Supplemental Material

CD70 Exacerbates Blood Pressure Elevation and Renal Damage in Response to Repeated Hypertensive Stimuli

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Running Head: T cell memory in hypertension

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METHODS

Animals and blood pressure measurement: Wild type, and interferon-gamma deficient mice (IFN-$\gamma^{-/-}$) on a C57Bl/6J background were purchased from Jackson Laboratories (Bar Harbor, Maine). CD70-deficient mice ($CD70^{-/-}$) on a C57Bl/6J background were obtained from Dr. Ross Kedl (University of Colorado). Mice were provided regular chow and water ad libitum. At 12 weeks of age, male mice were randomly selected for treatment. The animals initially received L-NAME (0.5mg/ml, Abcam 120136) in the drinking water for two weeks. LNAME was then stopped and the mice were allowed a two-week washout period, and then fed a high salt diet (4% NaCl, Teklad TD.92034) for three weeks. Other mice received a normal diet, two-weeks of L-NAME, or three weeks of high salt diet as controls. Blood pressure was measured invasively using radio-telemetry as previously described.[1, 2] After telemetry implantation, mice were allowed to recover for 10 days prior to starting L-NAME/high salt protocol. In another model, mice received infusion of ang II (490 ng/kg/min) via osmotic minipumps for two weeks, followed by a two-week washout and then a second infusion of angiotensin II at 140 ng/kg/min.

For adoptive transfer experiments, donor CD45.2 mice underwent the L-NAME/high salt protocol described above and T_{EM} cells were isolated from the bone marrow using cell sorting. Approximately 0.5 X 10^6 cells were then injected retro-orbitally into CD45.1 recipients. One half of these mice were then fed a 4% NaCl diet for the ensuing 3 weeks and the other half remained on normal mouse chow. As an additional control, T_{EM} cells from the bone marrow of CD45.2 mice fed a normal diet were transferred to CD45.1 recipients and these recipients were fed a high salt diet for 3 weeks.

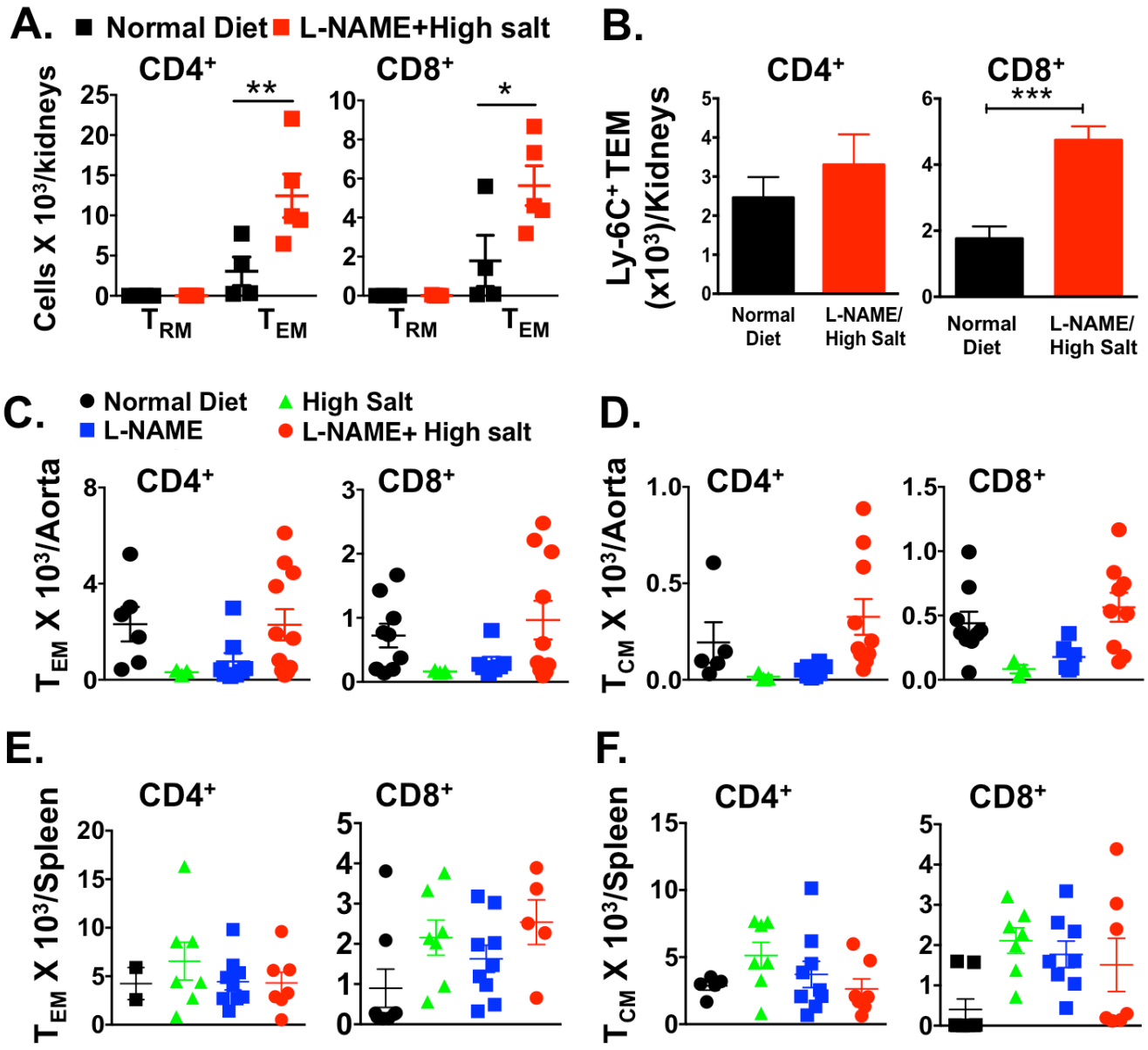
Mice were sacrificed at the end of all experiments by CO_2 inhalation. All animal procedures were approved by Vanderbilt University Institutional Animal Care and Use Committee (IACUC), and mice were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals.

