Supporting Information

Fassett et al. 10.1073/pnas.1200090109

SI Materials and Methods

Mice. C57BL/6.*Nur77*$^{-/-}$ mice (1) were a gift from J. Milbrandt (Washington University, St Louis, MO). Nur77KO were maintained as homozygotes (Nur77KO) or crossed to generate transgenic lines as follows: (*i*) to C57BL/6. A^{g7} [B6g7 (2)] to generate a B6g7.*Nur77*$^{-/-}$ line, which was (*ii*) further crossed to BDC2.5/B6g7 TCR transgenic mice (2) to generate the TCR heterozygous line BDC2.5/B6g7.*Nur77*$^{-/-}$ (BDC2.5/Nur77KO). The Nur77KO line was also crossed to C57BL/6 lines carrying both *OT-II* (3) and *RIP-mOVA* (4) transgenes (OTII/RIP-mOVA/B6) to create OTII/RIP-mOVA/B6.*Nur77*$^{-/-}$ (OTII.RIP-mOVA/Nur77KO) mice and OTII/B6. *Nur77*$^{-/-}$ (OTII/Nur77-KO) controls. C57BL/6.Bim$^{-/-}$ [BimKO (5)] were purchased from The Jackson Laboratory and crossed to OT-II/RIP-mOVA/C57BL/6 and OT-II/RIP-mOVA/C57BL/6.*Nur77*$^{-/-}$ to generate OT-II/RIP-mOVA/B6.Bim$^{-/-}$ and OT-II/RIP-mOVA/B6.Bim$^{-/-}$ Nur77$^{-/-}$ animals. All mice, including the previously described BDC2.5 TCR transgenic mice (2) on the NOD and B6g7 background [BDC2.5/NOD and BDC2.5/B6g7, respectively (6)] were bred in a specific-pathogen-free barrier facility at The Joslin Diabetes Center (protocols 99–19 and 99–20 approved by The Joslin Diabetes Center's International Animal Care and Use Committee).

Fetal Thymic Organ Cultures. BDC2.5/B6g7, BDC2.5/Nur77KO, and BDC2.5/NOD timed embryos were generated by breeding BDC2.5 transgenic males with nontransgenic females for 12 h overnight, where embryonic day 0 (E0) was the morning of separation. Fetal thymus lobes were dissected from E15.5 embryos and cultured as previously described (6). Fetal thymic organ culture (FTOC) media was peptide free until culture day 3, when a BDC-specific mimotope peptide [BDCmi, peptides 1,040–1,063) (7)] was added at 1, 3, or 10 ng/mL After 16 h, FTOCs with BDCmi (and no peptide controls) were disrupted for flow cytometric analysis.

Analysis of Lymphocyte Populations by Flow Cytometry. Single-cell suspensions of thymus were generated by mechanical disruption with frosted-glass slides. Samples were stained with the following antibody conjugates: anti-CD4, anti-CD8a, anti-CD25, anti-Va2, and anti-Vb5. Intracellular expression of Foxp3 was determined by monoclonal antibody staining (eBioscience) following fixation and permeabilization according to the manufacturer's protocol (eBioscience). Antibody-stained samples were collected with a Beckton Dickinson LSRII flow cytometer and analyzed using Flojo software. Cell numbers were determined with a hemocytometer.

TGFβ-Conversion Assay. This assay was performed as previously described (8). Briefly, naive CD4+ CD62L^{high} were purified by sorting on a Moflo flow cytometer and cultured at 0.5×10^6 cells/well/96-well plate in RPMI supplemented with 10% fetal calf serum and 20 U/mL IL-2 (Proleukin) and 25 ng/mL recombinant TGFβ. After 4 d, cultures were analyzed by staining for CD4 and Foxp3 according to the eBioscience intracellular fixation/permeabilization protocol.

In Vitro Thymocyte Stimulation. Single-cell suspensions of B6g7 and B6g7.Nur77$^{-/-}$ thymocytes were incubated at 10×10^6 cells/2 mL cRPMI for 3 h at 37 °C at 5% CO_2 in six-well plates precoated with anti-TCR (H57-597, 10 μg/mL), anti-CD2 (RM2-5, 10 μg/mL), and anti-CD28 (37.51, 50 μg/mL) or a PBS control, following a published protocol for Nur77 induction in ex vivo thymocytes (9).

