SUPPLEMENTAL INFORMATION

c-Cbl regulates dendritic cell activation

Shin-Heng Chiou[1], Payam Shahi[1,2,^], Ryan T. Wagner[3,^], Hongbo Hu[1,4], Natalia Lapteva[1], Mamatha Seethammagari[1], Shao-Cong Sun[4], Jonathan M. Levitt[1,5], and David M. Spencer[1*]

[1]Department of Pathology & Immunology, [2]Interdepartmental Program in Cell and Molecular Biology, [3]Department of Molecular and Cellular Biology, [4]Department of Immunology, The University of Texas MD Anderson Cancer Center, The University of Texas Graduate School of Biomedical Sciences at Houston, and [5]Scott Department of Urology, Baylor College of Medicine, Houston, TX 77030

Supplemental Data

Chiou *et al*., Figure S1

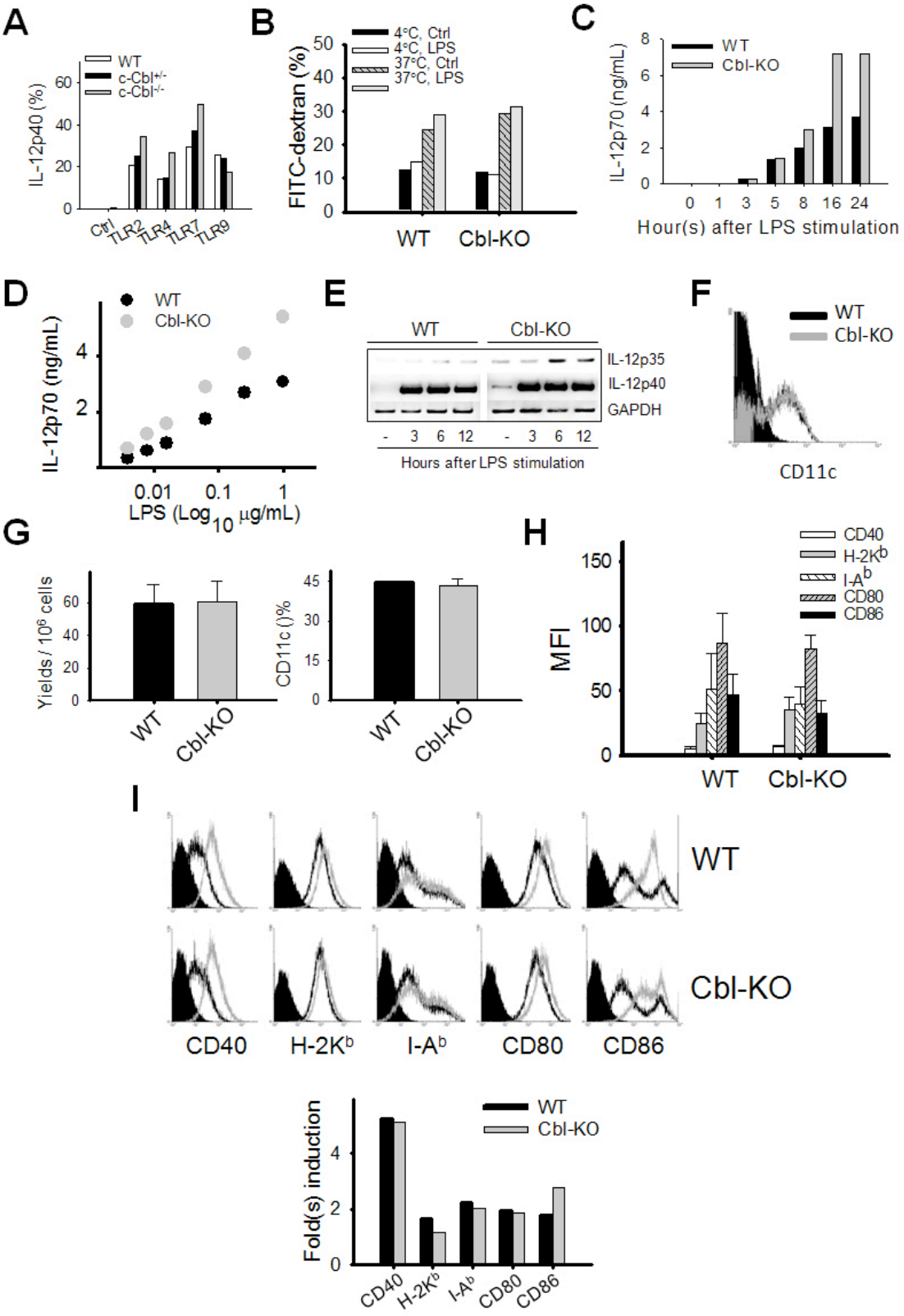

Figure S1.
(A) BMDCs with indicated genotypes were intracellularly stained for IL-12p40 after stimulation overnight with indicated TLR agonists. Data represent percentages of IL-12-expressing population. Data are representative of three independent experiments. (B) BMDCs stimulated with or without LPS overnight (1 μg/mL) were cultured at indicated temperatures for at least 30 minutes before assay. Subsequently, FITC-dextran (1 mg/mL) was added onto the media and allowed DC uptake for 1 hour before harvesting cells for FACS analysis. Data represent pooled results of two independent experiments. (C) WT and Cbl-KO BMDCs were stimulated with LPS for indicated time before quantification of IL-12p70 by ELISA. Data are representative of three independent experiments. (D) WT and Cbl-KO BMDCs were stimulated indicated amounts of LPS for 24 hours before collection of supernatants for ELISA. Data are representative of two independent experiments. (E) WT and Cbl-KO BMDCs were stimulated with LPS for indicated time before quantification of mRNA levels of IL-12p35, p40, and GAPDH by semi-quantitative RT-PCR. Data are representative of three independent experiments. (F, G) Bone marrow precursor cells derived from WT and Cbl-KO mice were cultured in the presence of mGM-CSF and mIL-4 as described in *Materials and Methods*. By day 6, BMDCs were collected and subjected to quantification of CD11c surface expression (F) and cellularity (G, left panel) by FACS analysis. Histogram, filled: isotype control; open: anti-CD11c Ab staining. Data represent pooled results from three pairs of mice (Average ± SD). (H) Quantification of surface expression of the indicated molecules on BMDCs was conducted by FACS analyses as described in *Materials and Methods*. Data represent pooled results from three pairs of mice (Average ± SE). (I) Fold(s) induction was calculated by normalizing the MFI of stimulated group against unstimulated control. Upper panel, representative histograms of LPS-induced upregulation of indicated molecules in (H). Lower panel, data represent pooled results from two pairs of mice. Filled histogram: isotype control; open black: unstimulated control; open grey: stimulated group.