Flow cytometry: Single cell suspensions of kidneys were prepared as follows. Briefly, kidneys were mechanically dissociated using a single gentleMACS C tube dissociator system (Miltenyi) followed by incubation at 37°C for 20 min with collagenase D (2 mg/ml) and DNAse I (100 µg/ml) in RPMI 1640 medium with 5% FBS. Kidney homogenates were filtered through a 70 mm cell strainer. The resultant cell suspensions were subjected to Percoll gradient centrifugation as described previously,[3, 4] and the enriched cells were washed and stained LIVE/DEAD® Fixable Violet dead cell stain (Invitrogen) and the following antibodies: Brilliant Violet 510 (BV510)-conjugated anti-CD45 antibody (BioLegend), peridinin chlorophyll protein-cyanin-5.5 (PerCP-Cy5.5)-conjugated anti-CD3 antibody (BioLegend), phycoerythrin-cyanin-7(PE-Cy7)-conjugated anti-CD8 antibody (BioLegend), allophycocyanin-Hilite-7 (APC-H7)-conjugated anti-CD4 antibody (BD Biosciences), APC-conjugated anti-CD44 antibody (BD Biosciences), PE-conjugated anti-CD62L antibody (BD biosciences), BV510-conjugated anti-LY-6C antibody (BD Biosciences), and FITC conjugated anti-Ki-67 antibody (eBioscience). Flow cytometry was performed on a BD FACS Canto II™ system and data analysis was performed using BD FACSDiva software (BD Biosciences). Gates were set using fluorescence minus one (FMO) controls. All lymphocyte subpopulations ($CD4^+$, $CD8^+$) were quantified within the $CD45^+CD3^+$ gate. The absolute number of infiltrating cells of each type was calculated by dividing the number of each population by the number of live cells obtained during flow cytometry and then multiplying by the total number of live cells counted on a

