mg kg^{-1} 5-Fluorouracil (APP Pharmaceuticals) three days prior to bone marrow harvest. After harvest, bone marrow was cultured for two days in X-Vivo 10 (Lonza) supplemented with 15% FCS, 1% penicillin/streptomycin, 100 ng mL^{-1} mouse SCF, 10 ng mL^{-1} mouse IL-3, and 20 ng mL^{-1} human IL-6 (all from Biolegend). Stimulated cells were infected with retrovirus in the presence of 4 μg mL^{-1} polybrene (EMD Millipore) by centrifugation at 800xg for 90 minutes at 30°C. After 24 hours of additional culture in medium as above, bone marrow cells were stained with antibodies against human CD4 or mouse Thy1.1, MACS-enriched (Miltenyi Biotec), and injected into lethally irradiated recipient C57BL/6 mice. In several experiments, mixtures of two bone marrow samples separately infected with different TCRα chains were injected and could be distinguished from each other by staining for congenic markers (CD45.1 *versus* CD45.2) and/or different retrovirally encoded proteins (human tail-less CD4 (RV^{cond1}) *versus* mouse Thy1.1 (RV^{cond2})). Chimeric mice were analyzed 3-8 weeks post reconstitution.

TCR Sequencing

Sorted cell populations were stored in Trizol (Life Technologies) prior to use. RNA was isolated using an RNeasy mini kit (Qiagen) and cDNA synthesized using SuperScript III (Life Technologies). TCR $V\alpha2^+$ TCRs were amplified using forward primer: 5'-ATGGACAAGATCCTGACAGCA-3' and reverse primer: 5'-TCAACTGGACCACAGCCTCAGC-3'. For cloning the transgenic TCR Vβ8 from unconventional iIEL, cDNA was amplified using two pairs of forward primers (able to amplify TCR Vβ8.1 and Vβ8.2): 5'-ATGGGCTCCAGACTCTTCTTTGTG-3' and 5'-ATGGGCTCCAGGCTCTTCTTCGTG-3' and reverse primer 5'-AGACCTTGGGTGGAGTCACATTTCTC-3'. PCR products were cloned into Zero Blunt TOPO (Life Technologies), transduced into XL-10 Gold Ultracompetent Cells (Agilent Technologies), and sequenced. TCR sequences were analyzed using IMGT (Lefranc et al., 2009).

Thymic Emigration Assays

Mice were anesthetized with ketamine/xylazine and then one or two lobes of the thymus were injected with 10 μL of a 0.5 mg mL^{-1} solution of Sulfo-NHC-LC-Biotin (Thermo Fisher Scientific) using a Hamilton syringe. 18-24 hours after injection, recent thymic emigrants (RTE) were enumerated by staining peripheral tissues with a fluorescently labeled streptavidin and comparing with uninjected control. Results were adjusted to the percentage of TCR^{hi} thymocytes that were biotin labeled.