Supplemental Experimental Procedures

Proliferation assays

For proliferation studies involving mitogens including concanavalin A (ConA, Sigma-Aldrich, St. Louis, MO) or agonistic monoclonal antibodies towards CD3ε (clone Himmunotherapy3a, eBiosciences, San Diego, CA) and CD28 (clone: eBiosciences), stimulants were serially diluted in 96 well round bottom plates and splenocytes or human PBMCs were added in triplicate. For MLRs, splenocytes, human PBMCs, or purified cell populations (responders) were plated in triplicate and serially diluted. Allogeneic splenocytes (stimulators) or human PBMCs were irradiated at 30 Gy prior to plating with responders. Plates were incubated at 37°C, 5% CO_2 and 16-18h prior to harvest, pulsed with 1μCi [^{3}H]-thymidine (Perkin Elmer, Santa Clara, CA). [^{3}H]-thymidine incorporation was analyzed on a Wallac scintillation counter (Wallac. Turku, Finland).

Fresh human samples

For some studies, human T cells were isolated from leukocyte filters obtained from healthy donors (Delta Blood Bank, Stockton, CA). Leukocyte filters were back-flushed with PBS and PBMCs were isolated using density centrifugation. T cells were further isolated from the above samples using RosetteSep™ Human CD4 and CD8$^+$ T cell Enrichment cocktail (STEMCELL Technologies, Vancouver, BC, Canada) per manufacturer's instructions for subsequent sorting of purified T cell subpopulations.

Adoptive transfer of naïve OT-II

CD4 T cells were enriched from the spleens and LNs of 7-8 week old OT-II Tg mice by negative selection using the EasySep mouse CD4 T cell isolation kit (Stem Cell Technologies, Vancouver, British Columbia, Canada). Purified CD4 T cells were then stained with surface markers and sorted using a FACSAria (Becton Dickinson) with the following sorting schema: CD8-CD4+, CD25-, $CD44^{low}$, and CD62L+. Following sorting, cells were rested overnight in complete medium at 37C prior to injection intravenously into resting congenic CD45.1+ C57BL/6 mice. $1\text{-}2x10^6$ cells were injected in 0.2ml of PBS. Treatment with control or immunotherapy regimen was initiated within 1-2 days of adoptive transfer.

Dendritic cell proliferation assays

Dendritic cells were differentiated from congenic CD45.1+ bone marrow cells as previously described[31]. Briefly, bone marrow was aspirated from femurs and tibias and plated at a low density in complete media supplemented with GM-CSF (Peprotech, Rocky Hill, NJ) at 20ng/ml for 6 days. The loosely adherent fraction containing dendritic cells was removed and either pulsed with media supplemented with GM-CSF and 100ug/mL chicken ovalbumin or with GM-CSF alone overnight at 37C. Following overnight OVA pulse, DCs were harvested, counted by trypan blue exclusion, and plated at $0.5x10^6$ per well with purified, CFSE stained, experimental naïve CD4 T cells in a 12 well plate. Concurrently, spleens and lymph nodes were harvested from mice receiving adoptive transfers and processed into single cell suspensions. CD4 T cells were enriched using EasySep mouse CD4 T cell enrichment kits (Stem Cell Technologies). Following enrichment, they were stained with 0.5μM CFSE (Molecular Probes, Life Technologies, San Diego, CA) in PBS for 5 minutes on ice, washed 3 times, and counted using a Coulter Z2 Particle Counter. Following counting, cells were adjusted and $1.5x10^6$ per well were

plated with media alone or OVA pulsed DCs. DCs and CD4 T cells were cocultured for 72hrs, stained for surface markers, and evaluated for proliferation by CFSE dilution.

Flow cytometry

In general, 10^6 cells for surface only stains or $2x10^6$ cells for stains investigating intracellular antigens were stained in round bottom 96 well plates. Surface antibodies were diluted with staining buffer (1% FBS, 1mM EDTA, and 0.02% NaN_3 in PBS) into cocktails containing Fc block (purified anti-mouse CD16/32, BD Pharmingen, San Diego, CA) and added to cells at 45µl per sample. Cells were washed and resuspended in staining buffer for ananlysis within 24 hours. Some stains utilized PhosFlow® (Becton Dickinson, San Jose, CA) per manufacturer's instructions. Briefly, cells were fixed at 37°C with 4% formaldhyde (Sigma Aldrich) in PBS for 10 minutes. Cells were then washed in staining buffer and permeabilized using Perm Buffer III (BD) for 30 minutes at 4°C or overnight at -20°C, washed 3 times in staining buffer, and stained for appropriate intracellular phosphorylated proteins for 1 hour at room temperature prior to analysis. Data were collected using a BD Fortessa instrument running FACS DIVA software. Data were analyzed using FlowJo v10 (TreeStar, Ashland, OR).

FACS-sorting

For sorting experiments, cells were stained for surface markers per protocol described above in sorting buffer (2% FBS, 1mM EDTA, 1X Penn/Strep) for 20 minutes at 4°C. Following surface staining, cells were washed, diluted to $4x10^7$ per ml and filtered through 35µm filter top tubes (BD Falcon) to ensure that cells were in single cell suspensions. Sorting was performed using a

FACS Aria II sorter (Becton Dickinson) with a 70um nozzle running FACS DIVA software (Becton Dickinson).