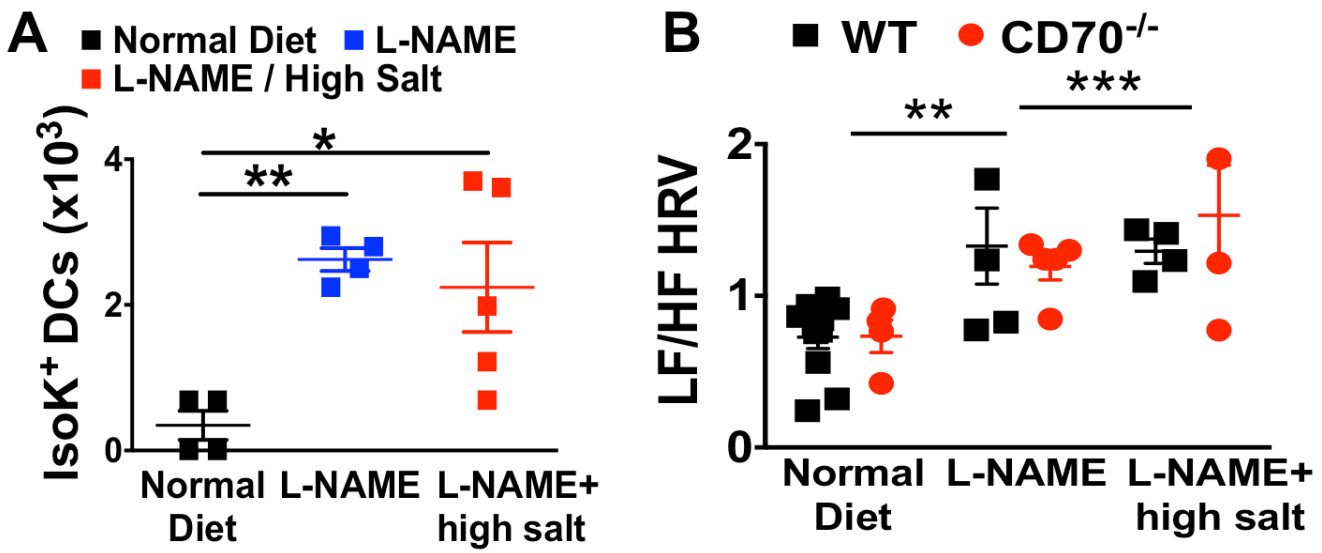
hemocytometer before staining (using trypan blue exclusion or counting beads). Results were expressed as number of cells per kidney. Intracellular staining for IL-17A or IFN-γ was performed as previously described. Briefly, $1x10^6$ kidney cells were resuspended in RPMI medium supplemented with 5% FBS and stimulated with 2 µl of BD Leukocyte Activation Cocktail (ionomycin and phorbol myristic acetate (PMA) along with the golgi inhibitor, brefeldin A) at 37°C for 5 hours. Surface staining was performed as described above followed by intracellular staining using Fluorescein isothiocyanate (FITC)-conjugated anti-IFN-γ antibody (eBioscience) or PE conjugated anti-IL-17A as previously described.[3] For studies of bone marrow cells, tibias and femurs of mice were flushed using the RPMI media and cells stained as above.

Measurements of renal injury: Neutrophil gelatinase-associated lipocalin (NGAL) mRNA was assessed by quantitative real-time PCR and normalized to GAPDH mRNA. Urinary albumin from 24-hour urine samples was determined with ELISA kits from Exocell. At the end of the L-NAME/high salt protocol, mice were placed in metabolic cages for 24 hours for acclimatization followed by 24 hours for urine collection. All concentrations were multiplied by total urine volume to obtain the daily excretion rate.

