Supplemental Data Figure 1

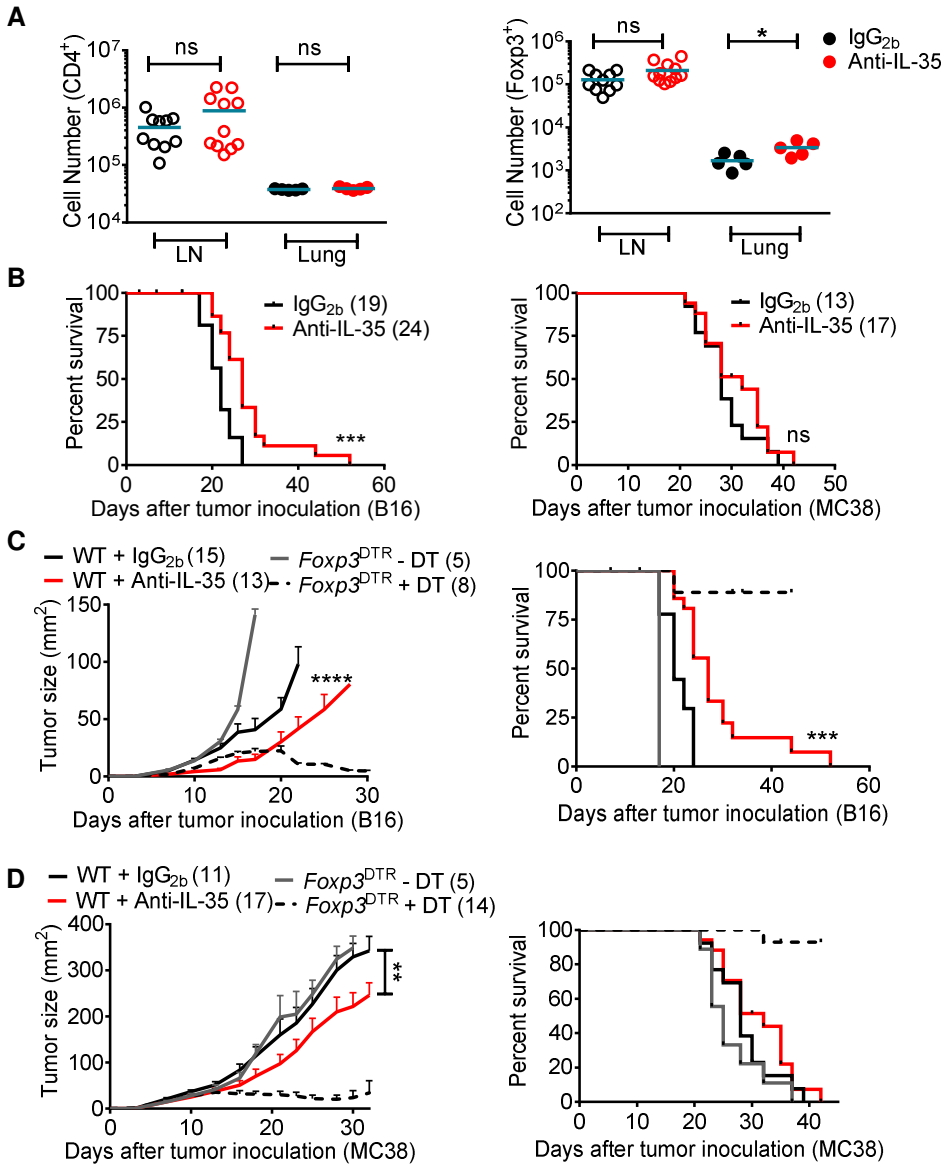

Figure S1, related to Figure 1. IL-35 neutralization reduces tumor growth and enhances survival of tumor-bearing mice

(A) To assess any depleting activity of anti-IL-35, sorted Treg and Teff cells were adoptively transferred into *Rag*1$^{-/-}$ mice followed by weekly anti-IL-35 or IgG_{2b} treatment starting on day 0 (100 µg first dose, 50 µg additional doses). LN and lungs were harvested and cells were counted. (B) Cumulative survival of i.d.-injected B16-bearing and s.c.-injected MC38-bearing mice (receiving weekly anti-IL-35 or IgG_{2b} as in part (A). (C) Tumor growth curve and survival plot of B16 tumor-bearing C57BL/6 mice (receiving weekly anti-IL-35 or IgG_{2b} as in part (A) and *Foxp3*^{DTR} mice (receiving 100 µg diphtheria toxin, DT, i.p. twice weekly). (D) Tumor growth curve and survival plot of MC38 tumor-bearing C57BL/6 mice (receiving weekly anti-IL-35 or IgG_{2b} as in part (A) and *Foxp3*^{DTR} mice (receiving 100 µg diphtheria toxin, DT, i.p. twice weekly). Data represent 2–4 independent experiments with number of mice in parentheses. Survival curves were analyzed for statistical significance by log-rank test. Tumor growth curves were analyzed by 2way ANOVA with multiple comparisons (panels B, C and D) and Unpaired Student's t-test (panel A). Error bars represent SEM; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, non-significant.