Antibodies for flow cytometry

All antibodies were purchased commercially and tested for optimal dilution in house based on lot, clone, and vendor. Antibodies purchased from BD Pharmingen included purified anti-mouse CD16/32, fluorescin isothiocyanate (FITC) conjugated anti-mouse CD4, phycoerythrin (PE) conjugated anti-mouse pSTAT5, Vα2, CD44, PD-1,and CD25, allophycocyannin (APC)-Cy7 conjugated anti-mouse CD25, PE-Cy5 conjugated anti-mouse CD44. Antibodies purchased from eBiosciences included FITC conjugated anti-mouse PD-1, PE conjugated anti-mouse Tim3, PE-Cy7 conjugated anti-mouse CD62L and NKG2D, PE-Cy5 conjugated anti-mouse CD62L, and PerCP-eFluor710 conjugated anti-mouse KLRG1. Antibodies purchased from Biolegend (San Diego, CA) include pacific blue conjugated anti-mouse CD44 and CD45.2, brilliant violet (BV) 605 conjugated anti-mouse CD8, BV711 conjugated anti-mouse CD4, BV785 conjugated anti-mouse CD3, FITC conjugated anti-mouse CD28, PE conjugated anti-mouse CD27, and alexa fluor (AF) 647 conjugated anti-mouse CD127.

RNA isolation and cDNA synthesis

Total RNA from mouse tissues was isolated using RNeasy mini kit (SABiosciences Qiagen, Valencia, CA) with mini spin columns. A minimum of 1 pg and a maximum of 1 ug of total RNA was reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, Life Technologies, Grand Island, NY) containing RT buffer, a random primer, reverse transcriptase and RNAse inhibitor. RNA was transcribed for 60 min at 37°C, for 5 min at

95°C, and then for 5 min at 4°C using AB Veriti Therma Cyler (Applied Biosystems) with 96-Well Aluminum Sample Block Module.

Microarray gene expression profiling

Microarray gene expression profiling was performed by whole-transcript analysis on Affymetrix GeneChip Mouse Gene 2.0 Sense Target (ST) Arrays. Biotinylated sense strand DNA targets were prepared from 100 ng total RNA using the Ambion WT Expression and Affymetrix GeneChip WT Terminal Labeling Kits according to the standard manufacturers' protocols. All downstream microarray processing procedures, including hybridization, washing, staining, and array scanning were performed according to manufacturer's instructions.

Microarray data analysis

Microarray data analysis was performed with GeneSpring GX analysis software (Agilent Technologies). Microarray probe intensity values (CEL files) were background-corrected, summarized, and normalized using the Robust Multi-array Average (RMA16) algorithm [32] and subsequently filtered on raw intensity for data in the upper 20-100th percentile. Comparison analysis was performed to identify genes that were differentially expressed (≥1.5-fold) in different CD4 and CD8 populations from experimental and control (PBS-treated) mice. Biological interpretation of the resulting gene list was performed using the functional annotation and clustering tools available at the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources 6.7 [33,34].

Quantitative Real Time PCR (qRT-PCR)

Quantitative real-time PCR was performed using AB Step-ONE Plus (Applied Biosystems) in the presence of SYBR Green Supermix (Applied Biosystems). Samples were run in triplicate. Primers for murine and human SOCS1, SOCS3, CISh, β-Actin, and GAPDH were purchased from Supperarray Qiagen (SABiosciences Qiagen). mRNA levels were calculated using the comparative threshold cycle method (C_t). Threshold cycle (C_t) values for the housekeeping gene (GAPDH) and for the genes of interest were determined and the difference between the C_t values of each gene of interest and the average GAPDH Ct was calculated (ΔC_t). Differences in ΔC_t ($\Delta\Delta C_t$) of genes of interest in treatment groups were normalized to indicated groups as shown in the equation below.

$$\Delta\Delta C_t = \Delta C_t(Sample) - \Delta C_t(average\ of\ control\ group)$$

RT-PCR data are presented as fold change expression = $2^{-\Delta\Delta Ct}$.

Enzyme-linked immunosorbent assay (ELISA) for SOCS3

Equal numbers of cells ($0.5\text{-}2x10^6$) were prepped for use in ELISA kits to quantify SOCS3 expression. ELISA kits for quantification of murine (Blue Gene Biotech, Shanghai, China) or human SOCS3 (Blue Gene Biotech) were performed per manufacturer's instructions.

In vivo vaccination with allogeneic stimulus

For *in vivo* vaccination studies, BALB/c mice were injected intraperitoneally with $5\text{-}10x10^6$ C57BL/6 splenocytes which were irradiated at 30Gy prior to injection on day 2 of immunotherapy. At day 35 post initiation of therapy, mice were harvested and analyzed for secondary responses. Secondary MLR assays were described in tritium assay section. CD8 recall responses involved restimulation of splenocytes with 30Gy irradiated allogeneic C57BL/6

splenocytes. After 4 days, restimulated T cells were subjected to a chromium release assay against allogeneic tumor cells. Briefly, C1498 (ATCC) were pulsed with [^{51}Cr] (Perkin Elmer) at 100uCi/10^6 cells at 37°C for 60 minutes. Restimulated T cells were then plated with [51Cr] labeled C1498 cells at indicated E:T ratios and incubated at 37°C for 4 hours. Supernatants were removed, mixed 1:1 with scintillation fluid, and analyzed on a Wallac scintillation counter (Wallac). Maximum release was determined by adding 100 μL of 1X-Triton X-100 detergent (Sigma-Aldrich) to target cells. Spontaneous release was determined by adding 100 μl of complete media to 100 μl of target cells. Specific ^{51}Cr release was calculated as: % lysis =100% x (Experimental-Spontaneous)/(Maximum–Spontaneous).

MCMV infection

MCMV Smith strain was obtained from American Type Culture Collection (Manassas, VA) and maintained by repeated salivary gland passage. MCMV (5×10^4 plaque-forming units) was administered intraperitoneally in 0.2ml RPMI (Gibco Laboratories) 1 day following initiation of high dose IL-2 regimen in C57BL/6 mice. Some groups were depleted of NK cells using anti-NK1.1 and/or CD4+ cells were depleted using anti-CD4 as indicated three days prior to, day of, and weekly post initiation of immunotherapy. Quantification of MCMV from DNA extracted from salivary glands using real-time–polymerase chain reaction was performed as previously described[35].

