Supporting Information

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SI Materials and Methods

Mice and Treatment. C57BL/6 mice were bred at Roswell Park Cancer Institute or purchased from the Jackson Laboratory. IL-10R2$^{-/-}$ mice were purchased from Jackson Laboratory. CD8$^+$ TCR transgenic Thy1.1$^+$ Rag1$^{-/-}$ OTI mice were generated as described (1). DC–β-catenin^{active} (CD11c-Cre$^+$β-catenin$^{Exon3/Exon3}$) and DC–β-catenin$^{-/-}$ (CD11c-Cre$^+$β-catenin$^{del/del}$) mice, whose DCs express either constitutively active or null alleles of β-catenin, were generated by crossing CD11c-Cre$^+$ transgenic mice to β-catenin$^{Exon3/Exon3}$ and β-catenin$^{del/del}$ mice, respectively (2). DC–β-catenin^{active} and DC–β-catenin$^{-/-}$ mice were maintained by self-crossing. Floxed mice (β-catenin$^{Exon3/Exon3}$ and β-catenin$^{del/del}$) were used as wild-type controls for DC–β-catenin^{active} and DC–β-catenin$^{-/-}$ mice, respectively. Immunization (vaccination) with anti–DEC-205-OVA plus CpG, and adoptive transfer of naïve OTI Thy1.1$^+$ CD8$^+$ T cells were carried out as described (2). Primary and recall responses were examined as described (2). In brief, cells from spleen and LN were stimulated for 5 h with OTI peptide (1–4 μg/mL, Pi Proteomics, LLC) in the presence of BFA (5 μg/mL), stained for cell surface protein like Thy1.1 followed by fixation and permeabilization, and staining for intracellular antigens like IFN-γ. Anti–IL-10 (200 μg per mouse), XAV939 (30 μg per mouse) and DMSO were injected intraperitoneally in PBS, at day −1, 0, 1, 3, and 5 after immunization. For maintenance experiments, immunized mice were treated with anti–IL-10 or XAV939 every other day after 5 d postimmunization. All procedures on animals followed protocols approved by the Institutional Animal Care and Use Committee at Roswell Park Cancer Institute.

Antibodies and Reagents. Anti-CD11c, anti-Thy1.1 magnetic microbeads, and CD8$^+$ T-cell isolation kits were purchased from Miltenyi Biotec. The constructs for anti–DEC-205-OVA were kindly provided by Warren Shlomchik, Yale University, New Haven, CT, with permission of Michel Nussenzweig, Rockefeller University, New York, and anti–DEC-205-OVA was produced as described (3). ELISA kits for mouse IL-10, IL-4, and IL-12p40 were purchased from eBioscience. Anti-mouse IL-10 was purchased from BioXcell (for in vivo experiments) and Biolegend (in vitro). Antibodies to Thy1.1, TCR Vα_2 and V$\beta_{5.1/5.2}$, CD4, CD8α, CD62L, CD44, B220, CD80, CD86, MHC class II I-A^b, MHCI, Annexin V, CD127, TNF-α, IL-2, IL-4, IL-10, and Granzyme B were purchased from Biolegend Inc. Brefeldin A (BFA), antibodies to IL-10, IL-4, Foxp3, KLRG-1, CD11c and IFN-γ were purchased from eBioscience. Anti–β-catenin, anti-mTOR, anti–4E-BP1, anti–phospho-S6, and isotype controls were obtained from Cell Signaling Inc. Anti–β-actin and anti-p70S6K was purchased from Santa Cruz. CpG was purchased from Invitrogen, XAV939 and rapamycin were purchased from www.Selleckchem.com. Staining for surface and intracellular antigen expression was performed as previously described (4). In brief, cells were stimulated for 5 h with OTI peptide followed by fixation and permeabilization, and staining for intracellular antigens. Where indicated, OTI CD8$^+$ T cells were labeled with 5-(6)-carboxyfluorescein diacetate succinimidyl diester (CFSE) and checked by flow cytometry before transfer. We used a FACSCalibur or Fortessa (BD Biosciences) with subsequent data analysis using FlowJo (Tree Star).

