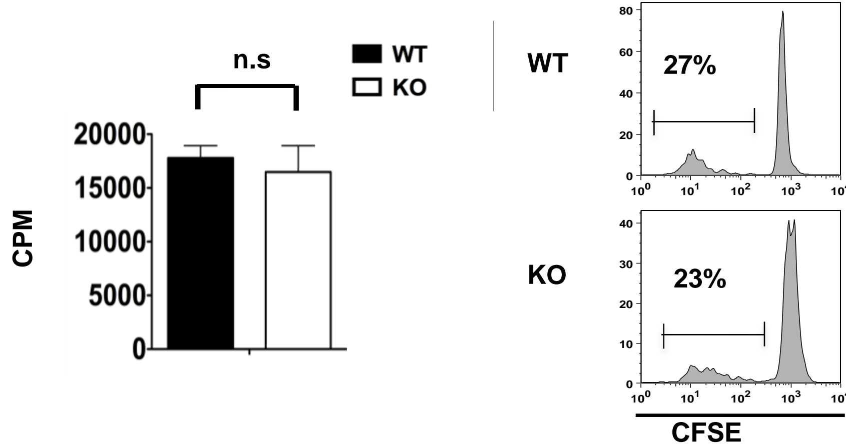
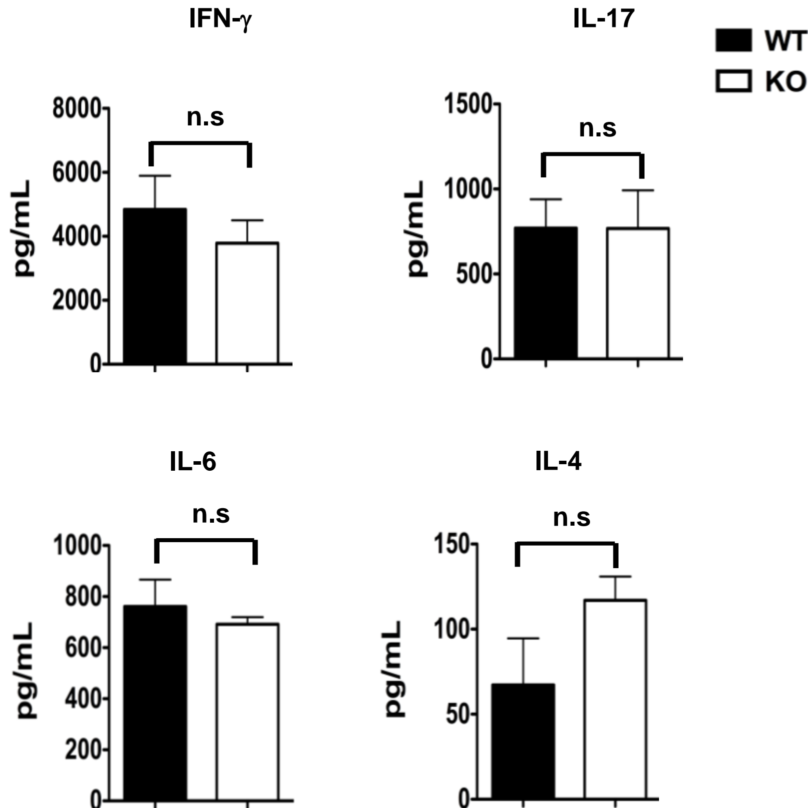
SUPPLEMENTAL INFORMATION

1. SUPPLEMENTAL FIGURES

SUPPLEMENTAL FIGURE 1.