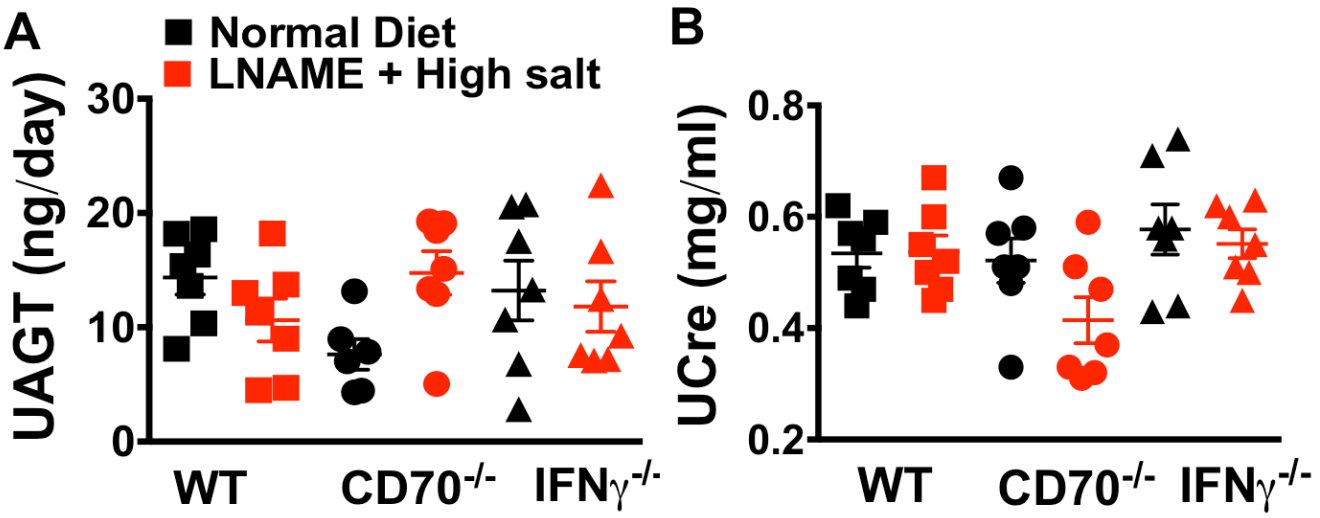
Statistics: Data are expressed as mean ± standard error of the mean. Blood pressures were analyzed by ANOVA for repeated measures. For comparisons of experiments involving a 2x2 design two-way ANOVA was used. When individual comparisons were made within this 2x2 design, Newman-Keuls or Holm-Sidak post-hoc tests were employed. When variances between groups were unequal, a Mann Whitney comparison followed by a Bonferroni correction was used.



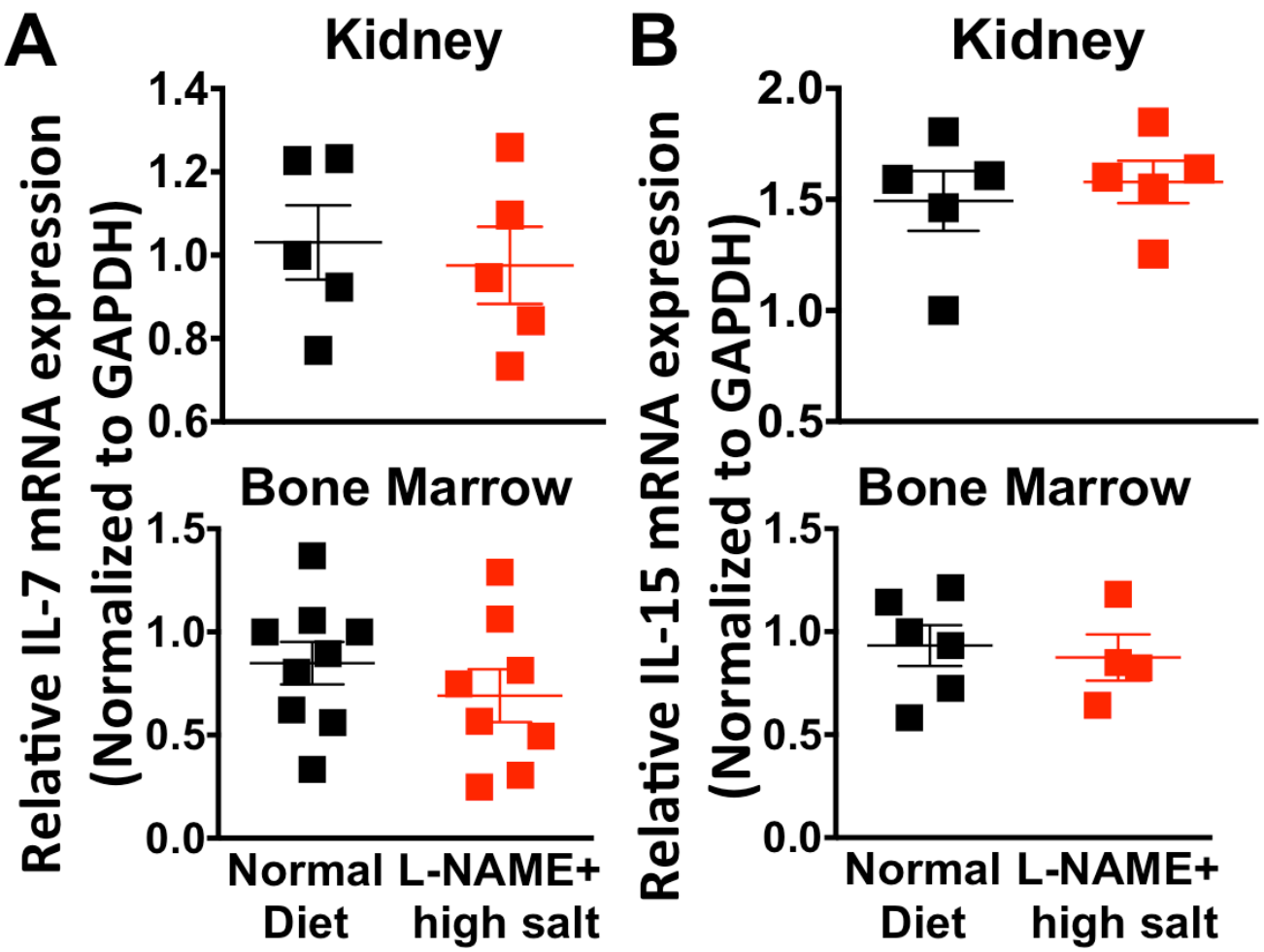
Online Figure I: Comparison of various populations of memory T cells in the kidney (A, B) aorta (C and D) and spleen (E and F). T_{RM} = resident memory T cells, T_{EM} = Effector memory T cells, T_{CM} = Central memory T cells. N = 5-8**.** Data were analyzed by ANOVA. ** < 0.01, *** <0.001.



