Isoketal-Modified Proteins in Dendritic Cells Activate T Cells and Promote Hypertension

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The authors have declared that no conflict of interest exists.

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MATERIALS AND METHODS

Immunization Assay: Splenocytes were obtained using a spleen dissociation kit (Miltenyi Biotec) from mice that had received either vehicle (sham) or angiotensin II infusion for 14 days. $CD11c^+/CD80^+/ CD86^+/ MHCII^+$ DCs were isolated from total splenocytes using a magnetic cell sorter and a commercially available kit (Miltenyi Biotec). This isolates all $CD11c^+$ cells and when used for mouse splenocytes, is an acceptable method to isolate essentially all DCs. One million DCs were injected into the tail vein of a C57Bl/6 male that had not received angiotensin II. Ten days later, pan T-cells ware isolated from the recipient mouse's spleen using a pan T cell isolation kit and a cell sorter (Miltenyi). These T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and placed in culture with DCs from another mouse that has been treated with either vehicle or Ang II at a ratio of 10 T cells to one dendritic cell for 7 days. This second, in vitro exposure acts as a boost to promote T cell activation. This assay is illustrated in figure 7A. In experiments to measure cytokine production by T cells, IL2 (40 µg/ml) was added to the media.

Flow cytometry: Immunostaining for flow cytometry was performed using the following antibodies; PerCP-Cy5.5 anti-CD45; Amcyan anti-CD45; FITC anti-CD3; APC anti-CD3; APC anti-CD4; PE-Cy7 anti-CD8a; PE-Cy7 anti-I-Ab; APC-Cy7 anti-CD11c; APC anti-CD11b (Becton Dickinson). $1x10^6$ cells were incubated with 1.5 µl of each antibody in 100 µl of FACS buffer for 25 minutes. The cells were then washed 3 times with FACs buffer and immediately analyzed on a FACSCanto flow cytometer with DIVA software (Becton Dickinson). Dead cells were eliminated from analysis using 7-AAD (BD Phermingen). For each experiment, we performed flow minus one (FMO) controls for each fluorophore to establish gates. In selected experiments, we confirmed accuracy of the FMO gating strategy using isotype controls (Supplemental figure 5). Data analysis was done using FlowJo software (Tree Star, Inc.).

Superoxide and cytokine analysis: $CD11c^+$ DCs were isolated from mice infused with angiotensin II or sham as described above. Superoxide was measured using dihydroethidium (DHE) and high-performance liquid chromatography (HPLC) assay as previously described (1). Cytokine production was measured using a Luminex-based assay. For the in vitro experiments, DCs were isolated from naïve wild type mice and treated with 100 nM of angiotensin II in RPMI 1640 culture medium containing 10% fetal bovine serum for 24 hours at 37 ^oC in a 5% CO_2 humidified atmosphere.

Isoketal Scavengers and Control Compounds: 2-hydroxybenzylamine (2-HOBA), 5-methyl-2-hydroxybenzylamine (5-Me-2-HOBA), 5-*O'*-pentylpyridoxamine (PnPM), 4-hydroxybenzylamine (4-HOBA) and *N*-Methyl-2-hydroxybenzylamine (*N*-Me-2-HOBA) were synthesized as previously described (2, 3). The rate constants for pyrrole formation were measured with reaction mixtures containing equal concentrations of amine and 4-oxopentanal (2 mM) in phosphate buffer (0.1 M, pH 7.4) at 25 °C as described previously (4). 1,1,3,3-Tetramethoxypropane (42 μl) was incubated with 1 M HCl (0.5 ml) at 40 °C for 5 min and mixed with 0.2 M Na_2HPO_4 (5 ml) and enough 0.1 M phosphate buffer, pH 7.4 to make the total volume 10 ml. The resulting 25 mM malondialdehyde (MDA) was prepared fresh. The reaction mixture containing 25 mM amine (160 μl), 0.2 M Na_2HPO_4 (80 μl), 0.1 M phosphate buffer, pH 7.4 (400 μl), and 25 mM MDA (160 μl) was incubated at 37 °C. At various time points an aliquot of 10 μl was diluted to 200 μl of solvent system A and analyzed by Waters liquid chromatograph 2695 with photo-diode detector 996. The column Phenomenex Synergi (150 × 2 mm, 4 μm) was held at 30 °C. Solvent system A (20% methanol in 5 mM formic acid) was held for 1 min, changed to solvent system B (100% methanol with 5 mM formic acid) in 7 min and held at system B for 8 min. The amines were eluted (flow rate of 0.2 ml/min) at 2-4 min while the adducts appeared after 10 min. Peak areas at 280 nm were used to calculate the ratio of amine to the adduct.