Isolation and Purification of CD8$^+$ T Cells and DCs. Naïve OTI Thy1.1$^+$ CD8$^+$ T cells were isolated from pooled LN of Thy1.1$^+$ Rag1$^{-/-}$ OTI mice and were routinely checked with Thy1.1, CD44, and CD62L for purity and activation. The percentages of Thy1.1$^+$ cells were around 85–95%. For cross-priming assays, either in vivo or in vitro, naïve OTI Thy1.1$^+$ CD8$^+$ T cells were labeled with 5 μM CFSE (Molecular Probes) in 5% FCS RPMI at room temperature for 15 min and washed three times before injection or plating. To purify CD11c$^+$ DCs from spleen and lymph nodes, we used anti-CD11c–conjugated beads and columns (Miltenyi Biotech) following the manufacturer's protocols. To purify primed Thy1.1$^+$ OTI cells from immunized mice, anti-Thy1.1–conjugated beads and columns were used on spleen and LN cells following the manufacturer's protocols. Total CD8$^+$ T cells were isolated with CD8$^+$ T-cell isolation kit following the manufacturer's protocols.

Phagocytosis. Splenic CD11c$^+$ cells were cultured with pHrodo Red *E. coli* Bioparticles conjugate (Life Technology) at either 37 °C or 0 °C (negative control) for 30, 60, and 90 min before staining with CD11c and subjected to flow analysis according to manufacturer's protocols.

In Vivo Cross-Priming Assay and DC–T-Cell Cocultures. For in vivo cross priming assay, mice were immunized with anti–DEC-205-OVA and CpG, and 1 × 10^6 CFSE labeled naïve Thy1.1$^+$ OTI cells were injected i.v. at day 3 after immunization. 3 d later LN and spleen cells were stimulated for 5 h in the presence of BFA and OTI peptide, stained for surface Thy1.1, CD8, and intracellular antigens like IFN-γ, and subjected to flow cytometry to evaluate proliferation by CFSE dilution and effector differentiation by IFN-γ production. In some experiments, unlabeled naïve Thy1.1$^+$ OTI cells were transferred and were analyzed 8 and 15 d after immunization (5 and 12 d after transfer). For cross-priming under the condition of IL-10 blockade, immunized DC–β-catenin^{active} or tumor-bearing WT mice were treated with anti–IL-10 antibody (200 μg per mouse) or PBS at day −1, 0, 1, 3, and 5 after immunization and cross-priming was examined as described. For XAV 939 treatment, immunized tumor-bearing mice were treated with β-catenin inhibitor XAV 939 (30 μg per mouse) or vehicle (DMSO in PBS) at day −1, 0, 1, 3, and 5 after immunization. For in vitro cross priming assays, purified CD11c$^+$ DCs were pulsed with anti–DEC-205-OVA (0.01–0.2 μg/mL) and stimulated with CpG (100-500 ng/mL), and after extensive washing were cultured with CFSE labeled naïve Thy1.1$^+$ OTI T cells. The cocultures were stimulated for 5 h with OTI peptide in the presence of BFA at day 3 or 4 and were subjected to intracellular staining and flow cytometry. For in vitro cross-priming in the presence of anti–IL-10, anti–IL-10 (10 μg/mL) was added to the cocultures and during in vitro restimulation. For cross-priming with rapamycin, rapamycin (10 ng/mL) was added to DCs 20 min before CpG treatment and DCs were extensively washed before plating. For cocultures with total CD8$^+$ T cells, antigen-pulsed DCs were cultured with total CD8$^+$ T cells purified from naïve mice.

Transfer Experiment Protocols. Mice were adoptively transferred with 2–5 × 10^6 Thy1.1$^+$ OTI cells followed with immunization with anti–DEC-205-OVA plus CpG. Thy1.1$^+$ OTI cells primed in these mice were then purified from LN and spleen and injected i.v. by tail veins in 200 μL PBS into host mice. For memory CD8$^+$ T-cell experiments, WT hosts were recalled with OVA (Worthington Biochemical) in CFA (Sigma-Aldrich) 40 d after the initial immunization and memory responses were examined 5 d later as described (2): briefly, cells from LNs (pooled inguinal, axillary, and brachial LN) were stimulated for 5 h in the

presence of BFA and OTI peptide, stained for surface Thy1.1, CD8 and intracellular cytokines (i.e., IFN-γ), and evaluated by flow cytometry. For maintenance experiments, Thy1.1$^+$ OTI cells primed in WT and DC–β-catenin$^{-/-}$ mice were purified at day 4 and transferred into WT and DC–β-catenin$^{-/-}$ mice that had been immunized at the same time.