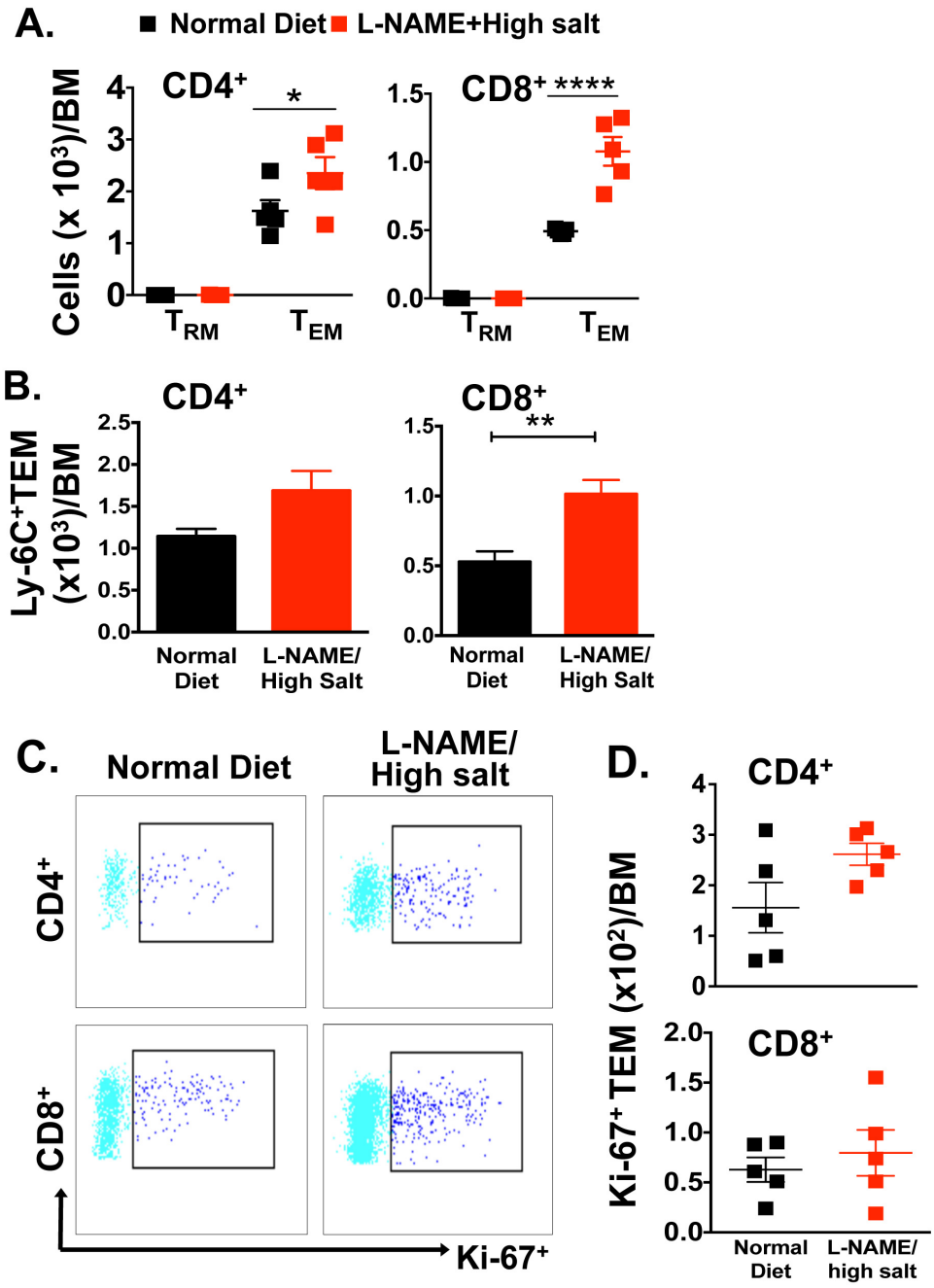
Online Figure II: Effect on L-NAME and L-NAME/high salt on DC isoketal-adduct formation and heart rate variability. (A) Splenic DCs were stained for isoketal adducts and analyzed by flow cytometry. (B) Telemetry recordings of blood pressure were analyzed for the ratio of low frequency to high frequency heart rate variability. N = 4 – 11. Data were analyzed by ANOVA. * < 0.05. ** < 0.01, *** <0.001.



