Supporting Information

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SI Methods

Mice. *Rag2*-deficient mice (1) were bred and maintained in the Sunnybrook Research Institute animal facility in specific pathogen-free conditions. *Id3*$^{-/-}$ mice were kindly provided by Y. Zhuang (Duke University, Durham, NC) (2) and backcrossed to C57BL/6 *Rag2*-deficient mice. All animal procedures were approved by the Sunnybrook Health Sciences Centre Animal Care Committee (Toronto, ON, Canada).

Retroviral Transduction and Culture. Retroviral constructs were generated, as previously described (3–5), by subcloning the cDNA of interest into the MigR1 or MIY plasmids, upstream of the internal ribosomal entry site, and stable retroviral-producing GP+E.86 packaging cell lines were generated for each construct. The KN6 T-cell receptor-γ (TCR-γ) and TCR-δ subunits were cloned into pMiY as a fusion protein linked by the 2A Tescovirus linker peptide, as described previously (5). OP9-DL1, OP9-DL4, and OP9-Ctrl cells were produced and maintained as previously described (6, 7), and cocultures were supplemented with 1 ng/mL mouse recombinant IL-7 and 5 ng/mL human recombinant Flt-3L (Peprotech). Fetal livers (FL) were obtained from timed-pregnant *Rag2*$^{-/-}$ or *Rag2*$^{-/-}$*Id3*$^{-/-}$ female mice on d 14 of gestation. Single-cell suspensions were generated by disruption through a 40-μm nylon-mesh screen using a syringe plunger, and FL cells were cocultured with OP9-DL1 as previously described to produce double-negative (DN)3 cells (4). For retroviral transduction of DN3 cells, cells from d 7 FL/OP9-DL1 cocultures were passaged for an overnight coculture with stable retrovirus-producing GP+E.86 packaging cells. Following this step, the transduced (GFP$^+$ and YFP$^+$) CD44$^-$ CD25$^+$ DN3 cells were purified by cell sorting and placed back onto OP9 cocultures, as previously described (4).

Quantitative Real-Time PCR. Thymocyte populations were purified by flow cytometry or selection using magnetic anti-CD45 beads (Miltenyi Biotech). Total RNA was extracted using TRIzol (Invitrogen) and converted to cDNA using Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Expression of the indicated genes was measured by quantitative real-time PCR using SYBR GreenER (Invitrogen). β-Actin was used to normalize cycle thresholds.

Generation of BALB/c Stromal Cells. Ear dermal fibroblasts from BALB/c mice were isolated by incubating the minced and exposed epidermis layer with 0.3% trypsin/PBS. The minced pieces were treated at 37 °C for 60 min, cut into smaller 2- to 3-mm squares, and subsequently incubated at 37 °C, in a 5% CO_2 incubator in medium (DMEM supplemented with 10% FBS) for 3–4 d, or until fibroblast outgrowth became confluent. The cells were then harvested, pelleted by centrifugation, and reseeded into a monolayer. Supernatants from retroviral-producing GP+E.86 packaging cell lines transfected with MigR1, MigR1-DL1, or MigR1-DL4 were used to transduce the BALB/c (B/c) fibroblasts and generate stable B/c-DL1, B/c-DL4, and B/c-Ctrl cell lines, as previously described (7, 8). Subsequently, BALB/c-DL4 and BALB/c-Ctrl lines were each retrovirally transduced to express pMiCherry or T22^b-MiCherry (9) creating the four new cell lines BALB/c-Ctrl, BALB/c-T22, BALB/c-DL4, and BALB/c-DL4-T22.