Supplemental Data Figure 2

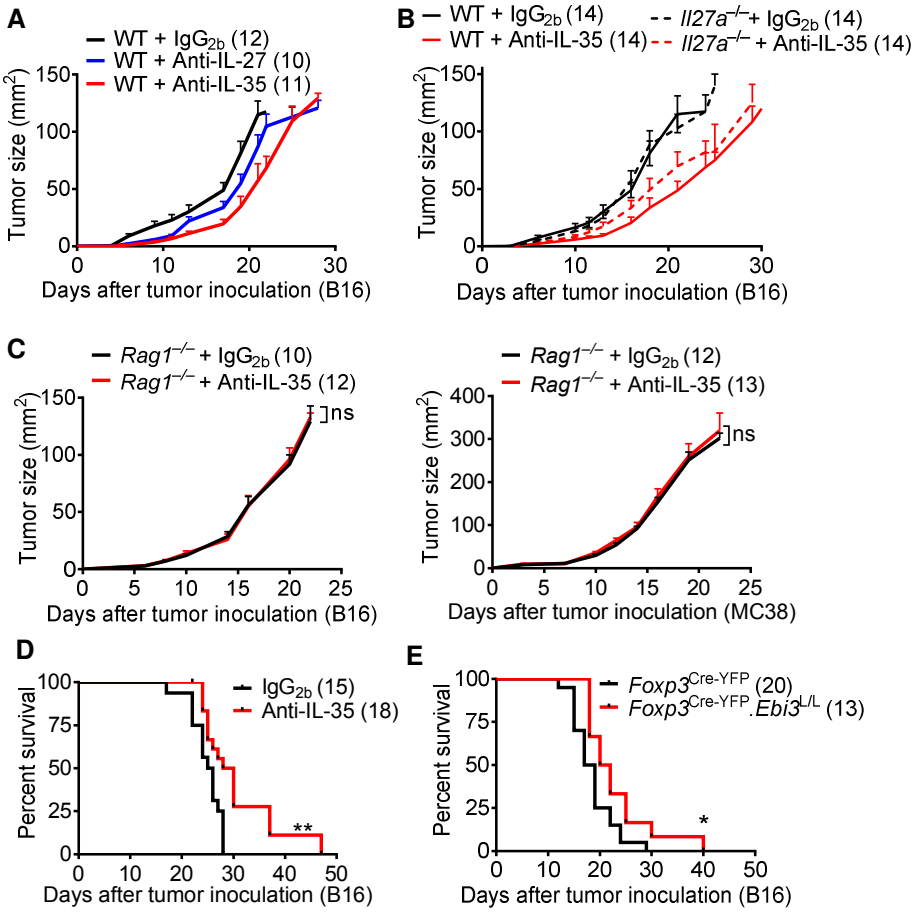

Figure S2, related to Figure 1. Characterization of anti-IL-35 antibody treatment
(A) C57BL/6 mice were injected i.d. with 1.25x10^5 B16 cells on day 0. Anti-IL-35, anti-IL-27, or IgG_{2b} was administered weekly (100 µg first dose, 50 µg additional doses). Tumor growth was monitored using a digital caliper. Anti-IL-35 vs IgG_{2b}: $p<0.0001$ at d21. Anti-IL-35 vs anti-IL-27: $p<0.01$ at d21. (B) C57BL/6 ("WT") or *IL-27a*$^{-/-}$ mice were injected i.d. with 1.25x10^5 B16 cells on day 0. Anti-IL-35 or IgG_{2b} was administered weekly as in part (A). Tumor growth was monitored using a digital caliper. *IL-27a*$^{-/-}$+IgG_{2b} vs *IL-27a*$^{-/-}$+anti-IL-35: $p<0.01$ at d21. WT+IgG_{2b} vs WT+anti-IL-35: $p<0.0001$ at d21. (C) *Rag1*$^{-/-}$ mice were injected i.d. with 1.25x10^5 B16 (left) or s.c. with 5x10^5 MC38 cells (right). Anti-IL-35 or IgG_{2b} was administered weekly as in part (A). Tumor growth was monitored using a digital caliper. (D) Cumulative survival of i.v.-injected metastatic B16-bearing mice. Anti-IL-35 or IgG_{2b} was administered weekly as in part (A) (E) Cumulative survival of i.d.-injected B16-bearing *Foxp3*$^{Cre\text{-}YFP}$*.Ebi3*$^{L/L}$ mice and *Foxp3*$^{Cre\text{-}YFP}$ control mice. Data represent 2-4 independent experiments with number of mice in parentheses; Survival curves were analyzed for statistical significance by log-rank test. Tumor growth curves were analyzed by ANOVA with repeated measures. Error bars represent SEM; *, $p < 0.05$; **, $p < 0.01$; ns, non-significant.