Chiou *et al*., Figure S2

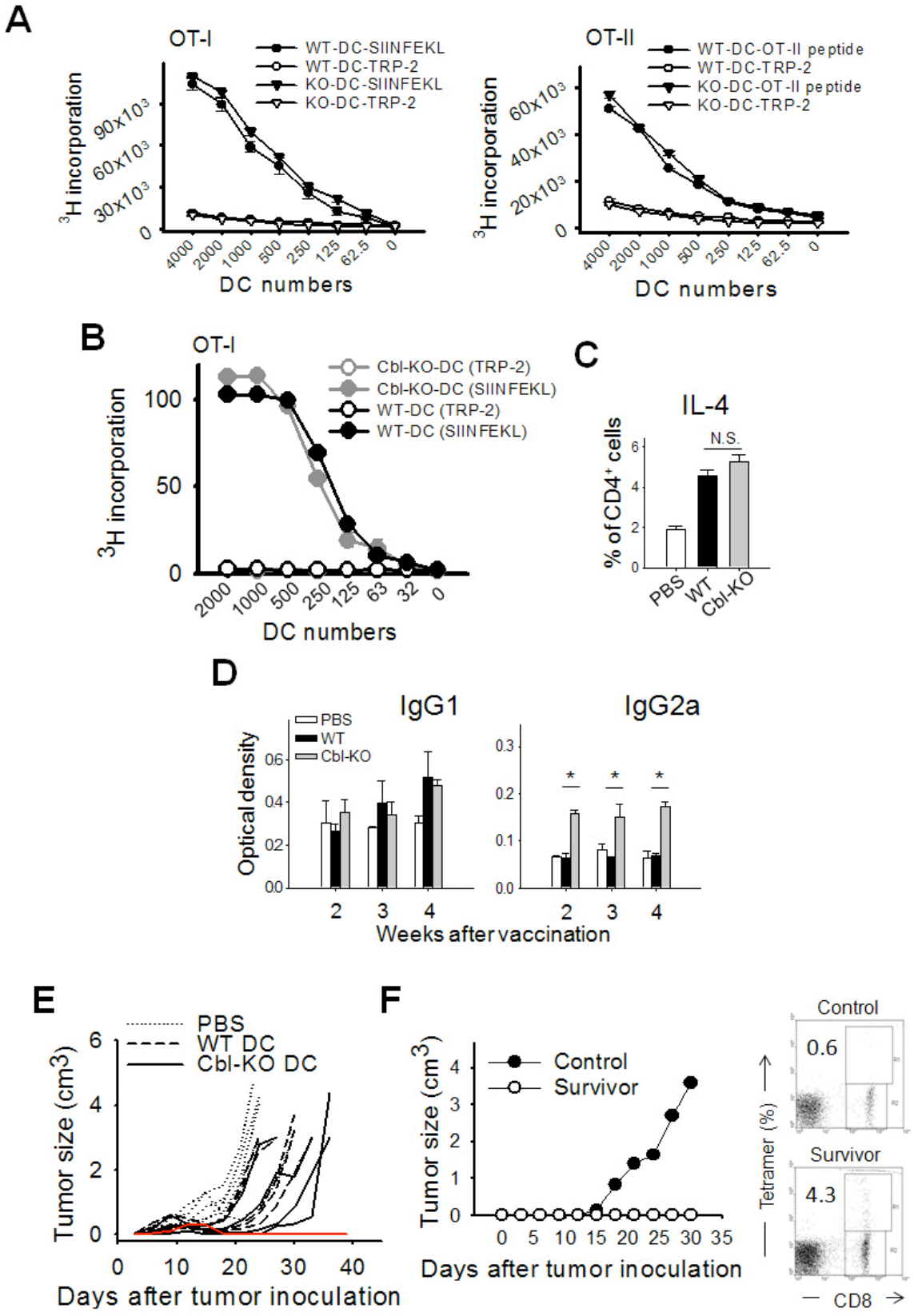

Figure S2.
(A, B) *In vitro* proliferation assay was conducted as described in *Supplemental Experimental Procedures*. Briefly, indicated numbers of BMDCs were stimulated with LPS only (A, 1 μg/mL) or LPS plus CD40L (B, 0.5 μg/mL) and pulsed with indicated peptides (50 μg/mL) before cocultured with splenocytes derived from OT-I or OT-II mice. TRP-2 (50 μg/mL) peptide was used as control in these experiments. Levels of peptide-specific proliferation were subsequently quantified by ^{3}H-thymidine incorporation. Data were triplicates derived from one experiment (Average ± SD) and are representative of three independent experiments. (C) Splenocytes isolated from mice vaccinated with PBS, WT, or Cbl-KO DCs for 2 weeks were stimulated with OT-II peptide overnight before quantification of IL-4 production by intracellular staining (data represent mean ± SE; n = 6; NS, not statistically significant). (D) Sera collected from vaccinated mice as in (C) at indicated time points were quantified for Ova-specific IgG1 (left panel) and IgG2a (right panel) by ELISA (n = 6). *P < 0.05. (E) E.G7-Ova tumor-bearing mice were vaccinated with indicated DC vaccines as described in *Materials and Methods*. Tumor size was monitored biweekly. Tumor sizes of individual animals were plotted against days. The red line represents the tumor growth of the tumor-free survivor. (F) Three months into the tumor study, both the naïve mouse (control) and the tumor-free survivor were injected with 5×10^5 E.G7-Ova cells and monitored for tumor growth over the course of a month. 10 days after tumor inoculation, the peripheral blood was collected retro-orbitally and stained for Ova-specific CD8$^+$ T cells with SIINFEKL-MHC I K^b-PE tetramer. The naïve mouse was sacrificed on day 30.