EAE induction model

EAE was induced as previously described[36]. Briefly, MOG peptide-EAE was induced by subcutaneous flank administration of 300 μg of rodent MOG peptide (amino acids 35–55, New

England Peptides) in CFA containing 5 mg/ml killed *Mycobacterium tuberculosis* (Difco) on day 2, with intraperitoneal administration of 75 ng of pertussis toxin on days 2 and 4 (days are post initiation of IT). Mice were weighed and examined daily. Neurological deficits were graded on a five-point scale (limp tail or waddling gait = 1; limp tail and waddling gait = 2; single limb paresis and ataxia 2.5; double limb paresis = 3; single limb paralysis and paresis of second limb = 3.5; full paralysis of 2 limbs = 4; moribund = 4.5; and death = 5)

Figure S1

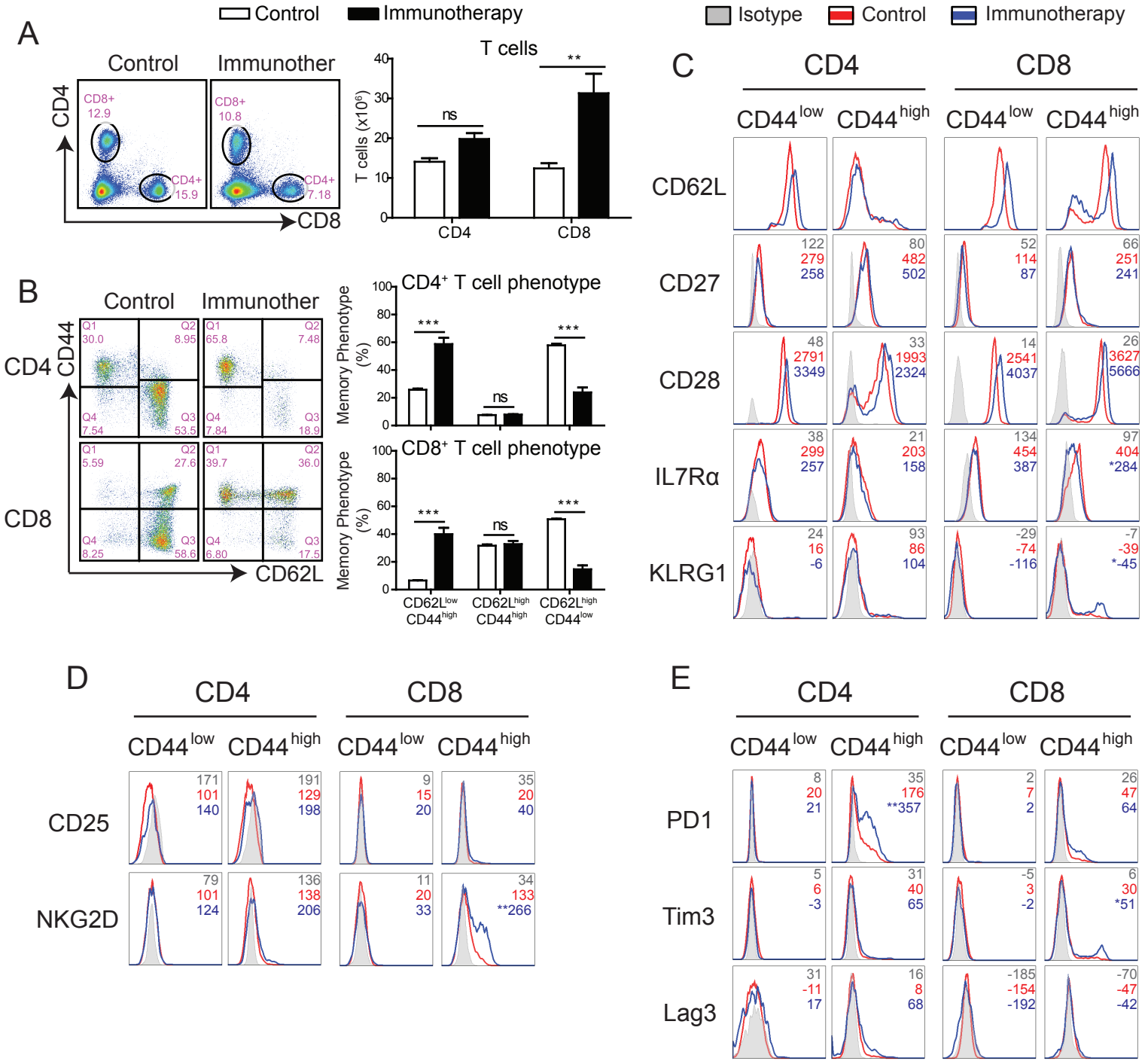

Figure S1 (related to Figure 1): *Characterization of CD4 and CD8 T cells following immunotherapy.* C57BL/6 mice (n=3 per group) were treated with control (rIgG/PBS) or immunotherapy (anti-CD40+IL-2) regimen. On day 12 of treatment, splenocytes were harvested into single cell suspensions and stained with various surface markers to evaluate T cell phenotype by flow cytometry. A) Representative dot plots (left panel) and corresponding absolute numbers (right panel) of CD4 and CD8 T cells from control and immunotherapy treated mice. B) Representative dot plots (left panel) and quantified frequencies (right panel) of different memory subsets based on CD44 and CD62L in $CD4^+$ (top panels) and $CD8^+$ (bottom panels) T cells in control and immunotherapy treated mice. Representative histograms of various C) memory, D) activation, and E) inhibitory markers in memory ($CD44^{high}$) and naïve ($CD44^{low}$), $CD4^+$ and $CD8^+$ T cells. Bar graphs indicate mean±SEM. Statistical analysis was performed by Two-way ANOVA with Bonferoni's post-test; *p<0.05, **p<0.01, ***p<0.001. For histograms, statistical significance is denoted by * if either MFI (shown on graph) or percentage (not shown on graph) or ** if both are significantly different in the immunotherapy group compared to control. These data are representative of 5-6 independent experiments.