Supplemental Data Figure 3

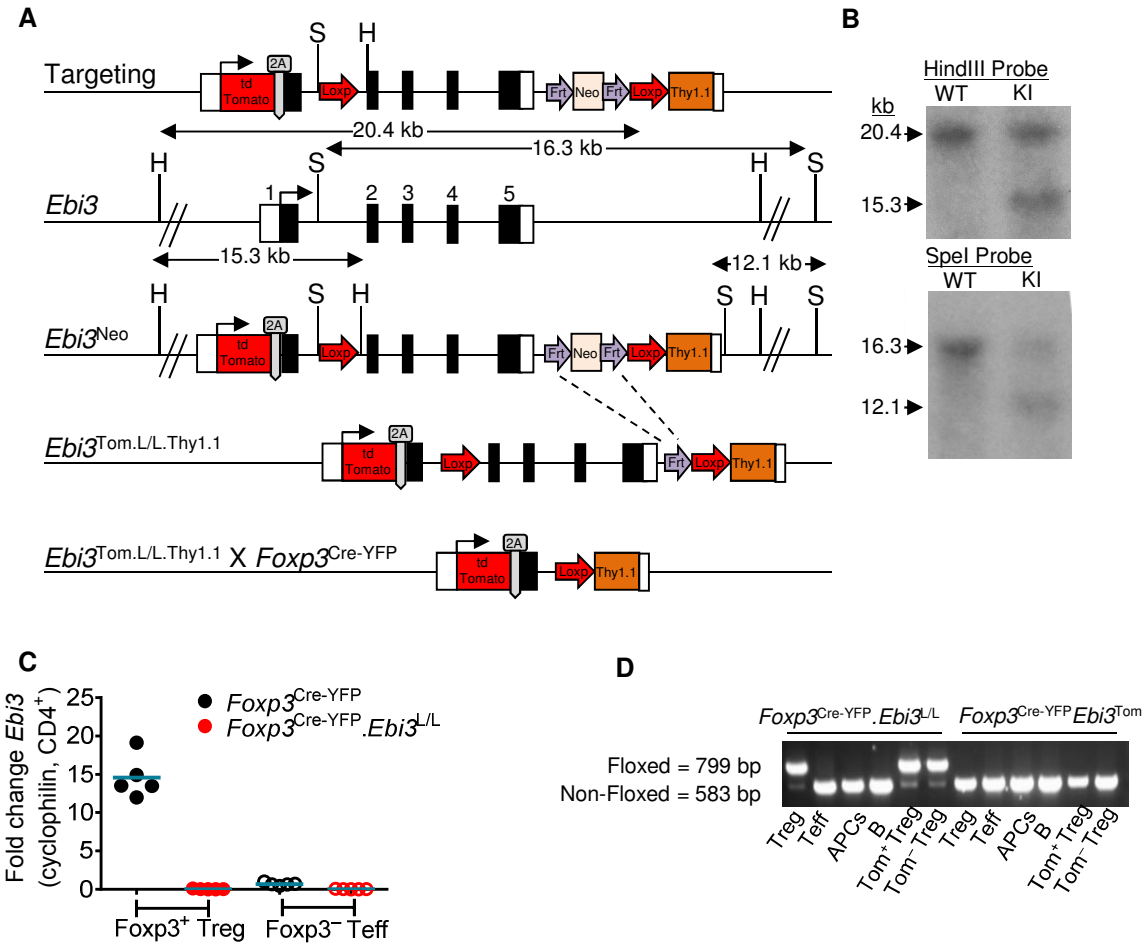

Figure S3, related to Figure 1. Tregs from *Ebi3*$^{Tom.L/L.Thy1.1}$x*Foxp3*$^{Cre-YFP}$ mice do not express Ebi3.

(A) Schematic of targeting construct, *Ebi3* locus and resulting *Ebi3*$^{Tom.L/L.Thy1.1}$ conditional reporter. When *Ebi3*$^{Tom.L/L.Thy1.1}$ mice are crossed to *Foxp3*$^{Cre-YFP}$ mice, Ebi3 is removed in Tregs by Cre-mediated deletion (B) Southern blot analysis indicating successful knock-in (KI) of targeting cassette. (C) YFP^{-}CD4^{+} Teffs and YFP^{+} Tregs from *Foxp3*$^{Cre-YFP}$*Ebi3*$^{L/L}$ mice and *Foxp3*$^{Cre-YFP}$ control mice were sorted, RNA was extracted, and cDNA was generated. Quantitative RT-PCR analysis assessing *Ebi3* expression with *cyclophilin* used as endogenous control. (D) PCR data validating genomic deletion of Ebi3 in Tregs sorted from *Foxp3*$^{Cre-YFP}$*Ebi3*$^{L/L}$ mice (total and segregated into Tom^{+} and Tom^{-} Treg fractions). Foxp3^{-} Teffs, TCRβ^{-} APCs (antigen-presenting cells) and B220^{+} B cells were used as controls. *Foxp3*$^{Cre-YFP}$Ebi3^{Tom} reporter mice were used as controls to verify intact Ebi3 expression in the IL-35 reporter mice.