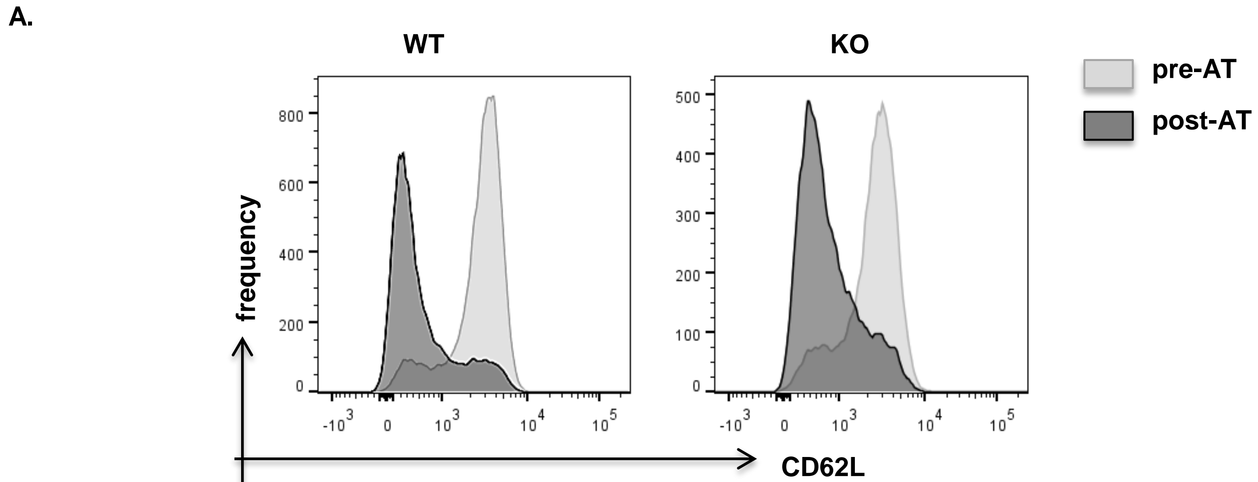




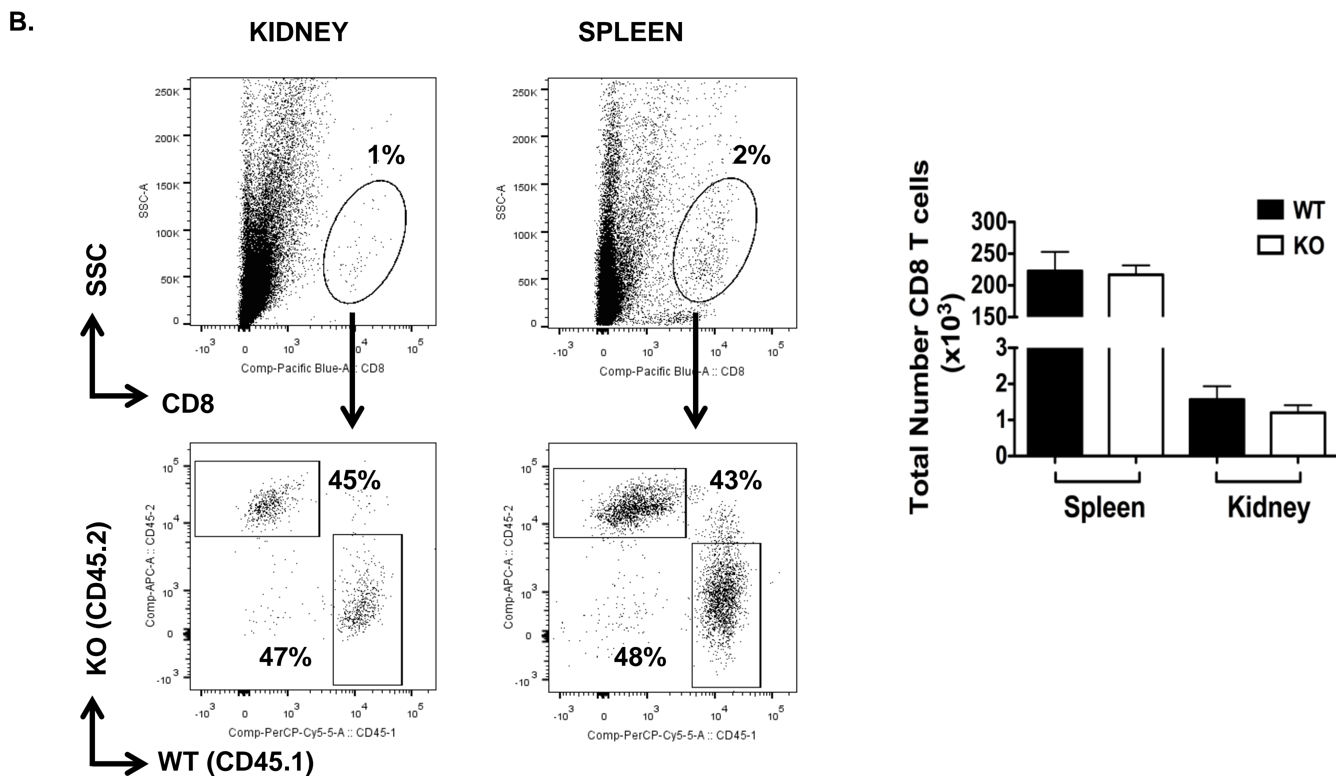
Supplemental Figure 1. Naïve T cells from WT or MyD88$^{-/-}$ recipients display similar proliferation potential and cytokine profiles in donor-stimulated MLRs. (A) MLRs were set up using T cells isolated from the spleens of naïve (untransplanted) WT or MyD88$^{-/-}$BALB/c mice. Responding T cells were stimulated with donor B6 APCs. Proliferation was assessed by [^{3}H] (left) as described above or by CFSE dilution (right). (B) Culture supernatants from the MLR were collected immediately prior to thymidine pulse and analyzed for the presence of IFN-γ, IL-17, IL-6, and IL-4 by Luminex bead assay.