Chiou *et al*., Figure S3

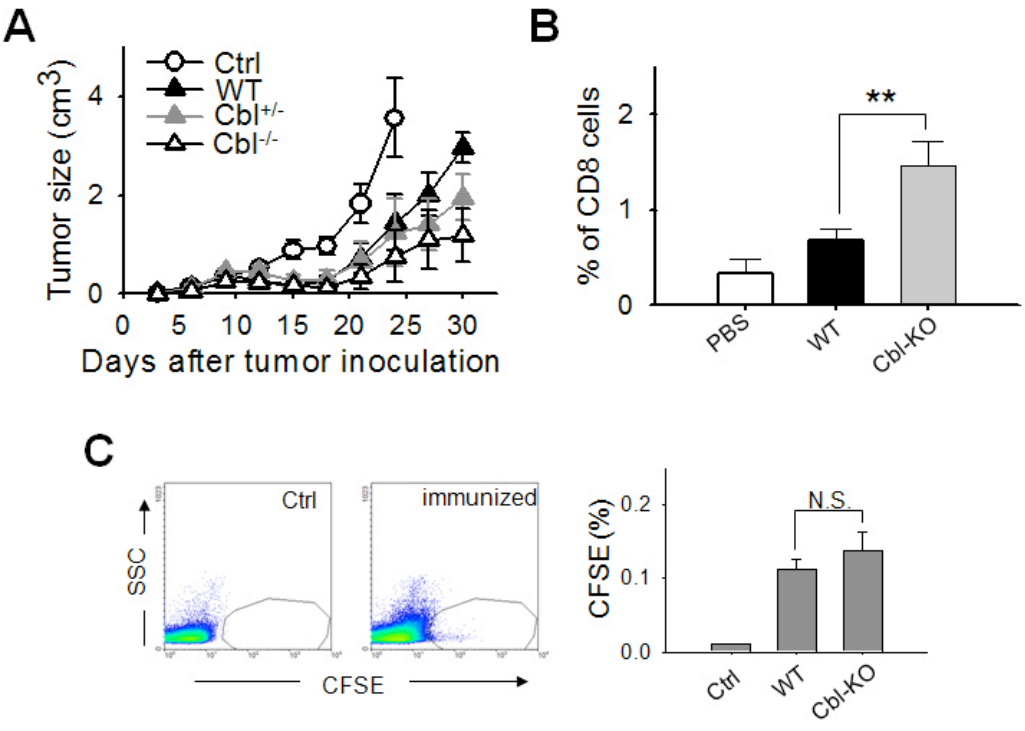

Figure S3.
(A) Naïve E.G7-Ova tumor-bearing mice aged 5-6 weeks old were vaccinated with indicated DC vaccines and subjected to measurements of tumor sizes as described in Materials and Methods (data represent mean ± SE; n = 5; *P < 0.05). (B) PBMCs derived from mice of tumor study in (A) were collected retro-orbitally and subjected to co-staining with MHC tetramer K^b-$Ova2_{57-264}$ and anti-CD8 by FACS 10 days post-vaccination. The percentages of $tetramer^+$ cells of all gated $CD8^+$ cells were plotted (data represent mean ± SE; PBS: n = 5; WT BMDCs: n = 12; Cbl-KO BMDCs: n = 12). **P < 0.01. (C) Paired BMDCs derived from WT and Cbl-KO mice were prepared as previously described. DCs were labeled with CFSE and subsequently stimulated with LPS for 1 hour before footpad-vaccination into naïve, female mice aged between 6-7 weeks old (n = 5; data represent mean ± SE; NS, not statistically significant). Labeled DCs that migrated into the popliteal lymph nodes were quantified by flow cytometry 24 hours post-vaccination by gating on the live (FSC-SSC/PI negative) and CFSE-positive population.