Supplemental Data Figure 4

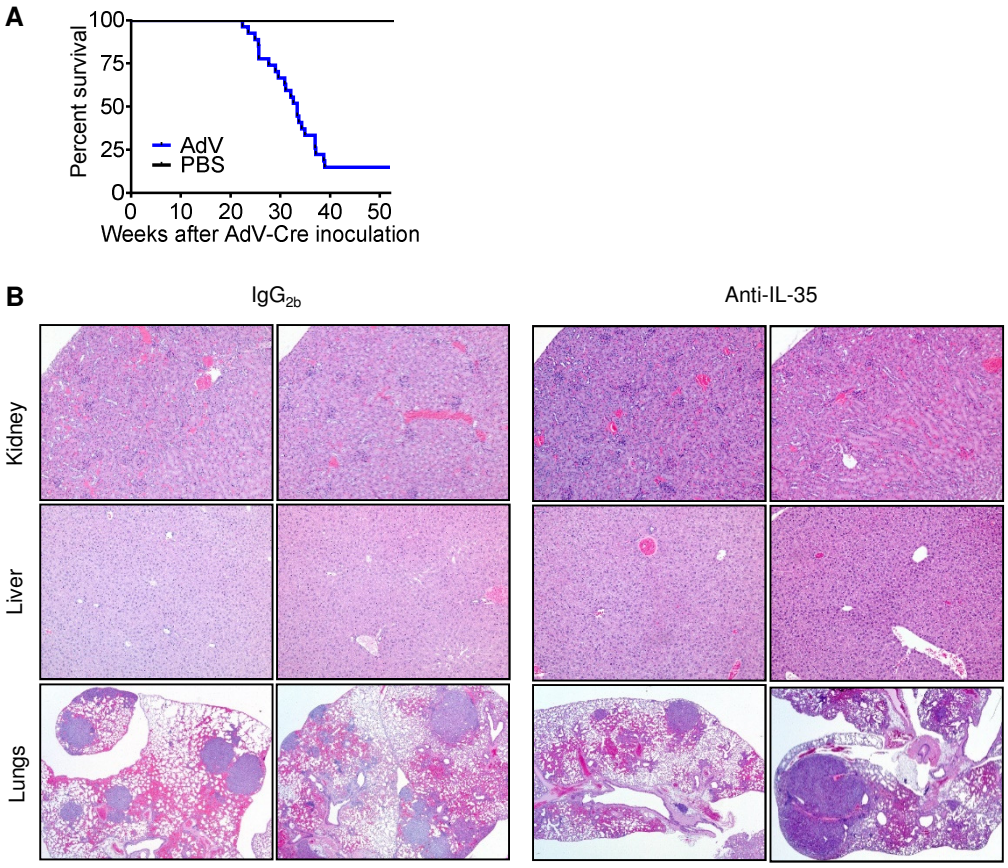

Figure S4, related to Figure 3. Absence of adverse events following long-term treatment with neutralizing IL-35 antibody

(A) Kaplan-Meier survival plot of KP mice (median survival=33.4 weeks) infected with $2.5x10^7$ PFU of AdV-Cre per mouse (B) Representative histological analysis of kidney and liver tissues of KP mice inoculated i.t. with AdV-Cre and then treated with anti-IL-35 or isotype control antibody starting at week eight for a total of ten injections (magnification - 10X). No notable inflammatory findings reported at any of these sites examined. Neoplasms noted in lung tissue and counts assessed by histology and MRI (magnification - 2X).