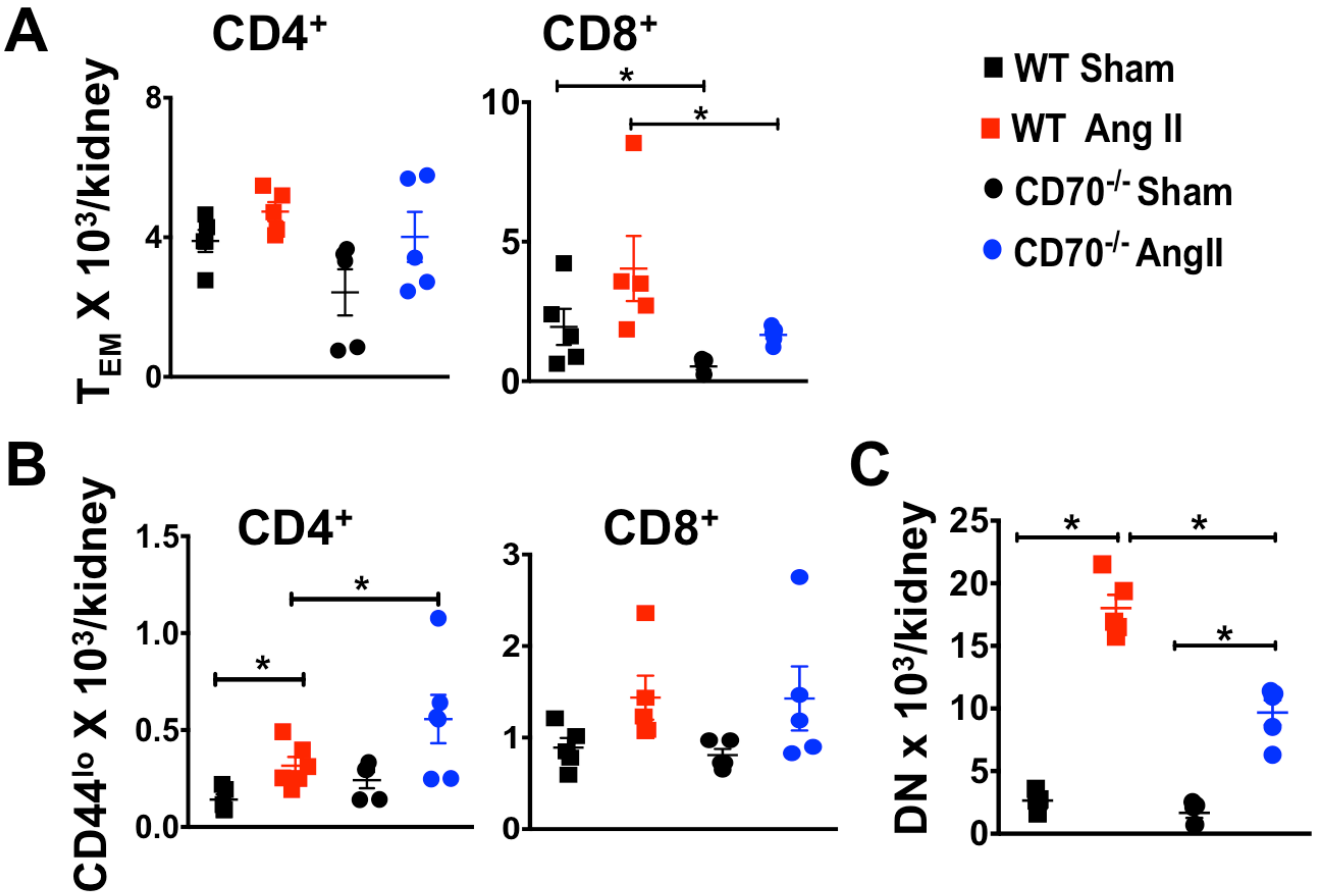
Online Figure III: Effect of L-NAME/high salt on urinary angiotensinogen and creatinine excretion. (A) Urinary concentrations of angiotensinogen (UAGT) and (B) Creatinine (UCre) were measured by ELISA among the three groups of WT, CD70$^{-/-}$, and IFN-$\gamma^{-/-}$ mice (n=7 per group). Data are expressed as means ± SEM.