Figure S2

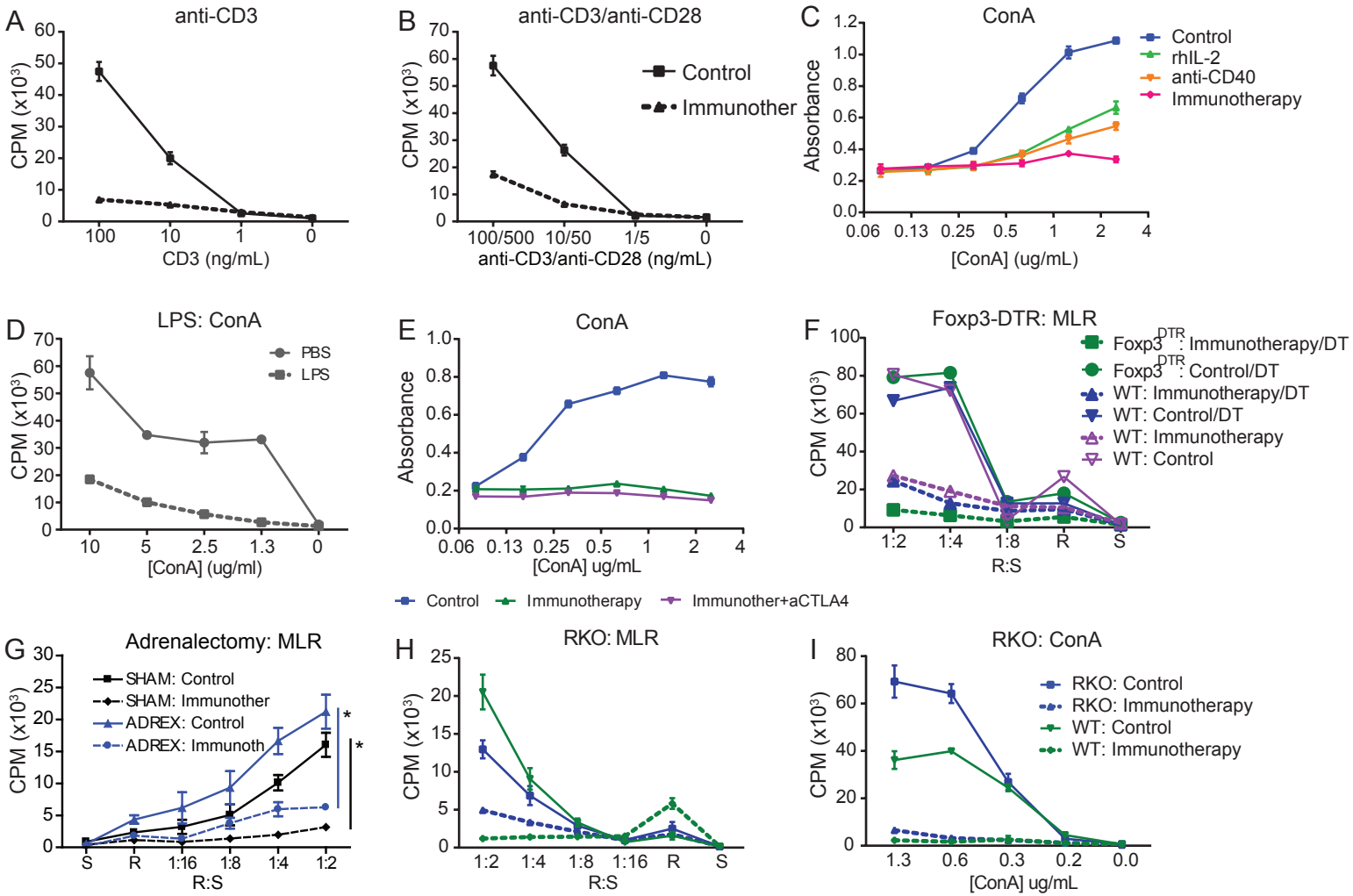

Figure S2 (related to Figure 1): *Persistence of paralysis through multiple regimens and in the absence of multiple inhibitors.* BALB/c (n=3-4 mice per group), C57BL/6 (n=2-3 mice per group), Foxp3-DTR (n=2 mice per group), or IFN-γ receptor KO (RKO, n=2-3 mice per group) mice were treated with control (rIgG/PBS), rhIL-2 alone (High dose IL-2), agonistic anti-CD40 alone, or immunotherapy (anti-CD40+IL-2) combination regimen. In some studies mice were adrenalectomized (or sham for controls) prior to initiation of therapy. In some studies, mice received anti-CTLA4 blockade or diphtheria toxin in vivo during treatment regimen. On day 2 (F-G), 7 (D), 11 (C, E) or 12 (A-B, H-I) of treatment, splenocytes were harvested into single cell suspensions and analyzed for proliferative responses by MTT assay (C, E) or [3H]-thymidine following indicated stimulation (A-B, D, F-I). (A-B) Proliferation in response to anti-CD3 or anti-CD3/anti-CD28 in C57BL/6 mice following indicated treatment regimens. (C-D) Proliferation in response to ConA in BALB/c mice following indicated treatment regimens. Proliferation in response to MLR in (F) the absence of Tregs or (G) adrenactomized mice following immunotherapy. Proliferation in response to (H) MLR or (I) ConA in wild-type (WT) or RKO mice. All graphs indicate mean±SEM. Statistical analysis was performed using Two-way ANOVA with Bonferroni's post-test; *p<0.05, **p<0.01, ***p<0.001. These data are representative of 1-2 independent experiments.