ELISA. Purified CD11c$^+$ DCs were stimulated with CpG (100 ng/mL) for 36 h, and cytokine were measured by ELISA according to the manufacturer's protocols. CpG was used at 75 and 50 ng/mL for DCs isolated at day 7 after immunization. For DC–T-cell co-cultures, purified DCs were pulsed with anti–DEC-205-OVA plus CpG for 4 h before cultured with naïve Thy1.1$^+$CD8$^+$ OTI cells, and IL-10 in the supernatants was measured by ELISA after 3 d. Thy1.1$^+$ CD8$^+$ OTI cells from immunized mice were purified with anti-Thy1.1 microbeads (Miltenyi Biotec) and cultured for 2 d to determine IL-4 and IL-10 production by ELISA. For rapamycin treatment, rapamycin (10 ng/mL) was added to DCs 20 min before CpG stimulation.

Western Blot. Splenic CD11c$^+$ DCs were purified with anti-CD11c–conjugated beads and columns (Miltenyi Biotech) following the manufacturer's protocols, with the exception of no BSA used. Cells were resuspended in PBS and treated with CpG, and cell lysates were subjected to Western blot analysis with anti–4E-BP1, anti-p70S6K and anti–β-actin according to the manufacturer's protocols.

Tumor Cell Lines and Treatment of Tumor-Bearing Mice. B16OVA melanoma cells were maintained as recommended and inoculated by s.c. injection with 1–20 × 10^5 cells. Tumors were measured every other day once they became palpable, and tumor sizes were calculated as (0.5 × short length × long length^2). Mice were euthanized when tumors reached 20 mm in any one dimension or when signs of illness were observed. For prevention experiments, WT and DC–β-catenin$^{-/-}$ mice were challenged with 2 × 10^6 B16OVA cells at 20 d after immunization. For treatment with XAV939, WT and DC–β-catenin^{active} mice bearing B16OVA tumors were treated with β-catenin inhibitor XAV 939 (30 μg per mouse) or vehicle (DMSO in PBS) at day −1, 0, 1, 3, and 5 after immunization, and increased tumor volume was calculated as tumor volume on the day of measurement minus the tumor volume at the time of immunization. As B16 tumors grow much faster in DC–β-catenin^{active} mice (2), a lower dose of B16OVA cells (1 × 10^5) were inoculated. For treatment with XAV939 without vaccination, B16OVA-bearing WT mice were treated with β-catenin inhibitor XAV939 alone in the same regiment but without immunization. Increased tumor volume was calculated as tumor volume on the day of measurement minus the tumor volume at immunization or XAV939 treatment (for treatment without vaccination).

Tumor Cell Growth in Vitro. B16OVA melanoma cells were seeded at 1–2 × 10^3 cells per well in a six-well plate, and were treated with Genistein (5), XAV939 (1–2 μM), or vehicle (DMSO in PBS). B16OVA cells were collected at day 5 or 6, mixed with a known number of CFSE-labeled B16OVA cells used for reference, and subjected to flow cytometry to calculate the numbers of B16OVA cells in each wells.

Detection of STAT3 Phosphorylation. WT and DC–β-catenin$^{-/-}$ mice adoptively transferred with naïve Thy1.1$^+$ OTI cells were immunized with anti–DEC-205-OVA plus CpG, and spleen and LN cells isolated at day 7 after immunization were treated with recombinant IL-10 (30–100 ng/mL) and subjected to intracellular staining of phosphorylated STAT3 and flow cytometry.

Statistical Analysis. The statistical significance of experimental results was evaluated with Excel or GraphPad Prism 6 using two-tailed unpaired two-sample Student's t test. P values less than 0.05 were considered significant and are denoted as NS > 0.05, *P < 0.05 and **P < 0.01.

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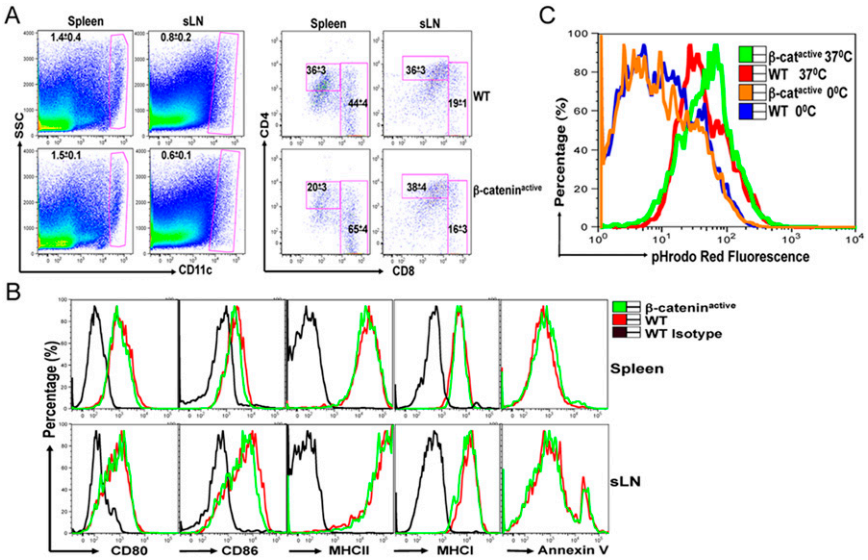