Chiou *et al*., Figure S4

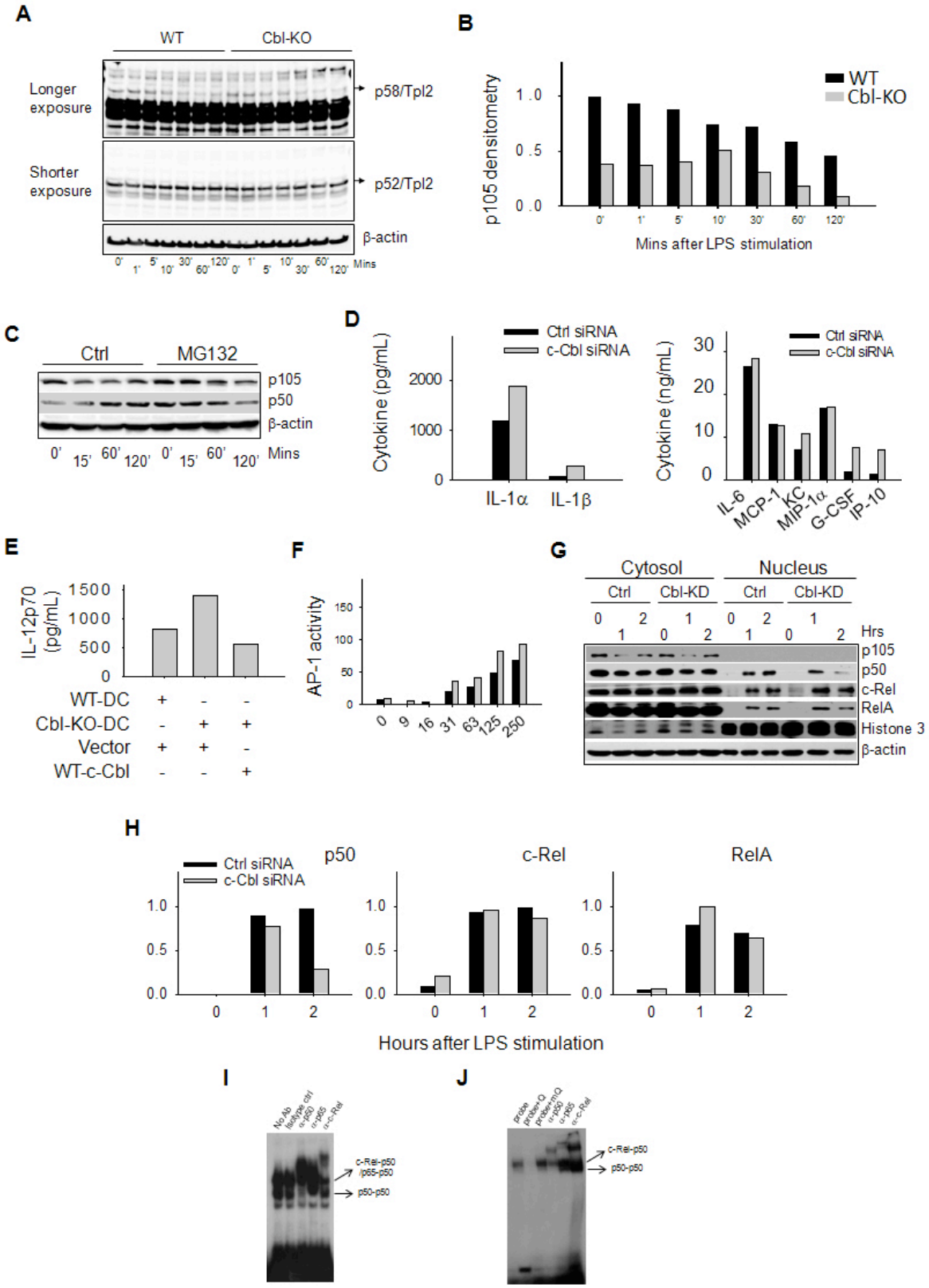

Figure S4.

(A) Paired WT and Cbl-KO DCs were stimulated with LPS for indicated times before lysis for western blot analysis for Tpl2. (B) Result of the densitometric analysis derived from the western blot of p105 (Fig. 3A, panel 6). (C) BMDCs derived from WT mice were stimulated with LPS for indicated time before lysis for western blot analysis. Before LPS treatment, cells were cultured in the presence of MG132 for 3 hours (10 μM). (D) WT BMDCs were nucleofected with indicated siRNAs and cultured overnight. Following LPS stimulation for 24 hours, supernatants were collected for quantification of indicated cytokine/chemokine by LINCOplex assay. (E) WT or Cbl-KO BMDCs were nucleofected with blank DNA vector or the construct encoding WT c-Cbl as indicated. 8-10 hours later, cells were stimulated with LPS overnight and expression of IL-12p70 was quantified by ELISA. (F) WT and Cbl-KO BMDCs were nucleofected with normalized amounts of AP-1-SEAP reporter and incubated for 8-10 hours. Subsequently, cells were stimulated with titrated amount of LPS before SEAP assay as described previously (Hanks *et al.*, 2005). (G, H) WT BMDCs were subjected to c-Cbl knockdown as in (D) before analysis. Subsequently, cells were stimulated with LPS for indicated time points before preparation of both cytosolic and nuclear lysates for quantification of p65, p50, and c-Rel by western blot (G) and densitometric analyses (H). (I, J) Supershift data for EMSA in Fig. 4E, F. Nuclear lysates were prepared and supershift was performed by using the indicated antibodies. Probes containing the conventional (I) or the TNF-α κB site (Baer *et al.*, 1998) (J) are used for the supershift experiments. Q (quencher), non-labeled probe; mQ, non-labeled, mutated probe. Data are representative of at least 3 independent experiments.