Cell Sorting, RNA Preparation, and Microarray Analysis. All cell sorting for microarray RNA preparation was performed as described (10), double-sorting 50,000 cells directly into 500 μL TRIzol on a BD Aria, with >95% purity attained determined by reanalyzing an aliquot of the sorted population. Sorted in vitro-stimulated thymocyte populations were stained and gated to be dump$^-$ (B220, CD11b, CD11c, CD49b, Ter119), CD4$^+$, and CD8$^+$. OT-II DP populations were dump$^-$ (B220, CD11b, CD11c, CD49b, Ter119), CD4$^+$, CD8$^+$, and Vα2-FITC Vβ5-PE TCR$^{intermediate-high}$. OT-II SP populations were (B220, CD11b, CD11c, CD49b, Ter119), CD4$^+$, CD8$^-$, Vα2Vβ5 TCR$^{intermediate-high}$, and CD25$^-$. Dead cells were excluded by propidium iodide staining. All cell populations analyzed for microarray were generated in duplicate or triplicate. RNA was prepared with TRIzol from sorted cell populations as described previously (10). Subsequent rounds of RNA amplification, cDNA preparation, and hybridization were performed by Expression Analysis using Affymetrix MoGene1.0ST microarrays. Raw data were normalized with the RMA algorithm implemented in the Expression File Creator module from the GenePattern software package (11). Data were visualized with the Multiplot module from GenePattern.

In Vitro Suppression Assay. This assay was performed as previously described (12). CD4+CD25− or CD4+GFP− target cells were labeled with 10 μmol/L carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes) and cultured at 2.5×10^4–10^5 cells/well in round-bottom, 96-well plates (Corning). Stimulation was effected by addition of 1.5 μg/mL anti-CD3 antibody in the presence of antigen presenting cells at a ratio of 1:1. Proliferation was measured by flow-cytometric analysis of CFSE dilution after 72 h.

1. Lee SL, et al. (1995) Unimpaired thymic and peripheral T cell death in mice lacking the nuclear receptor NGFI-B (Nur77). *Science* 269:532–535.
2. Katz JD, Wang B, Haskins K, Benoist C, Mathis D (1993) Following a diabetogenic T cell from genesis through pathogenesis. *Cell* 74:1089–1100.
3. Barnden MJ, Allison J, Heath WR, Carbone FR (1998) Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol Cell Biol* 76:34–40.
4. Kurts C, et al. (1996) Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J Exp Med* 184:923–930.
5. Bouillet P, et al. (1999) Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* 286: 1735–1738.
6. Zucchelli S, et al. (2005) Defective central tolerance induction in NOD mice: genomics and genetics. *Immunity* 22:385–396.
7. Judkowski V, et al. (2001) Identification of MHC class II-restricted peptide ligands, including a glutamic acid decarboxylase 65 sequence, that stimulate diabetogenic T cells from transgenic BDC2.5 nonobese diabetic mice. *J Immunol* 166:908–917.
8. Hill JA, et al. (2007) Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity* 27:786–800.
9. Cunningham NR, et al. (2006) Immature CD4+CD8+ thymocytes and mature T cells regulate Nur77 distinctly in response to TCR stimulation. *J Immunol* 177:6660–6666.
10. Yamagata T, Mathis D, Benoist C (2004) Self-reactivity in thymic double-positive cells commits cells to a CD8 alpha alpha lineage with characteristics of innate immune cells. *Nat Immunol* 5:597–605.
11. Reich M, et al. (2006) GenePattern 2.0. *Nat Genet* 38:500–501.
12. D'Alise AM, Ergun A, Hill JA, Mathis D, Benoist C (2011) A cluster of coregulated genes determines TGF-beta-induced regulatory T-cell (Treg) dysfunction in NOD mice. *Proc Natl Acad Sci USA* 108:8737–8742.

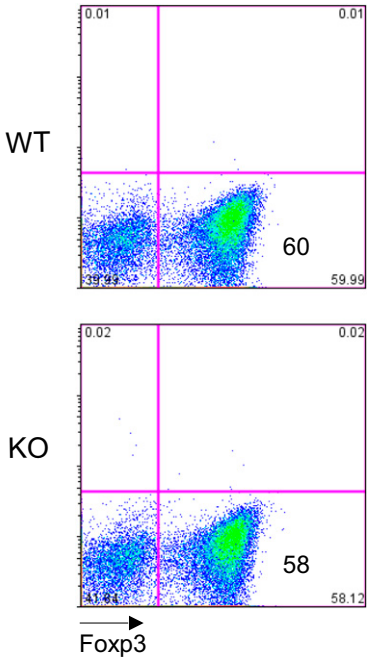

Fig. S1. TGFβ conversion assay generates similar frequency of Foxp3+ CD4+ T cells in Nr4a1KO. Representative FACS plot depicting Foxp3 expression in sorted WT and Nr4a1KO CD4+ peripheral T cells following 3 d in culture with IL-2/TGFβ.