Precursor Frequency Analysis. Limiting dilution analysis (LDA) was performed, as previously reported (3), using the automatic cell deposition unit function of the FACSAria cell sorter, such that 1, 3, 10, or 30, *Rag2*$^{-/-}$ DN3 (CD44$^-$CD25$^+$; in vitro-generated and retrovirally transduced as indicated above) YFP$^+$ GFP$^+$ cells were deposited into 96-well plates (48, 24, 24, and 12 well-replicates per dilution, respectively) containing OP9-DL4 or B/c-DL4 cells in OP9 medium supplemented with 1 ng/mL IL-7. After 7 d of culture, individual wells were scored for αβ- and γδ-lineage differentiation by flow cytometric analysis based on expression of CD45, and CD4$^+$ CD8$^+$, or CD4$^-$ CD8$^-$ CD73^{hi}, respectively. The αβ- and γδ-lineage precursor cell frequency was determined by the method of maximum-likelihood applied to the Poisson model (10).

PMA and Ionomycin Stimulation. CD45$^+$ GFP$^+$ cells were sorted from transduced DN3 cultured for 6 d on OP9-Ctrl, OP9-DL1, or BALB/c-DL4 cells. Sorted cells (5×10^4 cells per well of a 96-well plate) were subsequently cultured with 50 ng/mL PMA (Sigma) and 500 ng/mL Ionomycin (Sigma) in OP9 medium supplemented with 50 U/mL IL-2 (Peprotech). Supernatants were harvested after 36 h, and IFN-γ levels were quantified using the DuoSet ELISA Development System (R&D SysRtems) according to the manufacturer's protocol.

Statistics. The data and error bars are presented as mean ± SEM. To determine statistical significance a two-tailed unpaired Student *t* test was used for comparison between two experimental groups, using Prism software. Statistical significance was determined as $P < 0.05$ ($^*P < 0.05$; $^{**}P < 0.01$), and, n.s. as nonsignificant.

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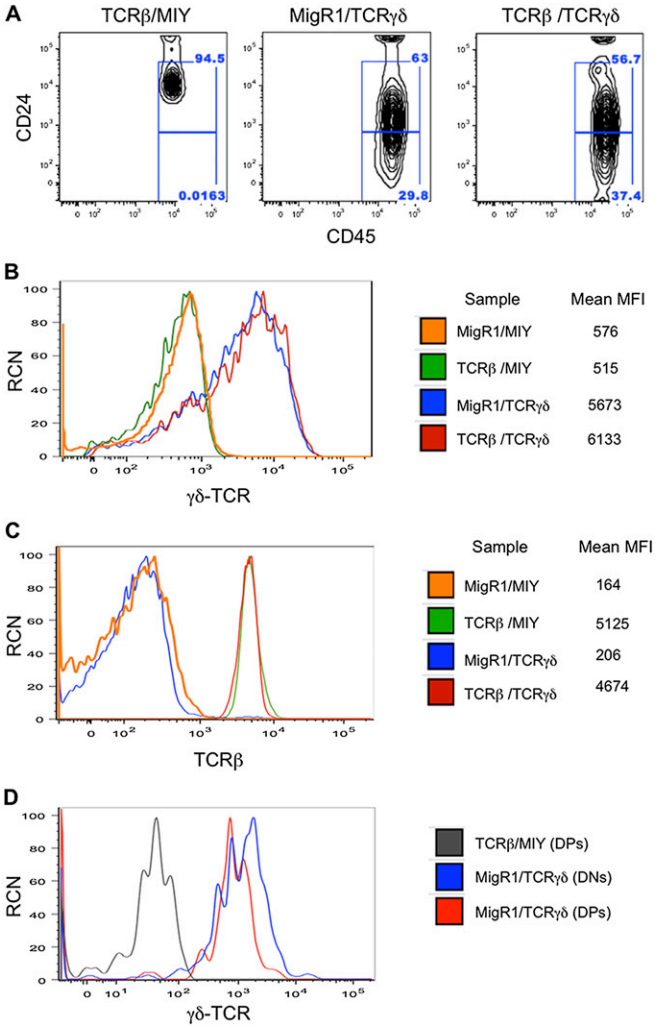