Chiou *et al*., Figure S5

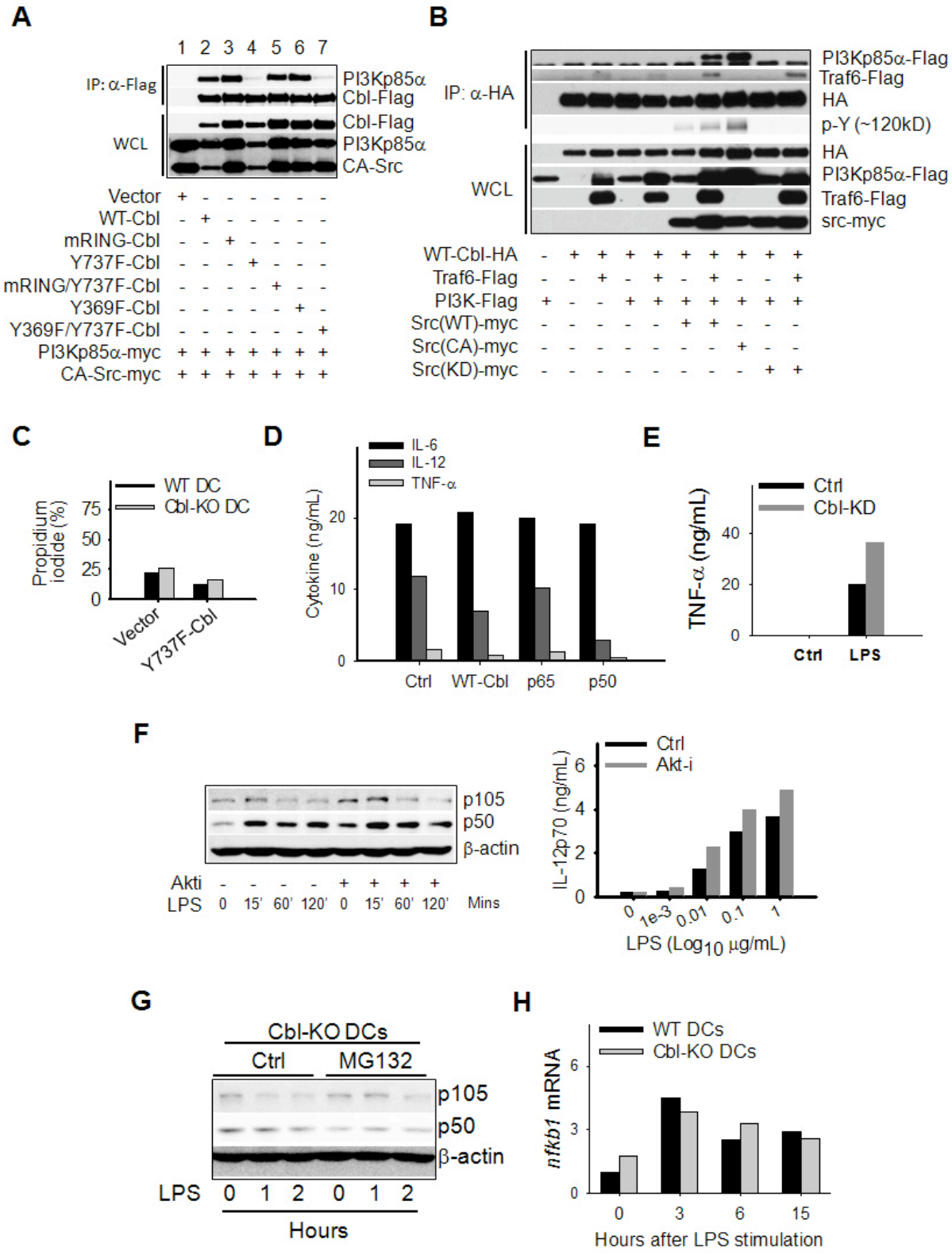

Figure S5.
(A, B) 293T cells were transfected with indicated constructs for 36 hours before lysis. Subsequently, transfectants were prepared for immunoprecipitation of Flag- (A) or HA-tagged (B) c-Cbl before western blot analysis. p-Y, phospho-tyrosines as quantified by the anti-p-Y antibody (clone 4G10). (C) WT or Cbl-KO BMDCs were nucleofected with control vector or Cbl-Y737F mutant 10 hours before LPS stimulation. After overnight stimulation, cells were harvested for analysis of death/necrosis by propidium iodide staining. (D) Cbl-KO BMDCs were nucleofected with either the control construct, WT c-Cbl, p65, or p50 as indicated and stimulated with LPS before collection of supernatants for quantifications of indicated cytokines by ELISA. (E) Control or Cbl-KD BMDCs were stimulated with LPS overnight and subsequently, the supernatants were collected for quantification of TNF-α. (F) WT BMDCs were treated with Akt inhibitor (Akt-i, 1 μM) for 1 hour before kinetic analysis for the assessment of LPS-induced p50 accumulation (left panel). To show the effect of Akt-i treatment, DCs were stimulated with titrated LPS in the presence or absence of Akt-i overnight before collection of supernatants for the quantification of IL-12p70 by ELISA (right panel). (G) Cbl-KO BMDCs were treated with proteasome inhibitor, MG132 (10 μM), for two hours before LPS stimulation. Following stimulation for indicated time, cells were harvested for quantification of p105 and p50/NF-κB1 total protein levels by western blot analysis. (H) WT and Cbl-KO BMDCs were stimulated with LPS for indicated times before RNA extraction for the quantification of *Nfkb1* mRNA by qRT-PCR. Data are representative of at least two independent experiments.

Supplemental Experimental Procedures

Mice and Cells

OT-I and OT-II TCR transgenic mice were from Jackson Laboratory (Bar Harbor, Maine). All bone marrow-derived dendritic cells (BMDCs) were derived from female (unless noted otherwise), 6-8 week old C57BL/6 background mice. All experimental procedures were conducted according to protocols approved by the Baylor Institutional Animal Care and Use Committee. BMDCs were cultured and harvested, essentially as described previously (Inaba *et al.*, 1992). Briefly, bone marrow cells were collected from tibias and femurs of both WT and Cbl-KO mice. Subsequently, red blood cells were depleted by Lympholyte-M (Cedarlane Labs, Burlington, NC). After washing, cells were cultured in complete RPMI 1640 (with 10% FBS and antibiotics). Murine granulocyte-macrophage colony-stimulating factor (mGM-CSF; 20 ng/mL, Invitrogen, Carlsbad, CA) and interleukin-4 (10 ng/mL, eBioscience, San Diego, CA) were used for differentiation into BMDCs. At day 6, BMDCs were further purified using anti-CD11c magnetic beads (Miltenyi Biotec, Inc., Bergisch Gladbach, Germany), if noted. 293T cells were maintained in Dulbecco's Modified Eagle medium (DMEM) containing 10% fetal bovine sera (FBS) and antibiotics.

Reagents

Flow cytometry: antibodies against CD11c, CD80, CD86, MHC-I K^b, MHC-II I-A^b, CD40, CD8, CD4, IL-12p40, and IFN-γ were from BD Biosciences, Inc. (San Jose, CA); APC-labeled anti-murine IL-4 antibody was from BioLegend, Inc. (San Diego, CA). For **TLR engagements**, Pam3CSK4, CL075, and imiquimod were from InvivoGen, Inc. (San Diego, CA); lipoteichoic acid (LTA), polyinosinic-polycytidylic acid (polyI:C), and LPS from Sigma-Aldrich, Inc. (St. Louis, MO); phosphorothioate-stabilized CpG oligonucleotide (TCCATGACGTTCCTGATGCT) was synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa) as described previously (Chen *et al.*, 2007). **For gene knockdowns**, all siRNAs were purchased from Sigma-Aldrich. For control siRNA, 5' *GGUAUUUCUUCGCUUGUUCUA*-UU 3'; murine c-Cbl (ID: 12402), 5' *GGCUUCUAUUUGUUUCCUGAU*-UU 3'. All oligonucleotides were purchased as double-stranded siRNAs with target gene homology (italicized) followed by a 3' UU overhang. For **the cytotoxicity and proliferation assays**, ^{51}Cr and ^{3}H-thymidine were from PerkinElmer, Inc. (Waltham, MA). For **the proliferation assay,** OT-I ($Ova_{257-264}$ SIINFEKL), OT-II ($Ova_{323-339}$ ISQAVHAAHAEINEAGR), and TRP-2 ($Trp\text{-}2_{180-188}$ SVYDFFVWL) peptides were purchased from Genemed Synthesis, Inc. (San Antonio, TX). For **western blot analyses**: anti-phospho-Akt (T308, #9275), phospho-Akt (S473, #9271), phospho-JNK (T183/T185, #9255), GAPDH (#2118), JNK (#9252), p-IKKα/β (S180/S181, #2681), p-p38 (#9228), p38 (#9217), p-ERK (T202/Y204, #4377), and RelA/p65 (#3034) antibodies were from Cell Signaling Technology, Inc. (Danvers, MA); anti-Akt1/PKBα (07-416), and anti-myc (4A6) antibodies from Millipore Corp. (Billerica, MA); anti-c-Cbl (sc-170), anti-IκB (sc-7182), anti-IKKα/β (sc-7607), anti-ERK (sc-93), anti-p105/p50 (sc-8414), anti-c-Rel (sc-71), rabbit anti-c-Cbl (sc-170), and anti-Tpl2 (p52/p58, sc-720) antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); secondary antibodies goat anti-rabbit IgG-HRP (sc-2004) and anti-mouse IgG-HRP (sc-2005) were from Santa Cruz Biotechnology; mouse anti-murine c-Cbl (17/c-Cbl) monoclonal antibody (MoAb) from BD Biosciences; anti-H3 antibody from Abcam Inc. (ab1791, Cambridge, MA); anti-HA