Supplemental Data Figure 5

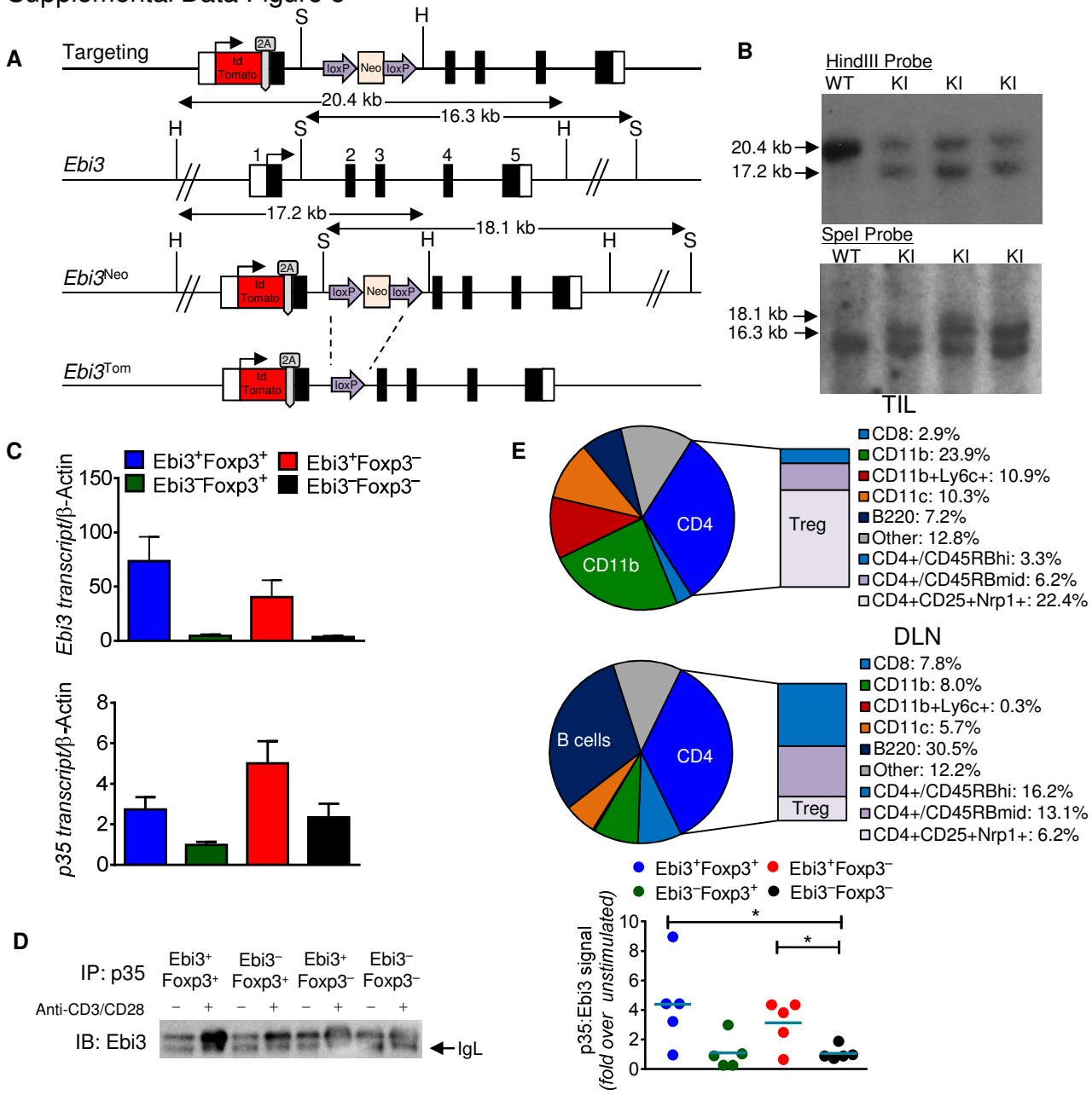

Figure S5, related to Figure 4. *Foxp3*$^{Cre\text{-}YFP}$*Ebi3*^{Tom} mice faithfully report IL-35 expression

(A) Schematic of targeting construct, *Ebi3* locus and resulting *Ebi3*tdTomato (*Ebi3*^{Tom}) reporter. (B) Southern blot analysis indicating successful knock-in (KI) of targeting cassette. (C) Ebi3/Foxp3-expressing T cell sub-populations were sorted from *Foxp3*$^{Cre\text{-}YFP}$*Ebi3*^{Tom} reporter mice, RNA was extracted, and cDNA was generated. Quantitative RT-PCR analysis of IL-35 subunits *Il12a* and *Ebi3,* with β-*actin* used as endogenous control. (D) Ebi3/Foxp3-expressing T cells from LNs of *Foxp3*$^{Cre\text{-}YFP}$*Ebi3*^{Tom} mice were expanded with PMA/Ionomycin and IL-2, then activated with anti-CD3/CD28 for 72 hours or left unstimulated; next supernatants were subjected to immunoprecipitation of IL12p35 followed by immunoblot analysis of Ebi3. Ebi3 band appears just above the light chain band, indicated by "IgL". Densitometry quantified at right. (E) Breakdown of tomato-expressing cells in DLN and TIL from B16 tumor-bearing *Foxp3*$^{Cre\text{-}YFP}$*Ebi3*^{Tom} reporter mice. Lymphocytes in DLN and TIL were gated on tomato^{+} cells followed by the markers indicated. Error bars represent SEM; *, $p < 0.05$; ns, non-significant (Unpaired Student's t-test).