SUPPLEMENTAL FIGURE 2.

A.

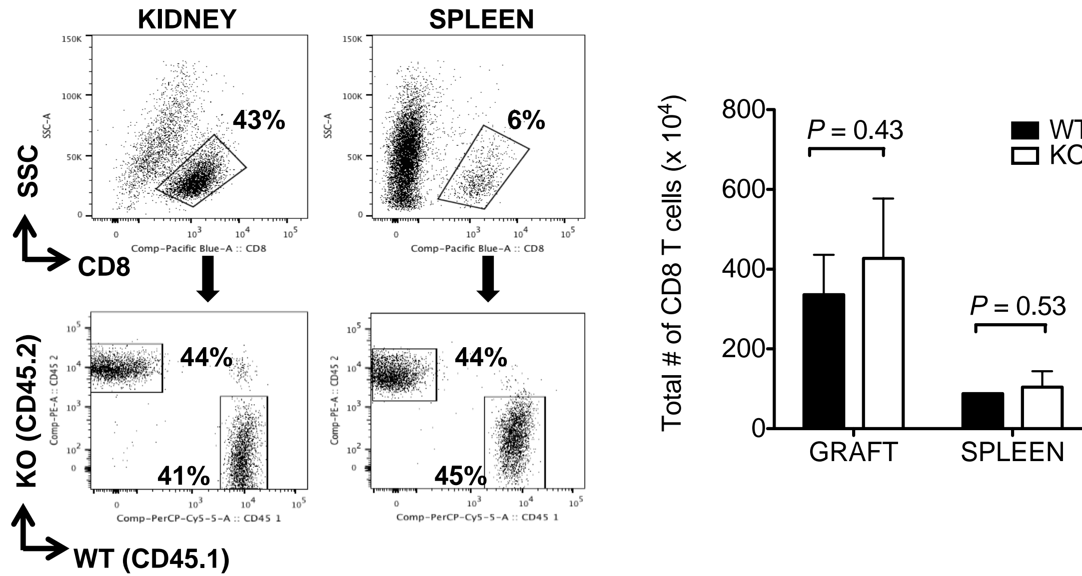


B.



Supplemental Figure 2. CD8 T cells from WT or MyD88$^{-/-}$ mice display equally down-regulated CD62L expression and accumulate in equal numbers in response to homeostatic proliferation. CFSE labeled congenic CD8 T cells derived from naïve WT (CD45.1, n=3) and MyD88$^{-/-}$ (CD45.2, n=3) mice were adoptively transferred into RAG$^{-/-}$ mice ($5x10^6$ WT and $5x10^6$ MyD88$^{-/-}$ cells/mouse i.v). 10 days post adoptive transfer, spleens and kidneys were harvested and analyzed via flow cytometry for (A) CD62L expression on the CD8 T cells prior to adoptive transfer (pre-AT) and 10 days post adoptive transfer (post-AT), and (B) The percentage and absolute number of congenically marked donor CD8 T cells in the native kidney and the spleen.