MoAb from Covance (clone 16B12, Princeton, NJ); and anti-FLAG (F1804) and anti-β-actin (AC-15) MoAb from Sigma-Aldrich. MG132 were from Sigma-Aldrich. **ChIP and supershift:** anti-c-Rel (sc-71X), anti-RelA (sc-372X), and anti-p50 (sc-1192X) antibodies were from Santa Cruz Biotechnology. **Co-immunoprecipitation:** anti-FLAG antibody (F1804) was from Sigma-Aldrich and anti-HA antibody (clone 16B12) was from Covance; protein G agarose beads were from Santa Cruz Biotechnology. The PE-conjugated, tetrameric H-2K^b-Ova$_{257-264}$ was from the Tetramer Core Facility, Baylor College of Medicine (BCM). Akt inhibitor II was from EMD Chemicals Inc. (Gibbstown, NJ). The constitutively active CD4-TLR4 construct was kindly provided by Sankar Ghosh (Zhang *et al.*, 2004). Detection of **serum anti-Ova isotypic antibodies**: horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG$_1$ (sc-2060) and IgG$_{2a}$ (sc-2061) antibodies were from Santa Cruz Biotechnology. **Macropinocytosis**: FITC-dextran was from Molecular Probes (Eugene, OR). The NF-κB-SEAP reporter plasmid was described previously (Spencer *et al.*, 1993). The WT TRAF6 construct was generously provided by Tse-Hua Tan (BCM). Human c-Cbl (HA-tagged) was a gift from Lawrence E. Samelson (NCI, Bethesda, MD). Plasmids encoding FLAG-tagged human *Pik3r1* gene (PI3Kp85α-Flag), murine p50, and p65/RelA (Sanjabi *et al.*, 2000) were obtained from Addgene (Cambridge, MA).

Plasmid constructions

Murine c-Cbl (mCbl) was cloned from two "tiling" pieces amplified by polymerase chain reaction (PCR), followed by TOPO ligation (Invitrogen). mCbl was subsequently cloned into a Flag-tagged vector, pcDNA3.1-Flag-MBNL1 (courtesy of Tom Cooper, BCM). The mCbl mutants were generated by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Oligonucleotides for different mCbl mutants were designed based on previous reports (see *Result*). Murine p105-myc was generated by cloning murine *Nfkb1* gene with two tiling pieces into the TOPO vector, pCR-Blunt vector (Invitrogen), before transfer into *Not*I/*Sal*I-digested pSH1-S-E-F$_{pk}$3-FADD-myc vector. To make myc-tagged murine PI3Kp85α, murine PI3Kp85α (*Pik3r1*) was cloned into the TOPO vector and subsequently digested with *Not*I/*Sal*I before subcloning into vector, pSH1-S-E-Fpk3-FADD-myc (Fan *et al.*, 1999). WT human c-Src (*SRC*) was similarly cloned into the pSH1-S-E-Fpk3-FADD-myc vector. The constitutively active and kinase-dead mutants of c-Src were created by site-directed mutagenesis (Davidson *et al.*, 1992; Destaing *et al.*, 2008). c-Cbl mutants containing inactivating mutations within various domains, including the PTB (G304E), RING (C379A), proline-rich domain (pro-R), tyrosine 369 (Y369F), implicated in RING function, and/or tyrosine 737 (Y737F), implicated in PI3K binding, were created by site-directed mutagenesis as described previously (Kassenbrock and Anderson, 2004; Sanjay *et al*., 2006; Standaert *et al*., 2004; Thien *et al*., 2003; Thien *et al*., 2001).

Western blots

For molecular kinetics studies in BMDCs, cells were generally seeded at 1×10^6 cells/mL the night before analysis. After treatment with indicated stimuli, reactions were terminated with ice-cold PBS before harvest. Cells were lysed with a Brij97-based lysis buffer containing protease inhibitor cocktail (1/100 dilution, Sigma-Aldrich) on ice for 30 min, followed by centrifugation. Subsequently, supernatants were collected and proteins were reduced and denatured with β-mercaptoethanol at 95°C for 5-10 min. Samples were then separated by PAGE, followed by

transfer to PVDF membranes. After transfer, membranes were blocked with 5% milk at RT. For phosphorylation detection, 4% BSA was used as blocking agent. Membranes were stained with 1° antibody overnight unless otherwise specified. For enhanced chemiluminescence, SuperSignal western blotting substrate was used (Thermo Fisher Scientific, Inc., Huntsville, AL). Images were created by X-ray film or using a 4000MM Gel Documenting System (Kodak, Rochester, NY).

Semi-quantitative- and real time-polymerase chain reaction (RT-PCR/qPCR)

BMDCs stimulated with LPS (1 μg/mL) at various time points were subjected to total RNA enrichment by using Trizol (Invitrogen). Reverse transcription was carried out by using the SuperScript III pre-amplification system (Invitrogen). For RT-PCR, the program consisted of 30 cycles with the initial denaturation at 95°C for 5 min, annealing at 58°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Primers used were as follows: murine GAPHD (*Gapdh*) sense (5'-ACTGGCATGGCCTTCCGTGT-3'), GAPHD antisense (5'-TTACTCCTTGGAGGCCATGT-3'), IL-12p40 (*Il12b*) sense (5'-CACACTGGACCAAAGGGACT-3'), murine IL-12p40 antisense (5'-AAAGCCAACCAAGCAGAAGA-3'); for quantification of murine IL-12p35 gene (*Il12a*), the anti-sense primer (5'-CTCAGATAGCCCATCACCCTGTTG-3') was adopted in combination with a previously described IL-12p35 sense primer (Homey *et al.*, 1998). For qPCR, TaqMan probes for the quantification of *Il10*, *Il12b*, *Il12a*, and *Nfkb1* were purchased from Applied Biosystems (Carlsbad, CA) and the assays were conducted according to the manufacturer's protocols.