Fig. S1. DC–β-catenin^{active} mice exhibited similar phenotype to wild type mice in their DC frequencies, composition, maturation and phagocytosis potential. (*A*) DC frequencies and composition of spleen and skin-draining LN of DC–β-catenin^{active} and WT mice. Cells were isolated from the spleen and skin-draining LN (sLN, pooled inguinal, axillary and brachial LN) of WT and DC–β-catenin^{active} mice ($n = 4$) and subjected to staining and flow cytometry. Mean and SD of the percentages of CD11c$^+$ cells is shown of representative plots on the left. Gated CD11c$^+$B220$^-$ cells were further analyzed for CD4 and CD8 expression, mean and SD of the percentages of CD4$^+$ and CD8$^+$ cells are shown. (*B*) Splenic and sLN DCs of DC–β-catenin^{active} and WT mice exhibited similar phenotypes in DC maturation and survival. Total spleen and sLN cells were stained for CD11c and maturation markers as in *A*. The expression of CD80, CD86, MHCI, MHCII and Annexin V of gated CD11c$^+$ cells is shown in the histogram, with isotype controls included for CD80, CD86, MHCI and MHCII. For Annexin V, total splenocytes and sLN cells were cultured for 2 d before staining. (*C*) β-Catenin^{active} DCs exhibited similar phagocytosis to WT DCs. Splenic CD11c$^+$ cells were purified from WT and DC–β-catenin^{active} mice, cultured with pHrodo Red *E. coli* Bioparticles conjugate at either 37 °C or 0 °C (negative control) for 30, 60, and 90 min before staining with CD11c and subjected to flow analysis. Representative overlay of Red staining from 60 min are shown. β-Catenin^{active} DCs showed similar or better phagocytosis to WT DCs in all time points measured. Data shown are representative of two independent experiments.