SUPPLEMENTAL FIGURE 3.



Supplemental Figure 3. CD8 T cells from MyD88$^{-/-}$ mice were recruited into the kidney allograft. RAG$^{-/-}$ mice on a BALB/c background (CD45.2^{+}) received kidney allografts from B6 donor mice. The mice were allowed to recover for 14 days before CFSE-labeled naïve BALB/c WT (CD45.1^{+}) and MyD88$^{-/-}$ (CD45.2^{+}) CD8 T cells were cotransferred at a 1:1 ratio (5x10^{6} WT and 5x10^{6} MyD88$^{-/-}$ cells/mouse i.v). Seven days post adoptive transfer, the kidney grafts and spleens were harvested and the percentage (representative dot plots on left) and absolute number (bar graph on right) of congenically marked WT and MyD88$^{-/-}$ CD8 T cells were determined by flow cytometry. Results are representative of three independent experiments. For each histogram, cells were first gated on total CD8 T cells followed by gating of the specific congenic markers CD45.1^{+} (WT) or CD45.2^{+} (MyD88$^{-/-}$). Results are representative of three independent experiments.

2. COMPLETE METHODS

Mice

Male BALB/c ($H2^d$), B6 ($H2^b$) donor, C3H ($H2^k$) and SJL ($H\text{-}2^s$) were purchased from Jackson Laboratories (Bar Harber, ME). $MyD88^{-/-}$ on the BALB/c background originally obtained from Dr. Shizuo Akira, M.D., Ph.D. (Osaka University, Osaka, Japan) and were bred in the animal care facility of Northwestern University. Breeders of CD45.1/Ly5.1 and $RAG\text{-}2^{-/-}$, both on the BALB/c background, were purchased from the Jackson Laboratories and bred in house. Male mice aged 10-14 weeks were used in all experiments. All mice were used according to protocols approved by the Internal Animal Care and Use Committee of Northwestern University.

Kidney Transplantation

Kidneys from wild-type (WT) B6 donor mice were transplanted into binephrectomized WT BALB/c or MyD88 deficient ($MyD88^{-/-}$) recipients, respectively, following the procedures previously described.[50] Briefly, the left kidney along with its vessels and ureter and bladder patch were removed *en bloc*. The donor aorta and inferior vena cava were then anastomosed end to side to the recipient abdominal aorta and inferior vena cava below the level of the native renal vessels, respectively. The donor bladder patch was anastomosed dome to dome to the bladder of the recipient. The native right kidney was removed before revascularization while the recipients' left kidney was removed immediately prior to the wound closure; therefore recipients' survival is depending upon the transplanted kidney. No immunosuppressive therapy was administered.

Assessment of renal graft function and rejection

Recipients were monitored daily for survival and renal function. Renal function was assessed by measuring whole blood creatinine (Cr) levels by using I-STAT handset analyzer (Abaxis, United City, CA). The endpoint of study was defined as >90 days of survival, recipient death, or when recipients developed clinical signs of renal failure or severe rejection, including deterioration of general health condition (weight loss; reduced spontaneous activity; piloerection and hunched posture) and increased Cr level (>90umol/l). Tissues and blood samples were collected at the end of the study for renal function and pathohistological examinations. Additional transplants were euthanized at pre-selected earlier time points for sequential functional, immunological and pathohistological analysis. Recipients that died of surgical complications within 4 days after transplantation surgery were considered as technical failure and were excluded from the study.

Skin Transplantation

Full-thickness tail skin grafts (from B6 donor or 3rd party SJL mice) were placed on the backs of MyD88$^{-/-}$ recipients that had accepted kidney allografts for ≥ 90 days. Grafts were covered with protective bandages for 7 days. Grafts were examined daily and considered rejected when >80% necrosis was observed.