Figure S3

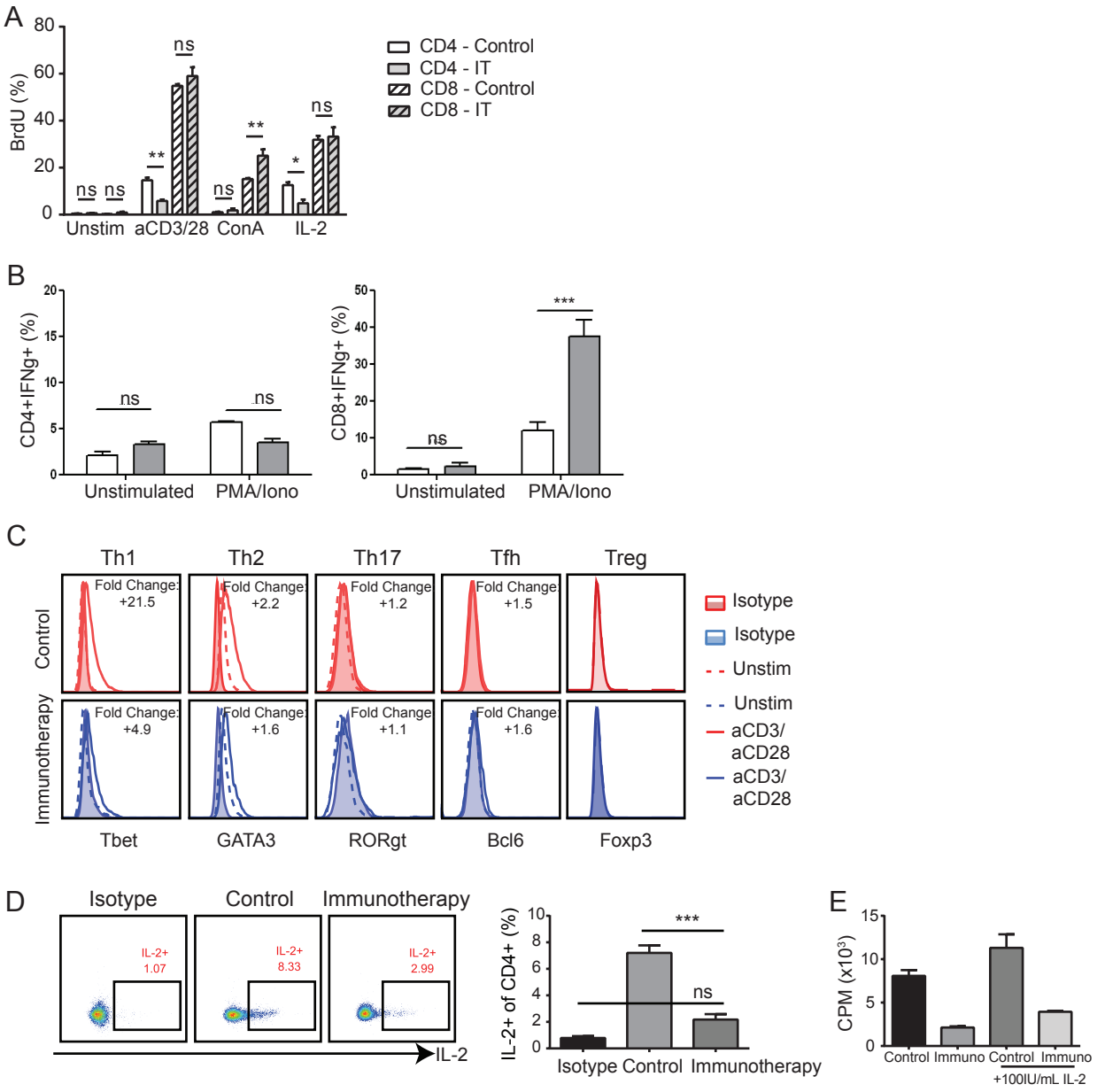

Figure S3 (related to Figure 1): *Impaired cytokine production and T cell polarization following ex vivo restimulation.* Mice were treated with immunotherapy and restimulated ex vivo with indicated reagents. (A) Proliferation as measured by BrdU incorporation ex vivo following 48hr restimulation. (B) Intracellular cytokine production following PMA+ionomycin restimulation. (C) Representative histograms depicting T cell transcription factor expression following 24hr anti-CD3+anti-CD28 stimulation. Fold change is over control (dotted lines) median fluorescence intensity (MedFI). (D) Representative dot plots (left) and bar graph (right) depicting IL-2 expression on $CD4^+$ T cells following 48hr anti-CD3+anti-CD28 stimulation. (E) Naïve $CD4^+$ T cells were flow cytometry sorted from control or immunotherapy treated mice and subjected to MLR with or without supplementation of 100IU/mL IL-2. Statical analysis was performed using Two way ANOVA where appropriate. *p<0.05, **p<0.001, ***p<0.001.