NF-κB Nuclear translocation assay

WT BMDCs were subjected to c-Cbl knockdown by nucleofection as described previously. Subsequently, nucleofectants were stimulated as indicated before preparation for cytosolic and nuclear extracts. Briefly, pelleted DCs were subjected to hypotonic lysis in the cytolysis solution (0.1% NP-40, 10 mM HEPES pH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 1/100 diluted protease inhibitor cocktail) for 15 mins on ice. Subsequently, nuclei were spun down at 14K rpm for 10 mins, followed by collection of supernatant (cytosolic sample) and nucleolysis by the hypertonic lysis buffer: 20 mM HEPES pH 8.0, 400 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, 1 mM DTT, 1 mM PMSF, and 1/100 diluted protease inhibitor cocktail. Nucleolysates were incubated on ice for 30 mins before centrifugation, followed by western blot analysis.

CTL (cell-mediated cytotoxicity) assay

Parental EL4 thymoma and E.G7-Ova cells (American Type Culture Collection) were used as targets. E.G7-Ova cells were cultured in the presence of G418 for at least 2 weeks before use. Target cells were loaded with ^{51}Cr and seeded before coculture with effector cells. Splenocytes harvested from mice vaccinated with DC vaccines for 10 days were used as effector cells. After harvest, splenocytes were cultured with target cells at various ratios. Data were collected 4-6 hours after coculture. Percentage (%) of CTL activity was determined as ($cpm_{experimental}$ – $cpm_{spontaneous}$ / $cpm_{maximum}$ – $cpm_{spontaneous}$). Maximum lysis of E.G7-Ova was achieved with 2% Triton X-100.

In vivo migration assay

WT and Cbl-KO BMDCs were prepared as described. Shortly before transfer, DCs were labeled with CFSE (6 μM) followed by LPS stimulation for an extra 2 hours (1 μg/mL). DCs were washed four times in PBS and then further concentrated for footpad injection into syngeneic, naïve mice (4×10^6 cell/mouse, n = 5). 24 hours post-vaccination, popliteal lymph nodes from both naïve, unvaccinated and vaccinated mice were collected and subsequently prepared for flow cytometric analysis. The level of migration was defined by the percentage of CFSE+ cells in the gated pool ($PI^-FSC^{hi}SSC^{lo}$).

Quantification of sera antibodies

For quantification of anti-Ova antibodies in sera samples from vaccinated mice, microtiter plates (Maxisorp, Thermo Fisher Scientific) were coated overnight at 4°C with 1 mg/ml of total Ova protein diluted in PBS. Plates were washed with a wash buffer and blocked with an assay diluent buffer provided with BD OptEIA Reagent Set A kit (BD Biosciences). 100 ml of serum diluted at 1:100 in assay diluent buffer was applied into the wells and incubated for 2 h at RT. Plates were washed 5 times and incubated for 1 h at RT with anti-mouse IgG_1-HRP or IgG_{2a}-HRP diluted at 1:1000. After washing again as above, plates were developed with a TMB substrate solution provided with BD OptEIA Reagent Set A and absorbance was read at 450 nm on a HTS 7000 Plus BioAssay Reader (PerkinElmer). The assays were performed in duplicates.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as previously described (Wagner *et al.*, 2010). Briefly, paired WT and Cbl-KO BMDCs (10^7 cells each) were cultured in 15 cm plates before assay. Subsequently, cells were stimulated with LPS (1 μg/mL) for indicated times before fixation with 1.1% formaldehyde. Crosslinked genomic DNA was subjected to enzymatic shearing by using the ChIP-IT Express Enzymatic kit (Active Motif, Carlsbad, CA). Lysate protein concentration was quantified post-shearing and subsequently diluted to 0.25 μg/mL. Antibodies used for the precipitation of lysate proteins are included in the Supplemental Experimental Procedures. Recovered DNA was quantitatively amplified with specific primers spanning the two pre-identified IL-12p35-κB sites (Grumont *et al.*, 2001) using QuantiFast SYBR Green PCR (Qiagen, Inc., Valencia, CA) and normalized against the input. Primers for the proximal IL-12-κB1: forward 5'-GGAATCTCCTACCCCAAACCTCC-3', reverse 5'-GAGTAGGTGGCAGGATATGGCC-3'; distal IL-12-κB2: forward 5'-CGAATCTCCTCAAACGGGCAC-3', reverse 5'-AACGCTGACCTTGGGAGACAC-3'.

IFN-γ ELISpot assay

Splenocytes harvested from mice vaccinated for 35 days were used in this assay. ELISpot assays were conducted as described previously (Park *et al.*, 2006). Briefly, cells were cultured for 24 hours in 96-well multi-screen-HA plates (Millipore) pre-coated with anti-murine IFN-γ (R4-6A2, eBioscience). Subsequently, a detection anti-murine IFN-γ was added and incubated for 2 hours after 5 washes. Spots were visualized with 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium alkaline phosphatase substrate (Sigma-Aldrich) and quantified in triplicate wells with a stereomicroscope.

Lentivirus preparation

Lentivirus was produced by co-transfecting 293T cells with pGIPZ (siCbl, pGIPZ-miRNA specific for human *CBL* gene product (Oligo ID: V2LHS_48408); NS: non-silencing control; siEG5, pGIPZ-miRNA specific for endogenous human EG5 or the gene product of *KIF11*; Thermo Fisher Scientific), pCMV-Δ8.9, and pMD.G (kindly provided by Si-Yi Chen, USC, Los Angeles, CA). Subsequently, virus was concentrated by ultracentrifugation (28K rpm for ≥ 2 hours; rotor SW28 (Beckman Coulter, Fullerton, CA)). Concentrated virus was resuspended in PBS and titered by directly transducing 293T cells followed by quantification via flow cytometry 2 days after transduction.