Supplemental Data Figure 6

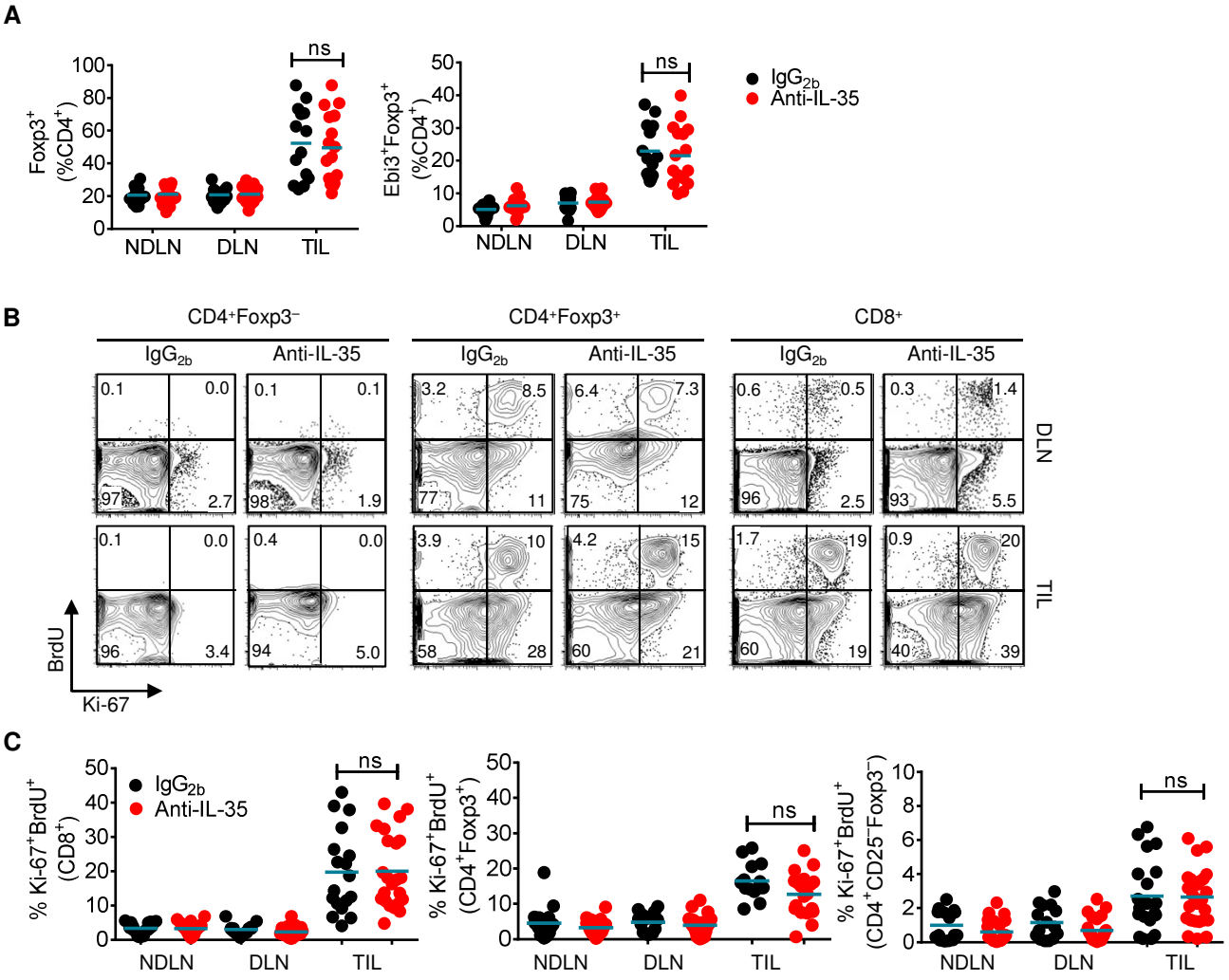

Figure S6, related to Figure 5. Proliferative phenotype in TILs of IL-35-neutralized mice.

(A) Tissues were harvested at day 16–18 from IgG_{2b}– or anti-IL-35–treated B16 tumor-bearing *Foxp3*$^{Cre\text{-}YFP}$*Ebi3*^{Tom} reporter mice. Percent of CD4$^+$Foxp3$^+$ and Ebi3$^+$Foxp3$^+$ Tregs analyzed by flow cytometry. (B) 18 hours prior to harvest, B16-bearing, IgG_{2b}– or anti-IL-35–treated C57BL/6 mice were injected with BrdU. Tissues were harvested and analyzed for BrdU/Ki-67 staining. Representative flow cytometric plots of BrdU/Ki-67 staining in DLN and TIL. (C) Percent of BrdU$^+$Ki-67$^+$ cells in NDLN, DLN or TILs. Plots are gated on CD8$^+$, CD4$^+$Foxp3$^+$ Tregs, or CD4$^+$CD25$^-$Foxp3$^-$ T cells as indicated. Data represent 3 independent experiments. Error bars represent SEM; ns, non-significant (Unpaired Student's t-test).