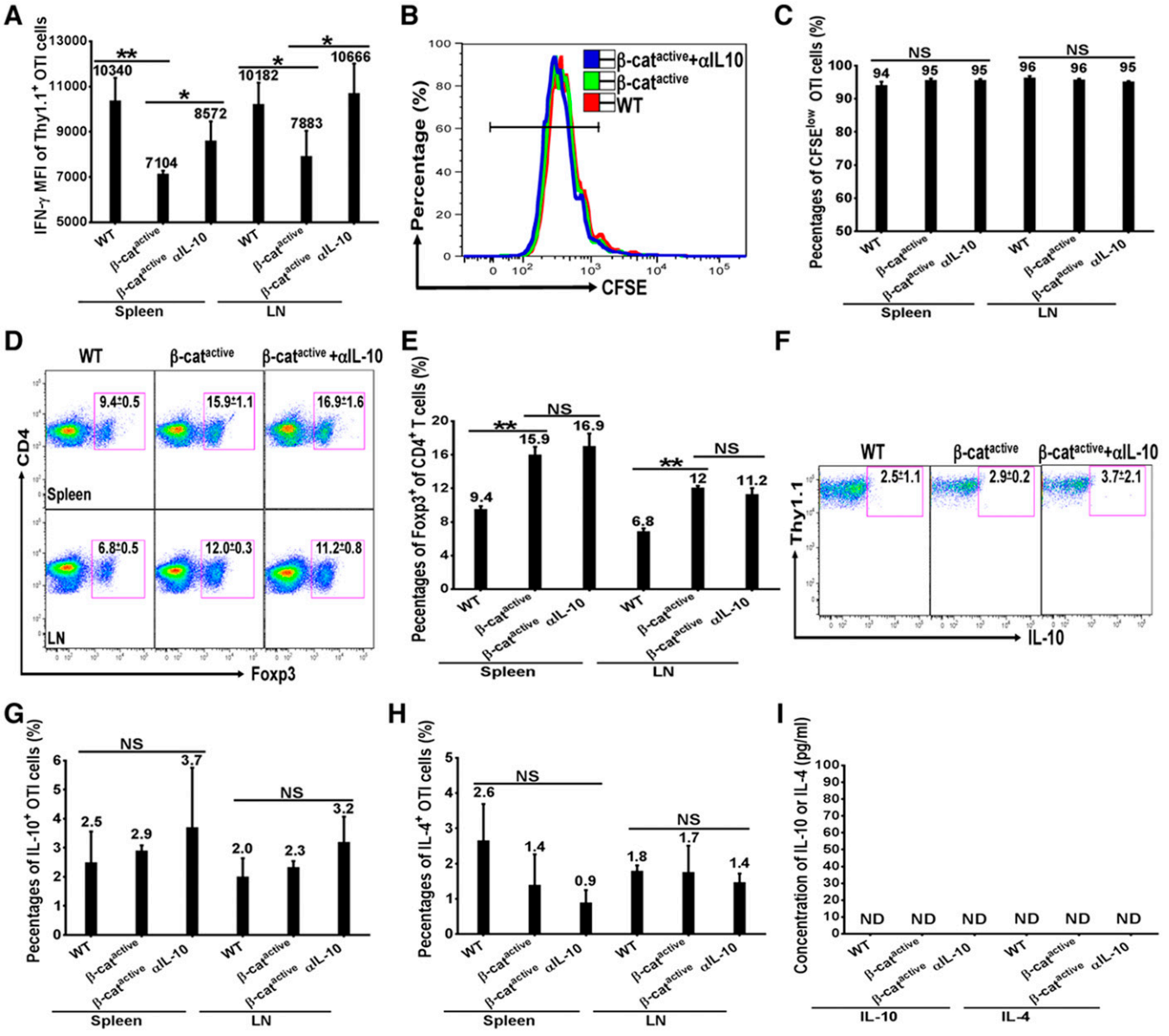

Fig. S2. Characterization of Thy1.1$^+$ OTI CD8$^+$ T cells primed in WT mice, DC–β-catenin^{active} mice and anti–IL-10 treated DC–β-catenin^{active} mice. WT and DC–β-catenin^{active} mice were immunized with anti–DEC-205-OVA plus CpG, and treated with anti–IL-10 or PBS. CFSE-labeled naïve Thy1.1$^+$ OTI cells were adoptively transferred 3 d after immunization. (*A*) IFN-γ$^+$ OTI cells from DC–β-catenin^{active} mice exhibited lower Mean Fluorescent Intensity (MFI) of IFN-γ expression. Mean and SD of IFN-γ MFI of IFN-γ$^+$ Thy1.1$^+$ OTI cells gated in Fig. 1*B* were shown. (*B* and *C*) Proliferation of Thy1.1$^+$ OTI cells was not significantly different in WT and DC–β-catenin^{active} mice. CFSE expression of total Thy1.1$^+$ OTI cells in Fig. 1*B* was shown in histogram overlays in *B*. Mean and SD of percentages of proliferated CFSE^{low} Thy1.1$^+$ OTI cells were shown in *C*. (*D* and *E*) Anti–IL-10 treatment did not significantly affect the percentages of Foxp3$^+$ regulatory T cells. WT and DC–β-catenin^{active} mice ($n = 4$) were treated as in *A*, and spleen and LN cells were analyzed for Foxp3 expression. Mean and SD of percentages of Foxp3$^+$ cells out of total CD4$^+$ T cells were shown. (*F–H*) Thy1.1$^+$ OTI cells primed in DC–β-catenin^{active} mice did not differentiate into IL-4- and IL-10-producing type 2 (Tc2) CD8$^+$ T cells. WT and DC–β-catenin^{active} mice ($n = 3$–4) were treated as in *A* but with unlabeled Thy1.1$^+$ OTI cells, and spleen and LN cells were isolated and stimulated with OTI peptide in the presence of BFA and subjected to flow cytometry. The percentages of IL-10-producing cells out of total Thy1.1$^+$ OTI cells in spleen are shown in dot plots (*F*). Mean and SD of percentages of IL-10$^+$ and IL-4$^+$ cells out of Thy1.1$^+$ OTI cells are shown in *G* and *H*, respectively. (*I*) Primed Thy1.1$^+$ OTI cells did not produce IL-10 or IL-4. Splenic Thy1.1$^+$ OTI cells were purified from mice in F and cultured for 2 d, and supernatants were assayed for IL-4 and IL-10 were by ELISA. ND = not detected. Data shown are representative of at least two experiments. Student's *t* test was performed, NS = $P > 0.05$, *$P < 0.05$ and **$P < 0.01$.