Fig. S1. γδ-TCR– and TCR-β/γδ–expressing DN3 cells express similar levels of γδ-TCR on the cell surface. Developmental progression of in vitro-derived *Rag2*$^{-/-}$ DN3 cells retrovirally transduced to express TCR-β, γδ-TCR, neither, or both, and cultured for 5 d with OP9-DL1 cells. (*A*) Flow cytometric analysis of CD24 and CD45 expression are shown for GFP^{+} YFP^{+} gated cells; (*B*) γδ-TCR expression, including mean fluorescence intensity (MFI) levels, as indicated; (*C*) intracellular staining for TCR-β expression, including MFI levels, as indicated. Results are representative of three independent experiments. (*D*) Flow cytometric analysis of γδ-TCR expression is shown for GFP^{+} YFP^{+}-gated cells from transduced DN3 cells, as indicated, cultured on B/c-DL cells for 5.5 d and gated for CD4^{+}CD8^{+} (double-positive, DP) or CD4^{-}CD8^{-} (DN) cells.

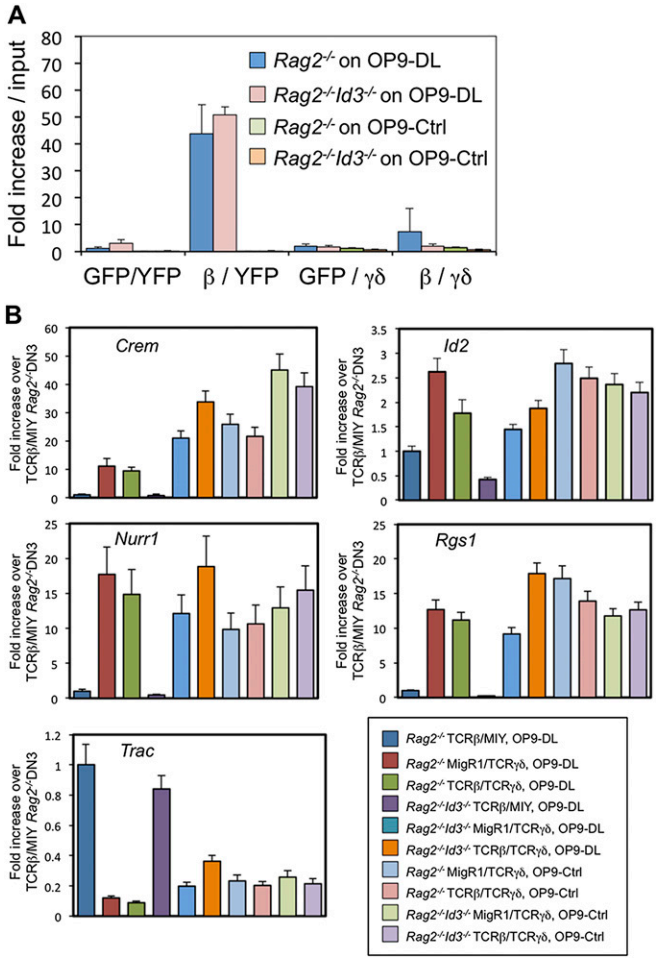

Fig. S2. Cellularity and gene expression analysis of pre-TCR or γδ-TCR–expressing cells. (*A*) Cellularity, fold-expansion over input, of in vitro-derived *Rag2*$^{-/-}$ DN3 or *Rag2*$^{-/-}$*Id3*$^{-/-}$ DN3 cells retrovirally transduced to express TCR-β, γδ-TCR, neither, or both, and cultured for 6 d with OP9-DL or OP9-Ctrl cells. (*B*) RNA was obtained from in vitro-derived *Rag2*$^{-/-}$ DN3 or *Rag2*$^{-/-}$*Id3*$^{-/-}$ DN3 cells retrovirally transduced to express TCR-β, γδ-TCR, neither, or both, and cultured for 6 d with OP9-DL1 or OP9-Ctrl cells. Quantitative RT-PCR analysis of γδ-biased genes: *Crem, Nurr1,* and *Rgs1*, and *Trac* (germ-line TCR-α constant region), in transduced *Rag2*$^{-/-}$ DN3 or *Rag2*$^{-/-}$*Id3*$^{-/-}$ DN3 cells cultured for 6 d on the indicated cells, with mRNA levels normalized to *β-actin*. Data are derived from at least three independent experiments. All data points for the γδ-TCR–expressing samples showed a significant difference ($P < 0.01$) over the TCR-β–transduced samples.