Cell Purification and Mixed Lymphocyte Reactions

To obtain single cell-suspensions from kidney allografts, kidneys were minced up before being subject to digestion with collagenase IV (Worthington Biochemical

Corporation). The resulting cell suspension was run through a 70μm filter and washed with PBS. After centrifugation the lymphocytes were purified using lymphocyte separation medium (Cellgro). Gradient separation was carried out at 2100 rpm for 20 minutes at room temperature. Cells were washed and filtered once more before being used in the assays as described. Spleen cells were also isolated using standard methods.[51]

Mixed lymphocyte reactions (MLRs) were set up using $1x10^5$ splenic T cells (responders) from $MyD88^{-/-}$ or BALB/c WT mice. To purify T cells, splenocytes from naïve mice were negative selected by exclusion of $CD25^+$, $NK1.1^+$, $Gr\text{-}1^+$, $CD11b^+$, $CD11c^+$ and $B220^+$ cells. The purity of purified T cells for MLRs was > 85%. $5x10^5$ irradiated C57BL/6 (donor) or SJL (3rd party) splenic APCs per well were added to responder T cells and co-cultured for 5 days in complete RPMI-1640 medium with 10% FCS. Alternatively, T cells were activated using anti-CD3/CD28 beads at a 1:1 ratio (DynaBeads Life Technologies). Proliferation was assessed by [^{3}H] thymidine uptake (1mCi per well, PerkinElmer) during the last 18 hours of a 3- or 5-day MLR or by 5,6-carboxyfluoresceine diacetate succinimidyl ester (CFSE) (Molecular Probes, Life Sciences) dilution. In some instances, 10 ng/mL mouse rIL-6 was added on day 0 of co-culture (R&D Systems). For IFN-γ, IL-4, IL-6 and IL-17 cytokine analysis, MLR supernatant was analyzed by Luminex bead assay (Millipore) as described below.

Adoptive Transfer

CD8 T cells from the spleens of un-manipulated congenically marked WT BALB/c (CD45.1 or CD45.2) or $MyD88^{-/-}$ mice (CD45.2) were purified through a MACS column

(Miltenyi) by excluding for $CD25^+$, $NK1.1^+$, $Gr\text{-}1^+$, $CD11b^+$, $CD4^+$, $CD11c^+$ and $B220^+$ cells. The purified BALB/c WT (CD45.1) or $MyD88^{-/-}$ (CD45.2) CD8 T cells (purity>90) were labeled with 5 μM CFSE (Molecular Probes) and were injected into WT BALB/c recipients (CD45.2), $5x10^6$ cells/mouse via the tail vein at 24 hr prior to receiving B6 kidney transplants, respectively. Ten days post-transplantation the recipient spleens and kidneys were harvested and analyzed by flow cytometry. In a separate experiment, 1:1 mixed naïve $MyD88^{-/-}$ (CD45.2) and CD8 WT T cells (CD45.1) were injected into either naïve $RAG\text{-}1^{-/-}$ mice or $RAG\text{-}1^{-/-}$ recipients with kidney transplant at 14 days post-transplant, and analyzed at 10 days post-transplant.

Real Time PCR based Super Array and Heat Map generation

Kidney grafts from WT or $MyD88^{-/-}$ recipients were harvested at 7 or 90 days post-transplant and snap-frozen in liquid nitrogen and stored at -80^oC for RNA analysis. Naïve non-transplanted kidneys were also included as controls. Total RNA was extracted with the RNeasy mini kit (Qiagen). The quality and quantity of the extracted total RNA were assessed using spectrophotometric UV absorbance ratios of 260/280 nm and 260/230 nm, respectively. cDNA was synthesized from DNase-treated RNA by reverse transcription, in accordance with the manufacture's protocol, using first strand cDNA synthesis kits (C-02, Super Array Bioscience Corporation). The delta CT values of the super array data were normalized to the intra-array Hprt1 control gene as this gene has the least variation among the three control genes. Principal component analysis and hierarchical clustering were performed to assess the relationship among samples and pinpoint potential outlier(s). Differential gene expression between the

treatment conditions was assessed based on a linear model in which the pooled standard error was estimated. Bonferroni-corrected p-values were computed and used to identify differentially expressed genes (p value < 0.05). For the differentially expressed genes between the control group and the other treatment groups, their expressions were visualized with a heatmap using the gene-wise mean delta CT values of the control groups as the reference. Specifically, the differences between the delta values of the genes in all the samples and the gene-wise mean delta CT values of the control groups were calculated and plotted in the heatmap.