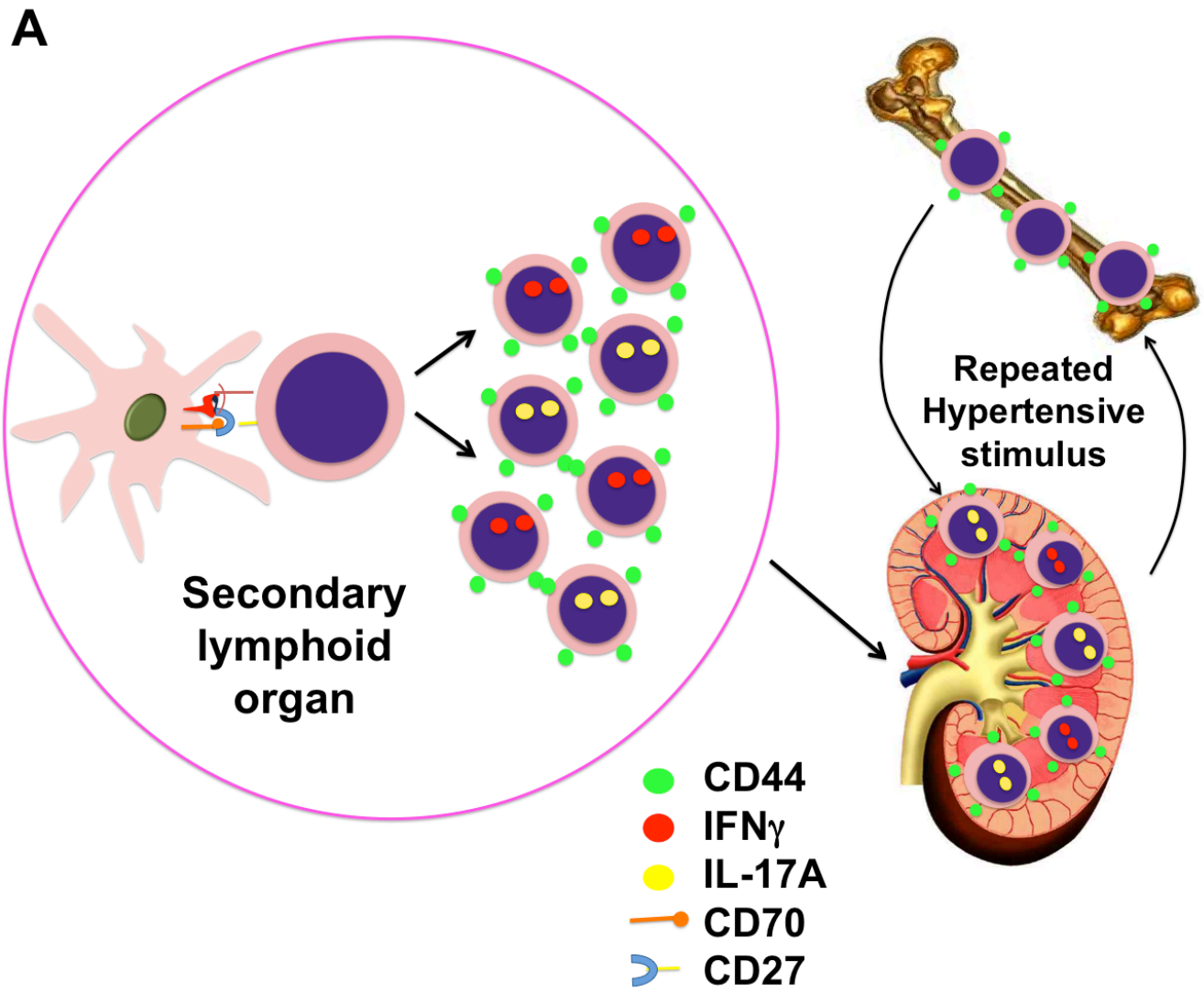
Online Figure IV: mRNA expression levels of interleukin 15 and 7 within the kidney and bone marrow of mice exposed to normal diet or the L-NAME/high salt protocol. N = 4-9.