Figure S4

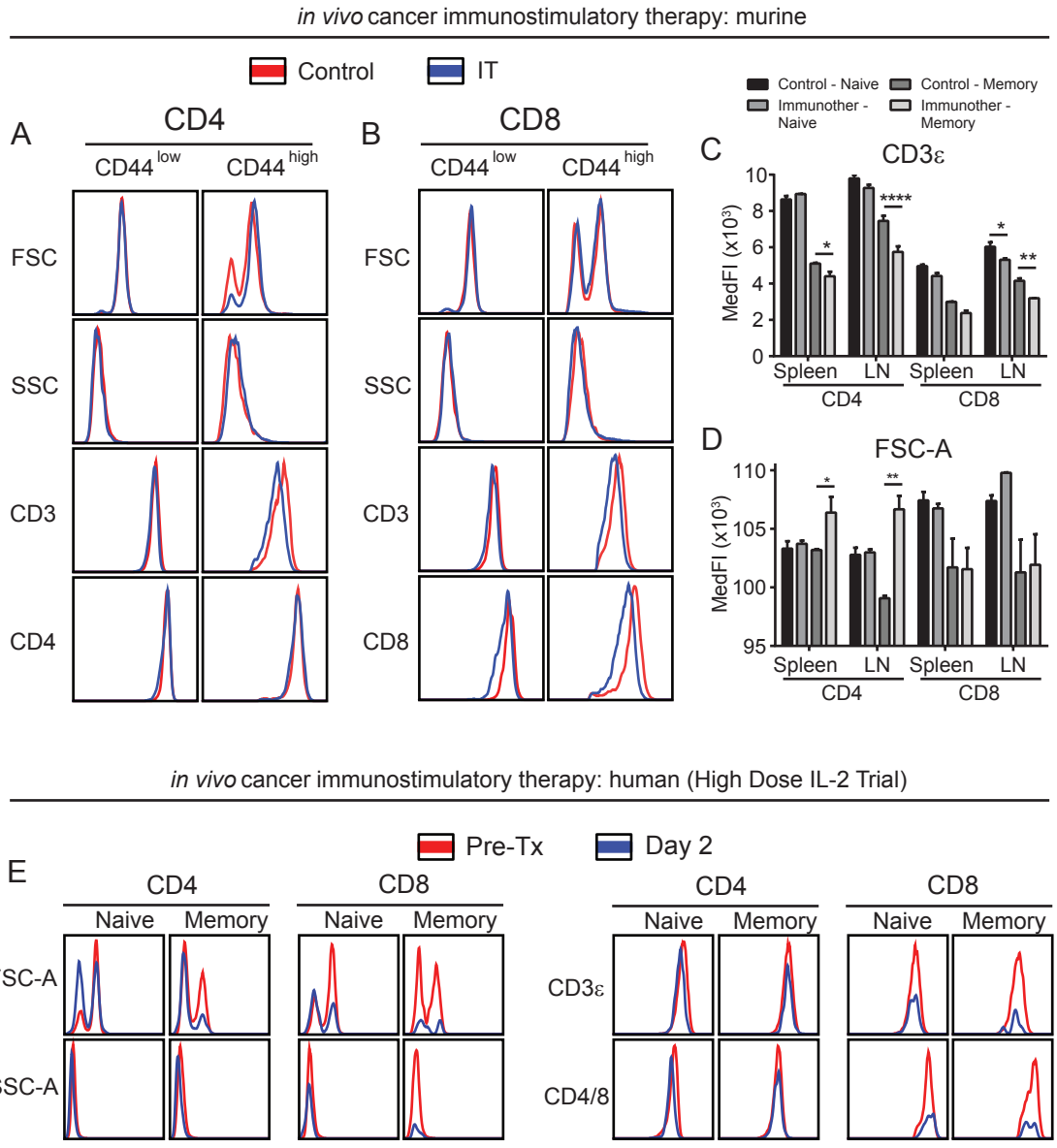

Figure S4 (related to Figure 1): *T cell size, granularity, and TCR expression in murine and human T cells following immunostimulatory therapy in vivo*. (A) Representative histograms of FSC, SSC, CD3, CD4, and CD8 expression in memory and naïve (A) $CD4^+$ and (B) $CD8^+$ T cells on day 12 of immunotherapy in mice. Median fluorescence intensity (MedFI) of (C) CD3 and (D) FSC in various T cell subpopulations in mice. (E) Representative histograms depicting size (FSC), granularity (SSC) and expression of CD3e and CD4 or CD8 on T cell subsets in humans. All graphs indicate mean±SEM. Statistical analysis was performed using Two-way ANOVA with Bonferroni's post-test; *p<0.05, **p<0.01, ***p<0.001. These data are representative of 2 independent experiments.