Antibodies and flow cytometry

At the indicated times after transplantation, lymphocytes were isolated and cell staining was performed with the specific indicated antibodies (1μg/10^6 cells) at 4°C for 30 min. Cells were analyzed using the FACSCantoII flow cytometer (BD Bioscience). Data was analyzed using FlowJo software (Tree Star Inc.).

Biotinylated mAbs for CD25 (7D4), Ly-76 (Ter-119), CD49b/Pan-NK (DX5), B220 (RA3-682), Gr1 (RB6-8C5), and CD11b (M1/70); PercpCy5.5 conjugated CD45.1 (A20), APC conjugated CD45.2 (104), PE conjugated CD4 (GK1.5), PE-conjugated Gr-1 (RB6-8C5), PE-CY7 conjugated CD11c (N418), and PE-CY7 conjugated CD11a (2D7) were purchased from BD Biosciences. PerCPCy5.5 conjugated CD8α (53-6.7), Pacific Blue conjugated CD4 (GK1.5), APC-eflour-780 conjugated CD11b (M1/70) and PE-CY7 conjugated CD44 (IM7) were all from eBiosciences.

Luminex Assay

Culture supernatants or homogenized tissue was collected and cytokines were quantitated using Liquichip Mouse 22-cytokine assay kit (Millipore). Assays were conducted according to the manufacturer's instructions and analyzed on a LiquiChip 200 (Qiagen). Results were normalized to total protein by Bradford analysis (Bio-Rad).

Histology and Immunohistochemistry

Kidney samples were bisected transversely and placed in phosphate buffered 10% Formalin for 10-12 hrs. The tissues were embedded in melted paraffin using plastic cassettes. The sections were stained with Hemotoxylin & Eosin or PAS or Trichome Masson for morphologic evaluation. The kidney sections were evaluated blindly by a pathologist for the morphologic characteristics of acute (tubulitis, interstitial mononuclear cell infiltration, vasculitis, glomerular hypercellularity) and chronic rejection (interstitial fibrosis, glomerular sclerosis and arterial Intimal hyperplasia). The severity of tubulitis was scored on the PSA staining sections of kidney allografts as 0 (normal) to 4+ (severest), depending upon the number of infiltrating monocytes per tubule in the kidney grafts.

Additional graft tissue was snap frozen in OCT compound with liquid nitrogen. All sections were 5 µm thick and blocked with either 10% donkey or goat serum (Sigma-Aldrich). Sections were stained with anti-mouse CD4 mAb (1:100, rat IgG2a, κ clone H129.19, BD Biosciences), or anti-mouse CD8 (1:100, rat IgG2aκ clone 53–6.7BD Biosciences), followed by goat anti-rat IgG Dylight 594 (1:500, JacksonImmunoResearch). For Gr-1 staining, frozen sections were blocked with Avidin/Biotin blocking kit (Vector Laboratories,) followed by staining with anti-mouse Ly-

6G/Ly-6C (1:2000, Rat IgG2b, clone RB6–8C5, Biolegend). Sections were stained with biotinylated goat anti-rat Ig (1:200, goat Ig clone polyclonal; BD Biosciences). Visualization was accomplished with Vectastain ABC-AP kit and Vector Blue substrate kit (Vector Laboratories).

Statistical Analysis

All data are expressed as the mean ± SD. Comparisons between graft survival times were calculated using Kaplan-Meier survival curves with the log-rank test. Statistical significances between the groups were determined by Wilcoxon nonparametric tests or by an unpaired Student's t-test with significance determined at $P < 0.05$ (***P=≤0.001; **P=<0.01; *P=<0.05). The data representing more than 2 groups was analyzed with one-way ANOVA analysis. Tueky's procedure was used for the post hoc multiple testing adjustment. Tubulitis score was analyzed by Fisher exact test. All analyses were done with GraphPad PRISM 5 software.