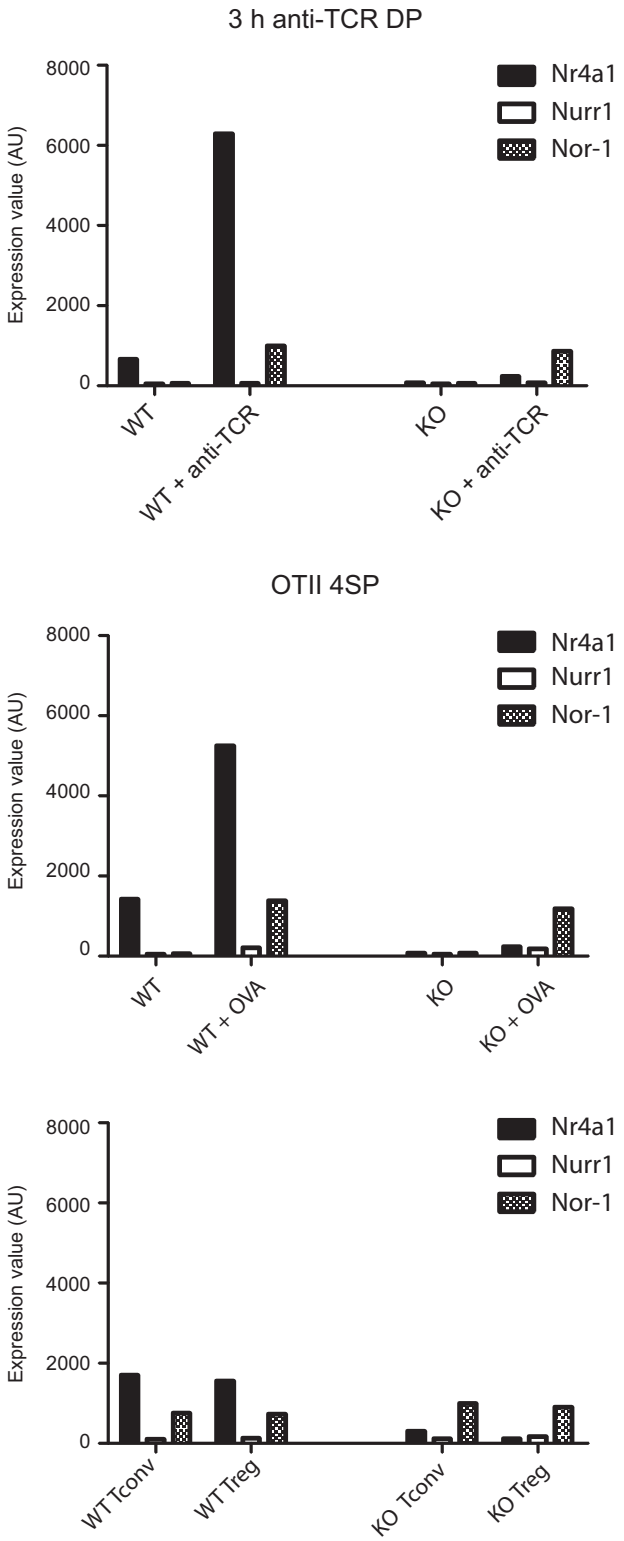

Fig. S2. Differences in expression of Nur77 family members during selection and in regulatory T-cells (Tregs). Mean expression values of Nur77 (*Nr4a1,* black bars), Nurr1 (*Nr4a2*, open bars), and Nor-1 (*Nr4a3*, hatched bars) from normalized ST1.0 microarray data of sorted populations of WT and Nur77KO DP thymocytes (*Top*), OTII-transgenic CD4SP thymocytes (*Middle*), and CD4SP CD25+ Tconv or CD25+ Treg thymocytes (*Bottom*).