Figure S5

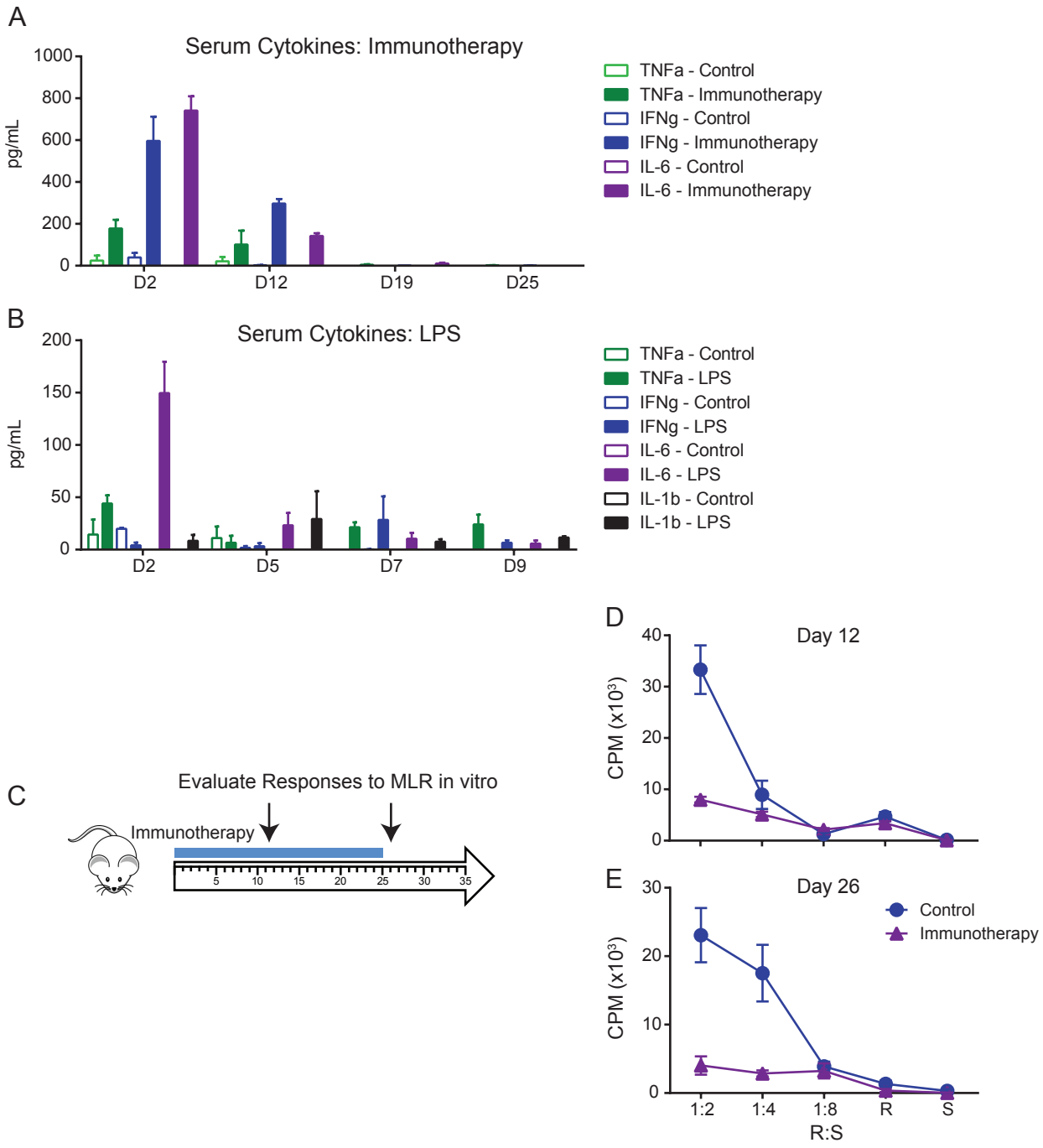

Figure S5 (related to Figures 2 and 3): *Systemic cytokine storm during inflammatory models.* Quantification of serum cytokines at indicated time points during (A) Immunotherapy (anti-CD40+IL-2) or (B) following systemic LPS administration. (C) Schema depicting treatment regimen. Mice were treated with 2 rounds of immunotherapy (anti-CD40+IL-2) treatment with the first round beginning on day 0 and the second round beginning on day 14. MLR responses from splenocytes harvested on (D) day 12 (post 1st round) or (E) day 26 (post 2nd round).