Supplemental Data Figure 7

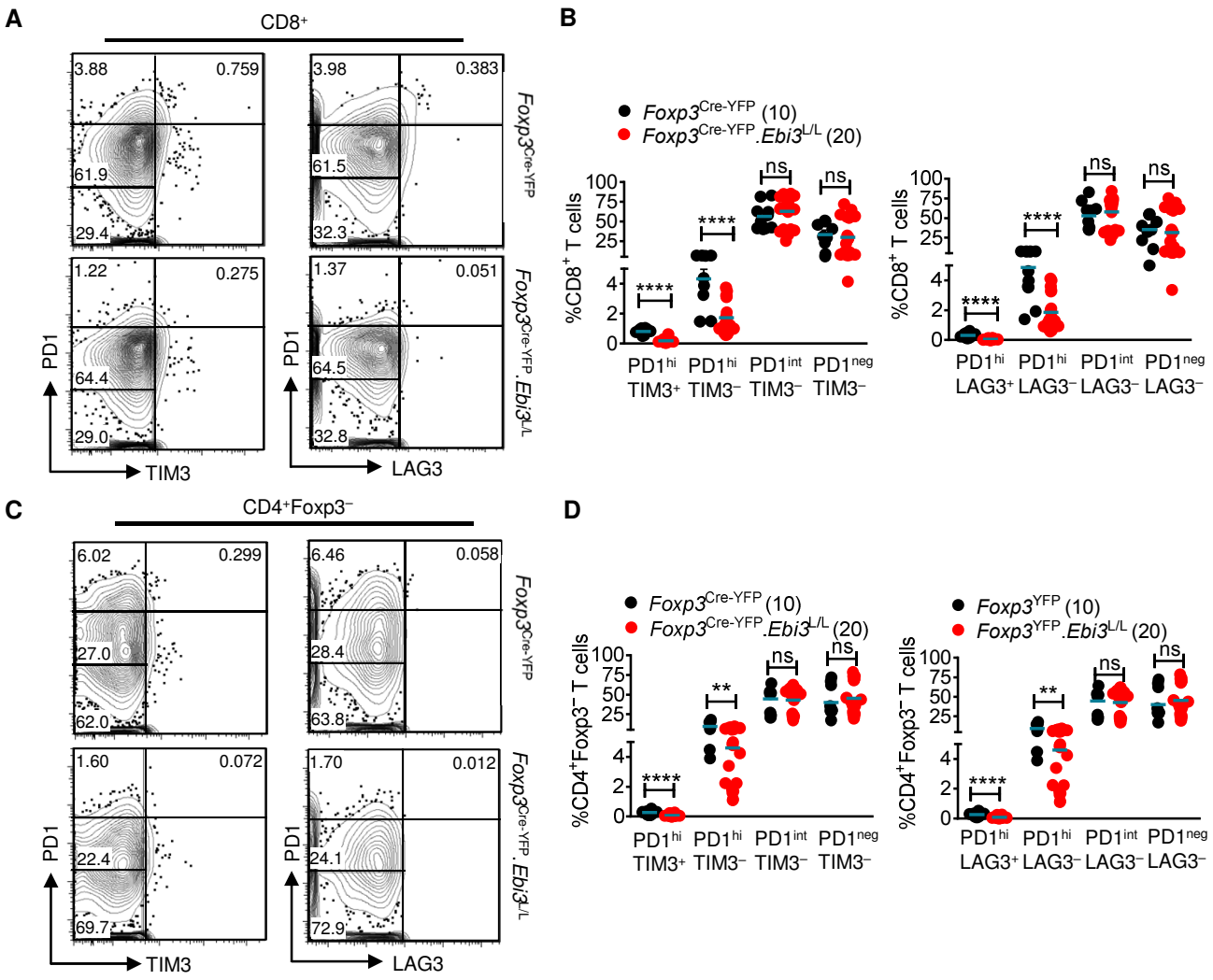

Figure S7, related to Figure 7. Absence of exhausted phenotype on *Foxp3*$^{Cre\text{-}YFP}$*Ebi3*$^{L/L}$ TILs in metastatic tumor model

Exhaustion marker analysis in TILs infiltrating *Foxp3*$^{Cre\text{-}YFP}$*Ebi3*$^{L/L}$ and *Foxp3*$^{Cre\text{-}YFP}$ mice in the B16 lung metastasis model (higher dose - 2.5x10^5 B16 injected i.v.). Flow cytometric plots depicting expression of inhibitory receptors (PD1/TIM3 and PD1/LAG3) on lung CD8$^+$ T cells, top (A) and CD4$^+$Foxp3$^-$ T cells, bottom (C). Cells gated on CD8$^+$ and CD4$^+$Foxp3$^-$ T cells, and assessed for percentages of PD1-high (PD1^{hi}), PD1-intermediate (PD1^{int}) and PD1-negative (PD1^{neg}) fractions co-expressing TIM3 or LAG3. (B and D) Scatter plots representing percentages of the four PD1/TIM3 and PD1/LAG3-expressing CD8$^+$ and CD4$^+$Foxp3$^-$ TIL populations in *Foxp3*$^{Cre\text{-}YFP}$*Ebi3*$^{L/L}$ mice and *Foxp3*$^{Cre\text{-}YFP}$ mice. Data represents 3 independent experiments. Error bars represent SEM; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, non-significant (Unpaired Student's t-test).

Supplementary Materials and Methods

Cell lines

B16-F10 cells (referred to as B16 throughout the manuscript) were purchased from ATCC (Manassas, Virginia). MC38 cells were obtained from J.P. Allison (Memorial Sloan-Kettering Cancer Center, New York), and B16-OVA cells were obtained from Greg Delgoffe (University of Pittsburgh).

Generation of *Ebi3*^{Tom} and *Ebi3*$^{Tom.L/L.Thy1.1}$ mice

Both *Ebi3*^{Tom} and *Ebi3*$^{Tom.L/L.Thy1.1}$ targeting constructs were generated using standard recombineering methods (Liu et al., 2003). Initially, 18.3-kilobases of the Ebi3 locus were retrieved from a BAC plasmid (BMQ386N2) and TdTomato cDNA with a 2A peptide linker (Szymczak et al., 2004) was inserted after the ATG start codon of *Ebi3* along with a Loxp-Neo-Loxp cassette and a *HindIII* restriction site (inserted into the 1st intron of the retrieved *Ebi3* locus). The resulting vector was used for the *Ebi3*^{Tom} targeting. For the *Ebi3*$^{Tom.L/L.Thy1.1}$ targeting vector, the Neo was removed via Cre mediated recombination leaving a single Loxp, then an *En2* splice acceptor followed by Thy1.1 cDNA (without a signal sequence), an SV40 late polyadenylation signal sequence and a *SpeI* restriction site together with a Frt-Neo-Frt-Loxp cassette was recombineered into the *Ebi3* locus following the last exon of *Ebi3*. The linearized targeting constructs were electroporated into JM8.N4 embryonic stem cells and neomycin-resistant clones were screened by Southern blot analysis using *HindIII* and *SpeI* digestions for the 5' and 3' ends, respectively. Clones that were correctly targeted and greater than 85% normal diploid by karyotype analysis were injected into C57BL/6 blastocysts. Chimeric mice were mated to C57BL/6 mice and transmission of the targeted allele verified by PCR. The mice were crossed with EIIA.Cre (*Ebi3*^{Tom}) and actin flipase (*Ebi3*$^{Tom.L/L.Thy1.1}$) mice to remove the Neo cassette.