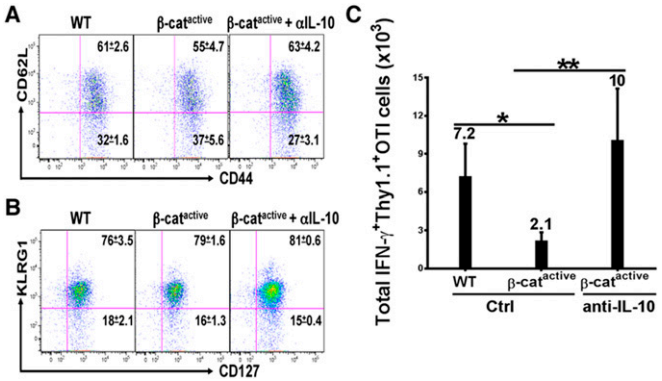

Fig. S3. DC–β-catenin^{active} mice produced significantly reduced IFN-γ$^+$ OTI effector cells that were restored by anti–IL-10 treatment. (*A* and *B*) Expression of CD44, CD62L, CD127 and KLRG1 on Thy1.1$^+$ OTI cells primed in WT mice, DC–β-catenin^{active} mice and anti–IL-10-treated DC–β-catenin^{active} mice. WT and DC–β-catenin^{active} mice were immunized with anti–DEC-205-OVA plus CpG, and treated with anti–IL-10 or PBS as in Fig. 1*B*. Naïve Thy1.1$^+$ OTI cells were adoptively transferred 3 d after immunization, and mice were analyzed 5 d after transfer. Mean and SD of percentages of indicated populations were shown and no significant difference was observed among the three groups. (*C*) DC–β-catenin^{active} mice produced significantly reduced number of IFN-γ$^+$ OTI effector cells, which were restored by anti–IL-10 treatment. WT and DC–β-catenin^{active} mice were treated as in *A*. Total numbers of IFN-γ$^+$ Thy1.1$^+$ OTI cells in skin-draining LN at day 15 after immunization are depicted. Data are representative of two experiments. Student's *t* test was performed, NS = $P > 0.05$, *$P < 0.05$, and **$P < 0.01$.

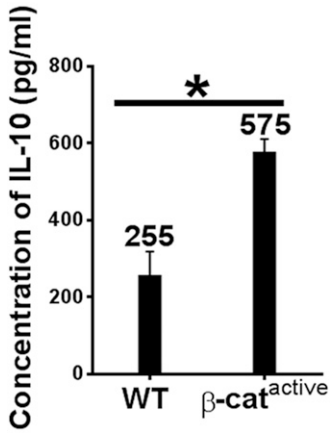

Fig. S4. Cocultures of naïve OTI cells with β-catenin^{active} DCs produced significantly more IL-10 than cocultures with WT DCs. Splenic DCs were pulsed with anti–DEC-205-OVA plus CpG for 4 h and cultured with naïve Thy1.1$^+$CD8$^+$ OTI cells. IL-10 was measured by ELISA after 3 d. Data shown are representative of two experiments. Student's *t* test was performed, *$P < 0.05$.