Online Figure V: Effector memory T cells in the bone marrow. Panel A compares effector (T_{EM}) and resident (T_{RM}) cells in the bone marrow of mice that received either a normal diet or L-NAME/high salt protocol. Panel B shows bone marrow Ly-6C$^+$ T_{EM} cells. Representative flow cytometry dot plots (C) and mean data (D) of bone marrow (BM) TEM cells stained for Ki-67. n= 5 -8 for each group. * < 0.05. ** < 0.01, **** <0.0001.



Online Figure VI: Effect of two-week ang II infusion on blood pressure and renal T cell infiltration. WT and CD70$^{-/-}$ mice were infused with either angiotensin II (490 ng/kg/min) or vehicle for two weeks. The accumulation of renal CD4^{+} and CD8^{+} T_{EM} cells and primary effector T cells is shown in panels A and B. Panel C shows presence of double negative (DN) cells in the kidneys of WT and CD70$^{-/-}$ mice. n= 5-8 for each. Groups were compared using a Mann-Whitney non-parametric analysis with a Bonferroni correction for multiple comparisons. * < 0.05.



Online Figure VII: Proposed model for memory T cell formation in hypertension. Hypertensive stimuli such as L-NAME/high salt or angiotensin II promote formation of neoantigens that are presented by DCs to naïve T cells. The latter proliferate and form $CD44^{high}$ effector memory T cells that accumulate in the kidney and produce cytokines such as IL-17A and IFN-γ, which alter renal sodium and volume reabsorption and promote hypertension. Long-lived effector memory T cells also reside in the bone marrow and likely can respond to additional hypertensive challenges.

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