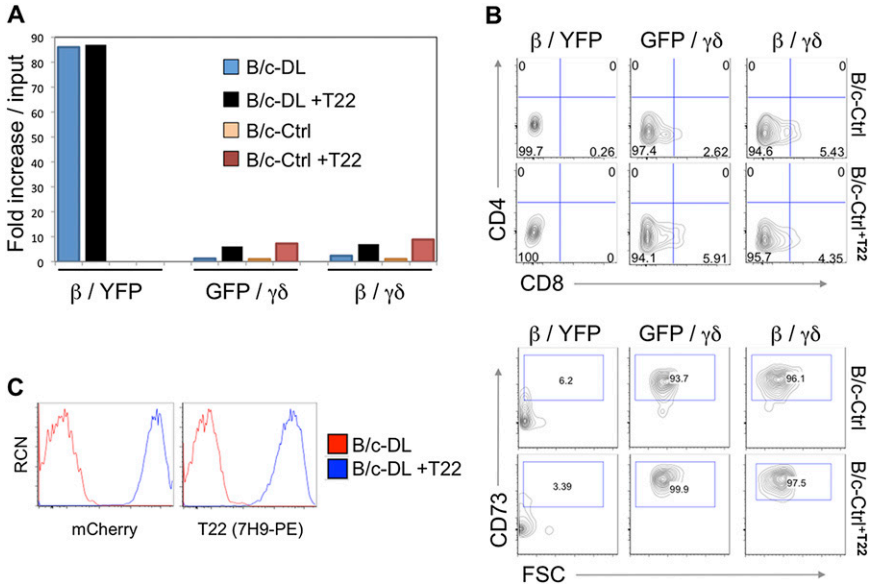

Fig. S3. Cellularity and flow cytometry analysis of TCR-β, γδ-TCR–, and TCR-β/γδ–expressing DN3 cells cocultured on B/c-DL and B/c-Ctrl cells. (*A*) Cellularity, fold-increase over input, of in vitro-derived *Rag2*$^{-/-}$ DN3 cells retrovirally transduced to express TCR-β, γδ-TCR, neither, or both, and cultured for 5.5 d with B/c-DL4 or B/c-Ctrl cells transduced to express T22^{b} or MICherry alone. (*B*) Development of in vitro-derived *Rag2*$^{-/-}$ DN3 cells retrovirally transduced to express TCR-β, γδ-TCR, or both, and cultured for 5.5 d with B/c-Ctrl cells expressing T22^{b} or MICherry alone Flow cytometric analysis of cell surface expression for CD4, CD8 and CD73, FSC. (*C*) Flow cytometric analysis of mCherry (*Left*) and T22^{b} (*Right*) expression in B/c-DL4 cells transduced to express T22-MICherry or MICherry alone. Data are derived from at least three independent experiments.