Figure S6

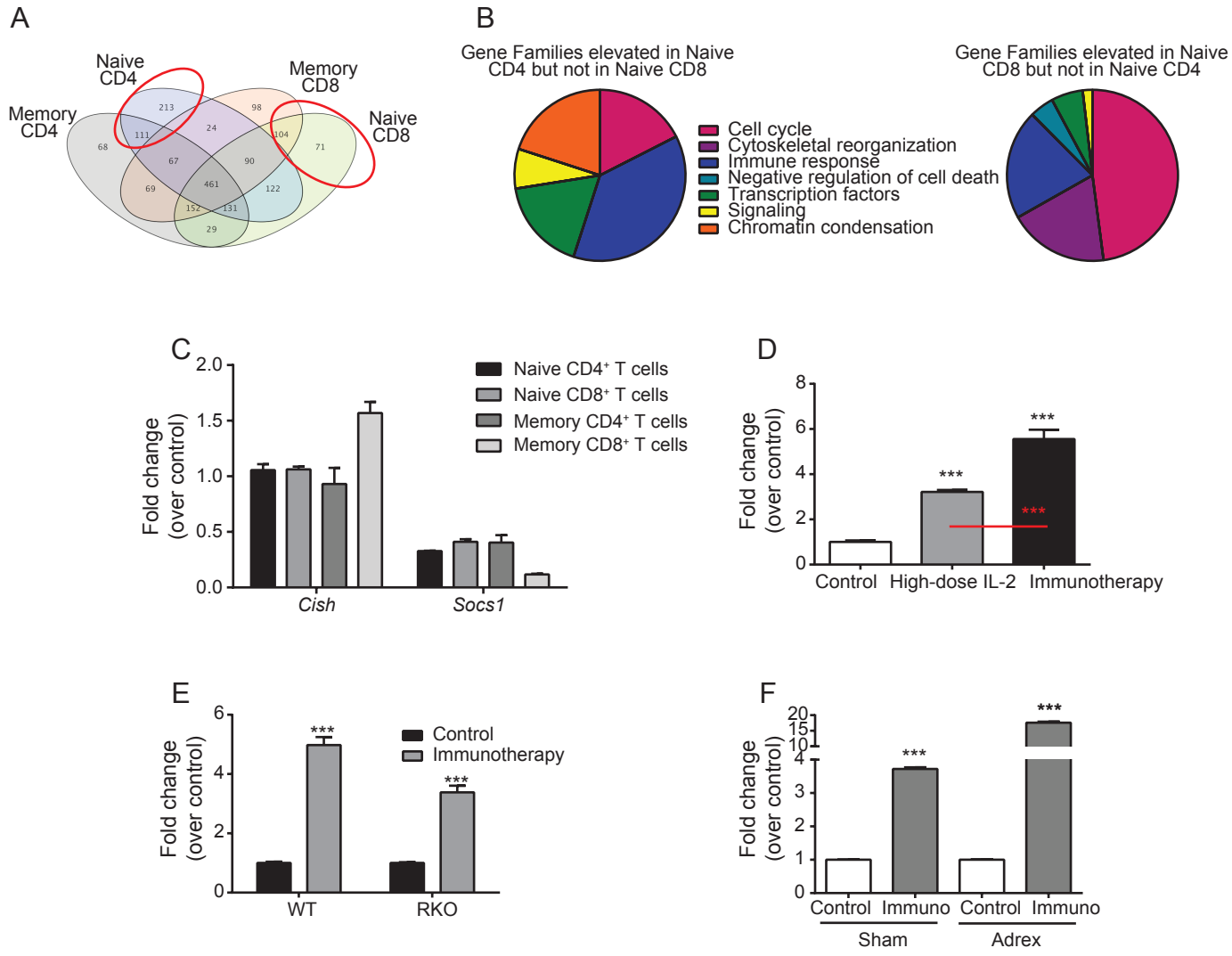

Figure S6 (related to Figure 6): *Differential gene family expression profiles in naïve T cells following immunotherapy*. Naïve and memory CD4 T cell were sorted from control or immunotherapy treated mice. RNA was isolated from sorted populations and subjected to microarray analysis. Each immunotherapy treated population was compared to the corresponding control population to determine fold change values. (A) Venn diagram depicting the number of genes differentially expressed in naïve CD4+ and CD8+ T cells following immunotherapy compared to controls. (B) Gene ontology (GO) analysis was performed on differentially expressed genes using the DAVID database. Genes were annotated into groups based on function and those that clustered significantly were summarized and depicted as pie charts. (C) Expression of SOCS family members *Socs1* and *Cish* by qPCR in flow cytometry-sorted T cell populations on day 5 of immunotherapy. *Socs3* expression in flow cytometry-sorted naïve CD4+ T cells (D) on day 12 of high dose IL-2 or immunotherapy, (E) from WT or IFNgR KO mice on day 12 of immunotherapy, or (F) from Sham or Adrex, control or immunotherapy treated mice on day 2 of immunotherapy. All graphs indicate mean±SEM. Statistical analysis was performed using One or Two-way ANOVA with Bonferroni's post-test where appropriate; *p<0.05, **p<0.01, ***p<0.001. These data are representative of 1-2 independent experiments.

Figure S7

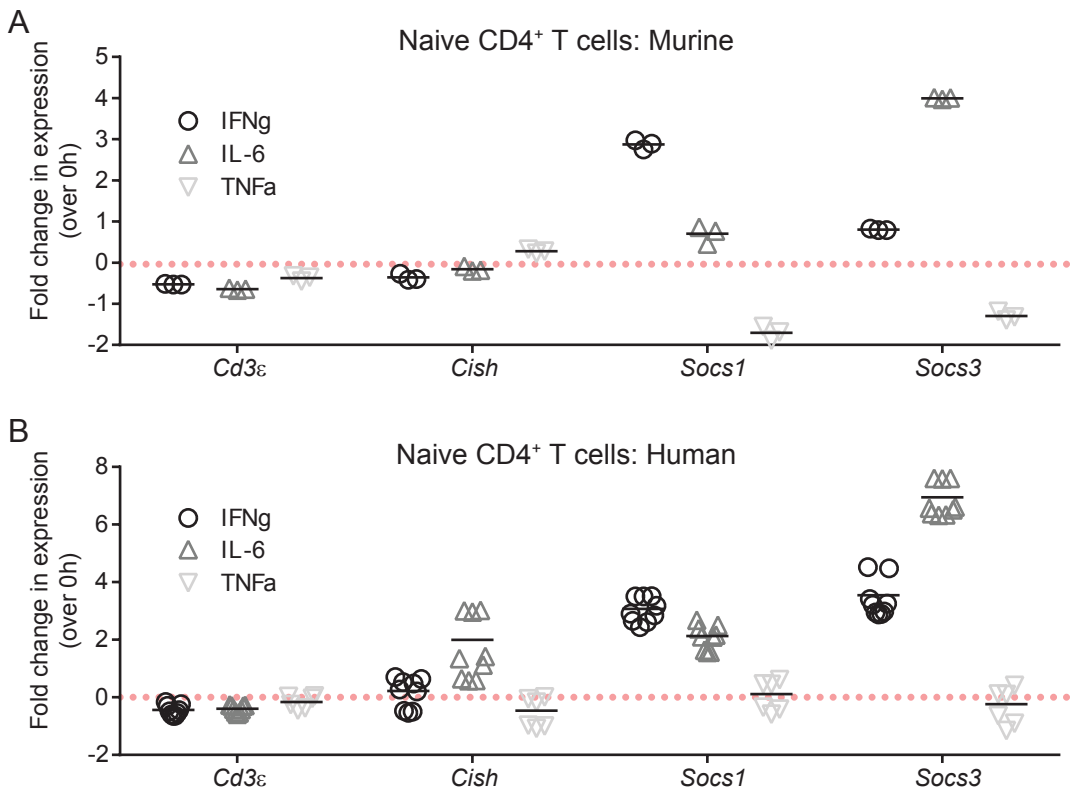

Figure S7 (related to Figure 6): *Induction of SOCS molecules in naïve CD4⁺ T cells following cytokine incubation in vitro.* Quantitative PCR analysis of SOCS family expression in sorted (A) murine or (B) human naïve CD4⁺ T cells following stimulation with indicated cytokines.