Flow cytometry

Tissues were processed, single cell suspensions prepared and cells were stained. 5% NMS (normal mouse serum) and vital dye (Live/Dead Fixable Aqua Dead Cell stain, Invitrogen) were included in all staining panels. Cells were stained with fluorescent-labeled antibodies, most of which were obtained from BioLegend, except few from eBioscience. The following clones were used: BrDU (3D4), CD4 (GK1.5), CD8a (53-6.7), CD11c (N418), CD25 (PC61), CD44 (IM7), CD45R/B220 (RA3-6B2), CD62L (MEL-14), CD69 (H1.2F3), IFNγ (XMG1.2), TNFα (MP6-XT22), IL-2 (JES6-5H4), Ebi3 (V1.4C4.29), CD279/PD-1 (RMP1-30), TIM-3 (RMT3-23), CD223/LAG-3 (C9B7W). Pentamer staining was performed using H2-K^b–restricted $TRP2_{180-188}$ (SVYDFFVWL) from ProImmune (Sarasota, Florida). For intracellular cytokine staining, cells were activated with phorbol 12-myristate 13-acetate (PMA; 100 ng/mL) plus ionomycin (500 ng/mL) as positive control, or with PepMix™ antigen-specific overlapping peptide pools corresponding to mouse tyrosinase (Tyr) and tyrosinase-related protein 2 (Trp-2) antigens for 12–16 hours (JPT, Acton, Massachusetts). Brefeldin A was added in the final 6 hours of culture. Cells were processed with BD Cytofix/Cytoperm kit. For BrdU labeling, mice were injected with BrdU in 0.2 mL sterile PBS (100 mg/kg body weight) i.p. 18 hours before harvest. Immunostaining for Ki-67 and BrdU was performed using eBioscience Foxp3 staining and BD Cytofix/Cytoperm kits. All flow cytometry data was acquired on an LSRII and Fortessa (BD Biosciences) and analyzed by FlowJo (Treestar, Inc.). Pie charts were created using Prism and SPICE programs (Roederer et al., 2011).

Tumor experiments

Briefly, on day 0, mice were injected with $1.25x10^5$ B16 cells intradermally (i.d.) on the back or $5x10^5$ MC38 cells subcutaneously (s.c) in the right flank. Tumor sizes (mm^2) were measured regularly with digital calipers. For metastatic tumor models, B16.F10 cells were injected intravenously (i.v.) at low dose ($1.25 x10^5$) for IL-35 antibody neutralization experiments and high dose ($2.5 x10^5$) for experiments with floxed mice. After 14–18 days, lungs were harvested,

inflated with H_2O_2 and metastases were counted using a microscope. The maximum tumor size permitted by our IACUC approved animal protocol is 20 mm x 20 mm for B16 and MC38 transplantable tumors and this was not exceeded in this study. Mice that had tumors that reached this maximal size were euthanized and considered moribund.

For memory experiments, the surgery group of mice were injected with $1.25x10^5$ B16 intradermally (i.d.). Tumors were resected at day 14, followed by a re-challenge with B16 intravenous (i.v.) injection 4 or 10 weeks later. The sham group of mice only received the B16 intravenous (i.v.) injection at 4 or 10 weeks. Both the sham and surgery groups received i.p. injections of anti-IL-35 or IgG_{2b} on days 0, 7 and 14. Lung metastases in the sham and surgery groups were assessed 3 weeks post–B16 i.v. injections. For NSCLC experiments, *K-ras*$^{LSL\text{-}G12D/+}$;*p53*$^{L/L}$ mice were inoculated i.t. with $2.5x10^7$ IU Ad-Cre while under avertin anesthesia. Antibody injections (100 µg/week) started 8 weeks later and continued for 10 weeks. Mice were either monitored for long-term survival or euthanized for harvest of lungs and LN at 18 weeks.Lung MRI studies for NSCLC model were performed using a 7 T Bruker ClinScan system (Bruker BioSpin MRI GmbH, Germany) equipped with a 12S gradient coil. A 2-channel surface coil was used for MR imaging. Each specimen was placed in 10% agarose gel for MR data acquisition. 3D T1-weighted Fast Low Angle SHot (FLASH) images were acquired (TR/TE = 500/5.04 ms, FOV = 20.6 x30mm, matrix = 528 x 768, number of acquisition = 32, thickness = 0.07 mm, scan time = 9.5 – 13.1 hr, depending on the number of slices which varied between 64-88). Segmentation of individual lesions and final tumor volume measurements were obtained using OsiriX (Pixmeo, Switzerland).

For adoptive transfer of OT-I TCR transgenic $CD8^+$ T cells, recipient mice were inoculated with $1.25x10^5$ B16-OVA cells i.d. Following development of palpable tumors (days 10–12), B16-OVA-bearing recipients received i.v. injection of $5x10^6$ OT-I $Thy1.1^+$ $CD8^+$ T cells. Transferred OT-I cells were assessed 4 days post injection by staining for Thy1.1 expression.