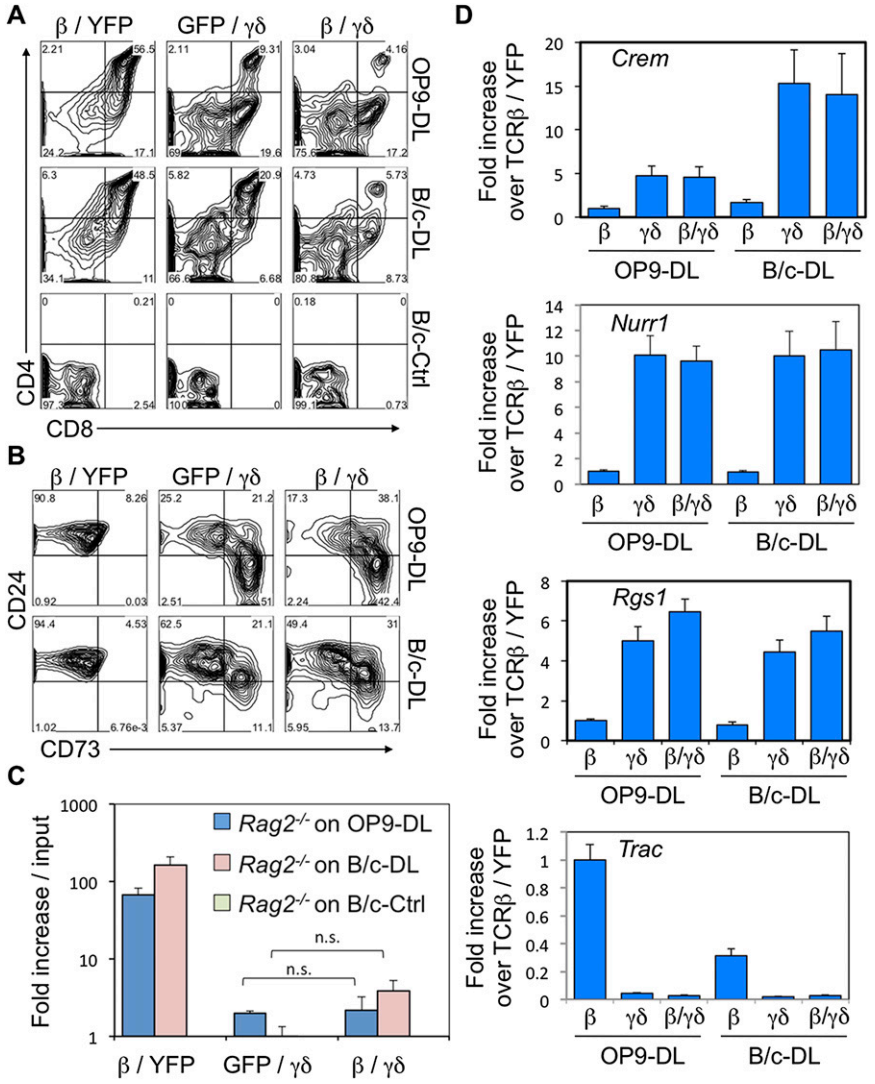

Fig. S4. Provision of a weak γδ-TCR ligand does not promote αβ-lineage choice in γδ-TCR–expressing DN3 cells, both in the presence and absence of Notch signals. (*A* and *B*) Development of in vitro-derived *Rag2*$^{-/-}$ DN3 cells retrovirally transduced to express TCR-β, γδ-TCR, neither, or both, and cultured for 6 d with OP9-DL4, B/c-DL4, or B/c-Ctrl cells. Flow cytometric analysis of cell surface expression for CD4 and CD8 (*A*), and CD24 and CD73 (*B*) are shown for GFP^{+} YFP^{+}-gated cells; *C* shows the corresponding fold-expansion in cellularity. (*D*) Quantitiative RT-PCR analysis of γδ-biased genes: *Crem*, *Nurr1*, and *Rgs1*, and *Trac* in transduced *Rag2*$^{-/-}$ DN3 cells cultured for 6 d on the indicated cells, with mRNA levels normalized to *β-actin*. β, γδ, and β/γδ represent TCR-β/MIY–, MigR1/TCR-γδ–, and TCR-β/TCR-γδ– transduced DN3 cells, respectively. Data are derived from at least three independent experiments. For *D*, all datapoints for the γδ-TCR–expressing samples showed a significant difference ($P < 0.01$) over the TCR-β–transduced samples.