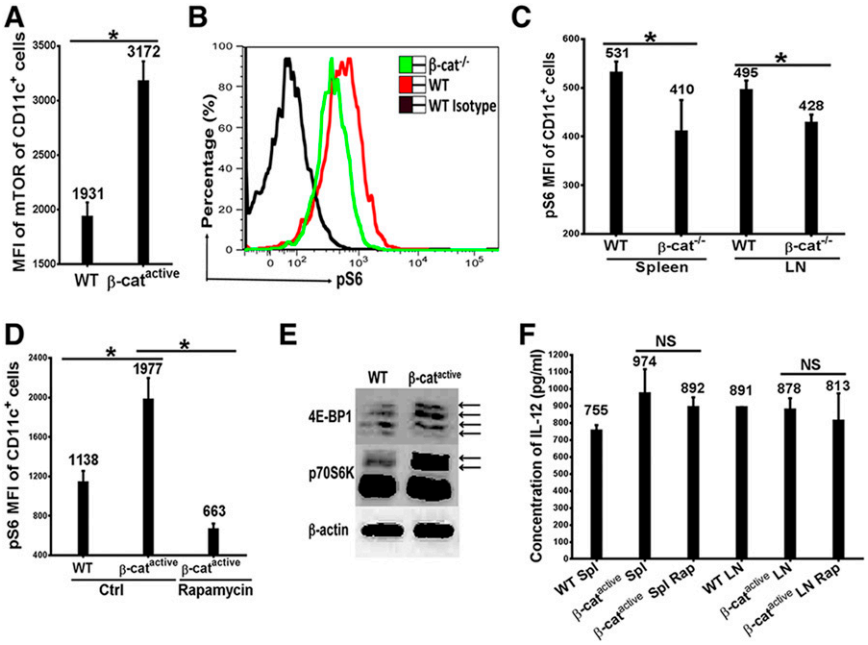

Fig. S5. β-Catenin up-regulates mTOR/IL-10 signaling in DCs. (*A*) β-Catenin up-regulates mTOR in DCs. Splenocytes were stained and subjected to flow cytometry, gated on $CD11c^+$ cells. Mean Fluorescent Intensity (MFI) of mTOR expression in $CD11c^+$ cells is shown. (*B* and *C*) DCs from immunized DC–β-catenin$^{-/-}$ mice exhibited reduced mTOR activity. Spleen and LN cells from immunized mice ($n = 4$) were isolated and subjected to flow cytometry. Expression of phosphorylated S6 (pS6) of splenic $CD11c^+$ cells is shown as histogram overlays in *B* and Mean Fluorescent Intensity (MFI) of pS6 is shown in *C*. (*D*) β-Catenin^{active} DCs exhibited augmented CpG-induced pS6 phosphorylation in vitro. Splenic DCs from naive mice were stimulated by CpG with or without rapamycin, and MFI of phosphorylated S6 expression of $CD11c^+$ cells is shown. (*E*) β-Catenin^{active} DCs exhibited increased phosphorylation of p70S6K and 4E-BP1. Purified splenic DCs from naïve WT and DC–β-catenin^{active} mice were stimulated by CpG and cell lysates were subjected to Western blot for p70S6K and 4E-BP1. The mobility shifts of p70S6K and 4E-BP1 caused by multiple phosphorylation sites were observed. (*F*) Inhibition of mTOR by rapamycin did not affect IL-12p40 production by β-catenin^{active} DCs. DCs were treated as in Fig. 2*E*, and IL-12p40 was measured by ELISA. Student's *t* test was performed between untreated and rapamycin-treated β-catenin^{active} DCs, NS = $P > 0.05$. Representative data from two or more experiments are shown. Student's *t* test was performed, NS = $P > 0.05$ and *$P < 0.05$.