Proliferation assay (*in vitro* T cell proliferation assay)

Before coculture with splenocytes, freshly harvested DCs were stimulated with LPS (0.5 - 1.0 μg/mL) ± CD40L (0.5 μg/mL) and pulsed with specific peptide for either OT-I or OT-II splenocytes. Subsequently, cells were washed three times before seeding onto U-bottomed, 96-well plates. Two hours later, splenocytes from both OT-I and OT-II mice were harvested and cocultured with BMDCs pulsed with OT-I or OT-II peptide, respectively, at a density of 10^5 cells per well. A day later, ^{3}H-thymidine was added to the media (1 μCi/well) for 16 hours prior to data collection.

Survival assay

BMDCs were cultured in complete RPMI 1640 medium (with 10% FBS) without mGM-CSF and mIL-4. Subsequently, cells were harvested on indicated days and stained with propidium iodide (1 μg/mL) shortly before flow cytometric analysis.

Macropinocytosis assay

BMDCs were stimulated with LPS (1 μg/mL) overnight before assay. Subsequently, cells were either incubated at 37°C or pre-cooled at 4°C, 1 hour before use. FITC-dextran (1 mg/mL) was added and incubated for 1 hour before washing with PBS. Intracellular retention of FITC-dextran was quantified and analyzed by flow cytometry.

Supplemental References

Baer, M., Dillner, A., Schwartz, R.C., Sedon, C., Nedospasov, S. and Johnson, P.F. (1998) Tumor necrosis factor alpha transcription in macrophages is attenuated by an autocrine factor that preferentially induces NF-kappaB p50. *Mol Cell Biol*, **18**, 5678-5689.

Chen, M., Huang, L. and Wang, J. (2007) Deficiency of Bim in dendritic cells contributes to overactivation of lymphocytes and autoimmunity. *Blood*, **109**, 4360-4367.

Davidson, D., Chow, L.M., Fournel, M. and Veillette, A. (1992) Differential regulation of T cell antigen responsiveness by isoforms of the src-related tyrosine protein kinase p59fyn. *J Exp Med*, **175**, 1483-1492.

Destaing, O., Sanjay, A., Itzstein, C., Horne, W.C., Toomre, D., De Camilli, P. and Baron, R. (2008) The tyrosine kinase activity of c-Src regulates actin dynamics and organization of podosomes in osteoclasts. *Mol Biol Cell*, **19**, 394-404.

Fan, L., Freeman, K.W., Khan, T., Pham, E. and Spencer, D.M. (1999) Improved artificial death switches based on caspases and FADD. *Hum Gene Ther*, **10**, 2273-2285.

Grumont, R., Hochrein, H., O'Keeffe, M., Gugasyan, R., White, C., Caminschi, I., Cook, W. and Gerondakis, S. (2001) c-Rel regulates interleukin 12 p70 expression in CD8(+) dendritic cells by specifically inducing p35 gene transcription. *J Exp Med*, **194**, 1021-1032.

Hanks, B.A., Jiang, J., Singh, R.A., Song, W., Barry, M., Huls, M.H., Slawin, K.M. and Spencer, D.M. (2005) Re-engineered CD40 receptor enables potent pharmacological activation of dendritic-cell cancer vaccines in vivo. *Nat Med*, **11**, 130-137.

Homey, B., Assmann, T., Vohr, H.W., Ulrich, P., Lauerma, A.I., Ruzicka, T., Lehmann, P. and Schuppe, H.C. (1998) Topical FK506 suppresses cytokine and costimulatory molecule expression in epidermal and local draining lymph node cells during primary skin immune responses. *J Immunol*, **160**, 5331-5340.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S. and Steinman, R.M. (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med*, **176**, 1693-1702.

Kassenbrock, C.K. and Anderson, S.M. (2004) Regulation of ubiquitin protein ligase activity in c-Cbl by phosphorylation-induced conformational change and constitutive activation by tyrosine to glutamate point mutations. *J Biol Chem*, **279**, 28017-28027.

Park, D., Lapteva, N., Seethammagari, M., Slawin, K.M. and Spencer, D.M. (2006) An essential role for Akt1 in dendritic cell function and tumor immunotherapy. *Nat Biotechnol*, **24**, 1581-1590.

Sanjabi, S., Hoffmann, A., Liou, H.C., Baltimore, D. and Smale, S.T. (2000) Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. *Proc Natl Acad Sci U S A*, **97**, 12705-12710.

Sanjay, A., Miyazaki, T., Itzstein, C., Purev, E., Horne, W.C. and Baron, R. (2006) Identification and functional characterization of an Src homology domain 3 domain-binding site on Cbl. *Febs J*, **273**, 5442-5456.

Spencer, D.M., Wandless, T.J., Schreiber, S.L. and Crabtree, G.R. (1993) Controlling signal transduction with synthetic ligands. *Science*, **262**, 1019-1024.

Standaert, M.L., Sajan, M.P., Miura, A., Bandyopadhyay, G. and Farese, R.V. (2004) Requirements for pYXXM motifs in Cbl for binding to the p85 subunit of phosphatidylinositol 3-kinase and Crk, and activation of atypical protein kinase C and glucose transport during insulin action in 3T3/L1 adipocytes. *Biochemistry*, **43**, 15494-15502.

Thien, C.B., Scaife, R.M., Papadimitriou, J.M., Murphy, M.A., Bowtell, D.D. and Langdon, W.Y. (2003) A mouse with a loss-of-function mutation in the c-Cbl TKB domain shows perturbed thymocyte signaling without enhancing the activity of the ZAP-70 tyrosine kinase. *J Exp Med*, **197**, 503-513.

Thien, C.B., Walker, F. and Langdon, W.Y. (2001) RING finger mutations that abolish c-Cbl-directed polyubiquitination and downregulation of the EGF receptor are insufficient for cell transformation. *Mol Cell*, **7**, 355-365.

Wagner, R.T., Xu, X., Yi, F., Merrill, B.J. and Cooney, A.J. (2010) Canonical Wnt/beta-catenin regulation of liver receptor homolog-1 mediates pluripotency gene expression. *Stem Cells*, **28**, 1794-1804.

Zhang, D., Zhang, G., Hayden, M.S., Greenblatt, M.B., Bussey, C., Flavell, R.A. and Ghosh, S. (2004) A toll-like receptor that prevents infection by uropathogenic bacteria. *Science*, **303**, 1522-1526.