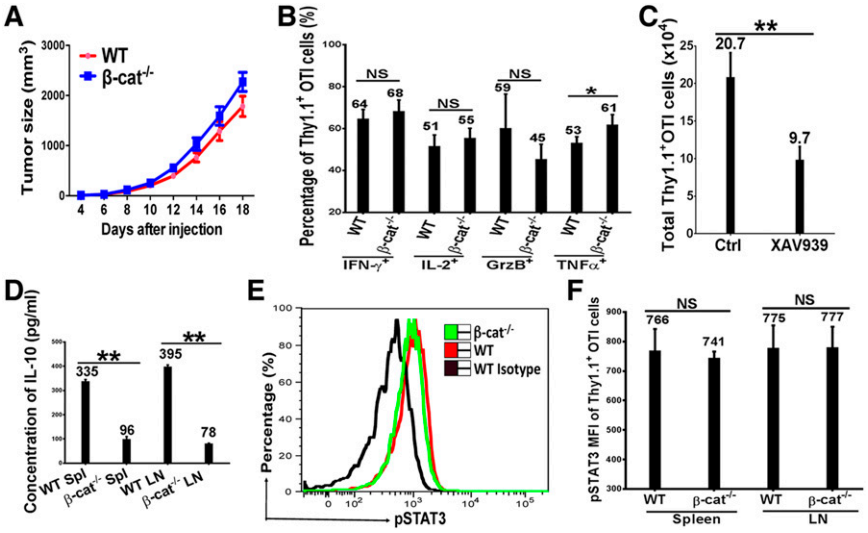

Fig. S6. β-Catenin in DCs plays a positive role in $CD8^+$ T-cell maintenance postclonal expansion. (*A*) WT and DC–β-catenin$^{-/-}$ mice exhibited similar tumor growth. Wild type (WT) and DC–β-catenin$^{-/-}$ mice ($n = 10$) were inoculated with B16OVA cells, and tumors were measured every other day once they become palpable. Student's *t* test was performed and $P > 0.05$ (not significant) on all time points. (*B*) DC–β-catenin$^{-/-}$ mice exhibited normal primary $CD8^+$ T-cell responses. LN cells from immunized mice ($n = 5$) were analyzed at day 4 after immunization as in Fig. 3*B*. Percentages of IFN-γ^+, IL-2^+, Granzyme B^+ ($GrzB^+$) and $TNF\alpha^+$ cells are shown. (*C*) Treatment with a pharmacological β-catenin inhibitor XAV939 after priming phase led to reduced $CD8^+$ effector cells. WT mice adoptively transferred with naïve $Thy1.1^+$ OTI cells were immunized with anti–DEC-205-OVA plus CpG, and treated with XAV939 or vehicle control ($n = 4$–5) every other day starting from day 6, and LN cells were analyzed at day 15. (*D*) DCs from immunized DC–β-catenin$^{-/-}$ mice produced significantly less IL-10. DCs purified from immunized mice at day 7 after immunization were cultured with CpG (50 ng/mL) and IL-10 was measured by ELISA. (*E* and *F*) $Thy1.1^+$ OTI cells primed in DC–β-catenin$^{-/-}$ mice were not deficient in IL-10R-mediated signaling. WT and DC–β-catenin$^{-/-}$ mice adoptively transferred with naïve $Thy1.1^+$ OTI cells were immunized with anti–DEC-205-OVA plus CpG, and spleen and LN cells from immunized mice at day 7 were treated with recombinant IL-10 and subjected to intracellular staining of phosphorylated STAT3 and flow cytometry. Expression of phosphorylated STAT3 (pSTAT3) of splenic $Thy1.1^+$ OTI cells is shown as histogram overlays in *E* and pSTAT3 MFI of gated $Thy1.1^+$ OTI cells in *F*. Data representative of two to three experiments are shown. Student's *t* test was performed, NS = $P > 0.05$, *$P < 0.05$, and **$P < 0.01$.

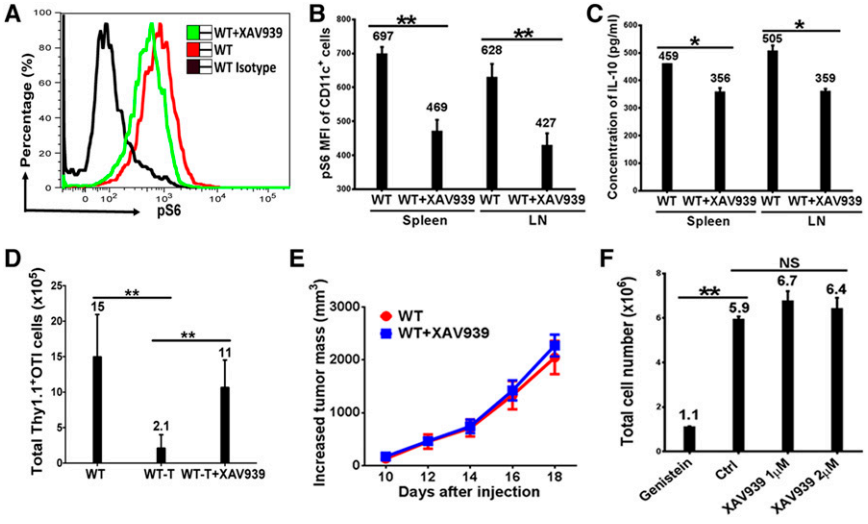

Fig. S7. Treatment with β-catenin inhibitor XAV939 led to similar effects to inhibition of β-catenin genetically in DCs (DC–β-catenin$^{-/-}$ mice). (*A* and *B*) DCs from XAV939-treated WT mice exhibited reduced mTOR activity. WT mice were immunized with anti–DEC-205-OVA plus CpG with or without XAV939 treatment ($n = 3$), spleen and LN cells were isolated at day 3 and subjected to flow cytometry as in Fig. 2*B*. Expression of phosphorylated S6 (pS6) of $CD11c^+$ cells is shown as histogram overlays in *A* and Mean Fluorescent Intensity (MFI) in *B*. (*C*) DCs from XAV939-treated WT mice exhibited reduced IL-10 production. DCs were purified and cultured with CpG, and IL-10 was measured by ELISA after 36 h. (*D*) Blocking β-catenin during priming phase rescued tumor-suppressed $CD8^+$ T-cell immunity. Tumor-free and B16OVA-bearing WT mice ($n = 4$–5) were treated as in Fig. 4*D* and were recalled at day 15. Total numbers of $Thy1.1^+$ OTI cells in draining LN are depicted. (*E*) Treatment of XAV939 alone did not affect tumor growth of B16OVA in vivo. B16OVA-bearing WT mice ($n = 5$) were treated with XAV939 as in Fig. 4*E* except without vaccination, and increased tumor sizes are shown. Student's *t* test was performed and no significant difference is observed at all time points. (*F*) B16OVA cells were cultured with or without XAV939 and total cell numbers were counted as described in *Materials and Methods*. Genistein (10 μM) was used a positive control for its inhibition of B16 growth. Data representative of two to four experiments are shown. Student's *t* test was performed, NS = $P > 0.05$, *$P < 0.05$, and **$P < 0.01$.