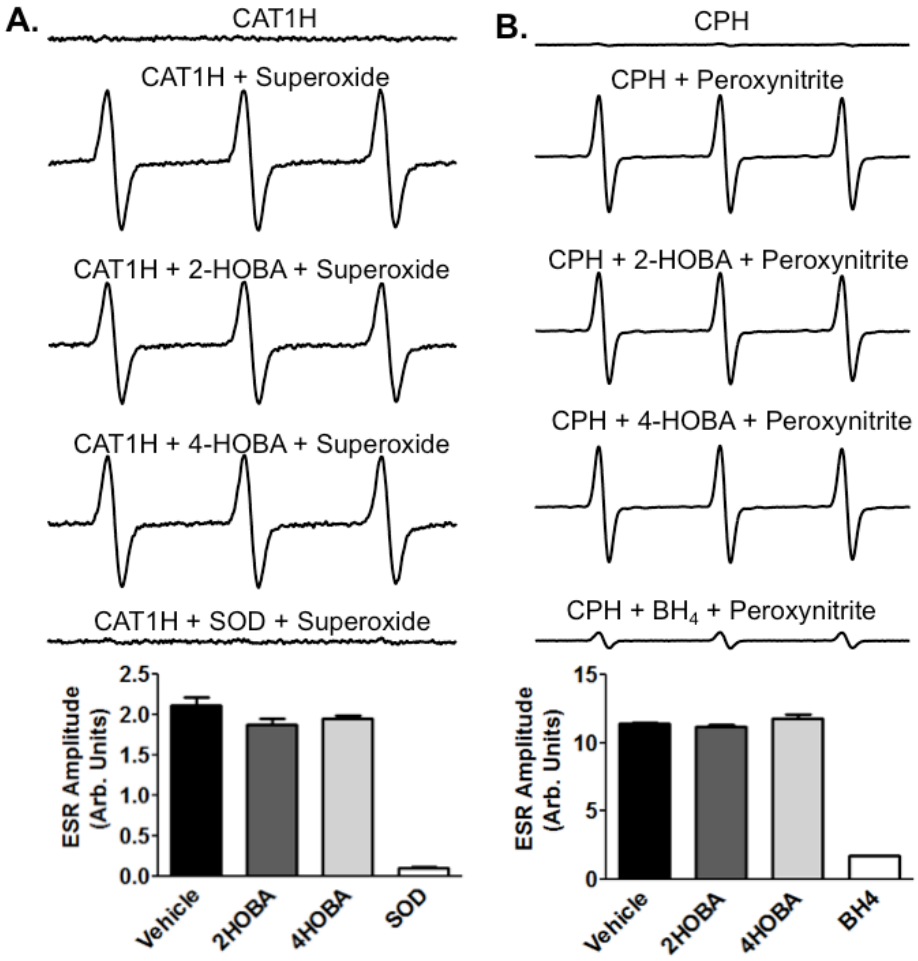
Isoketal measurements: Isoketal protein adducts were analyzed by immunohistochemistry using a D11 ScFv antibody that identifies isoketal-lysine adducts independent of the protein backbone as previously described (5), and quantified using the NIH ImageJ software. For flow cytometry, the D11 ScFv antibody was labeled with a fluorochrome using the APEXTM Alexa Fluor 488 Antibody Labeling kit (Invitrogen). The cells labeled with surface antibodies were then fixed and permeabilized for intracellular detection of isoketals using a cell permeabilization kit (Invitrogen). Specificity of the flow cytometric signal was confirmed by using an isotype control (Supplementary Figure 8A) and by successful competition using an exogenously added isoketal-adducted peptide (Supplementary Figure 8B). In contrast, peptides adducted by MDA, HNE or MGO did not reduce the flow cytometric signal detected by fluorescently labeled D11 (Supplemental Figure 8B).

We also measured isoketals in tissues and DCs by measuring the isoketal-lysyl-lactam adducts using mass spectrometric analysis essentially as previously described (6, 7). Dendritic cells from ten mice each were pooled together for each sample, and three samples per group were analyzed. Cells were homogenized in ice-cold buffer containing butylhydroxytoulene and tris-carboxyethyl phosphine to prevent oxidation, and proteins precipitated with 20% trichloroacetic acid followed by centrifugation (4000 g at 4ºC for 20 minutes). The proteins were washed with cold methanol/diethyl ether, re-suspended in 2.7 N potassium hydroxide, hydrolyzed under argon for 0.5 h at 40ºC, pH neutralized with concentrated hydrochloric acid, and then heated at 95ºC for 10 min. After cooling, they were incubated in pronase overnight at 37ºC. The digest was heated at 95ºC for 10 min to inactivate the pronase, cooled, and incubated with aminopeptidase M for 18 h at 37ºC. The adducted peptides were eluted on C18 solid phase extraction cartridges and then further purified by HPLC. Presence of the isoketal–lysyl-lactam adducts was determined by HPLC coupled to electrospray ionization (ESI) mass spectrometry

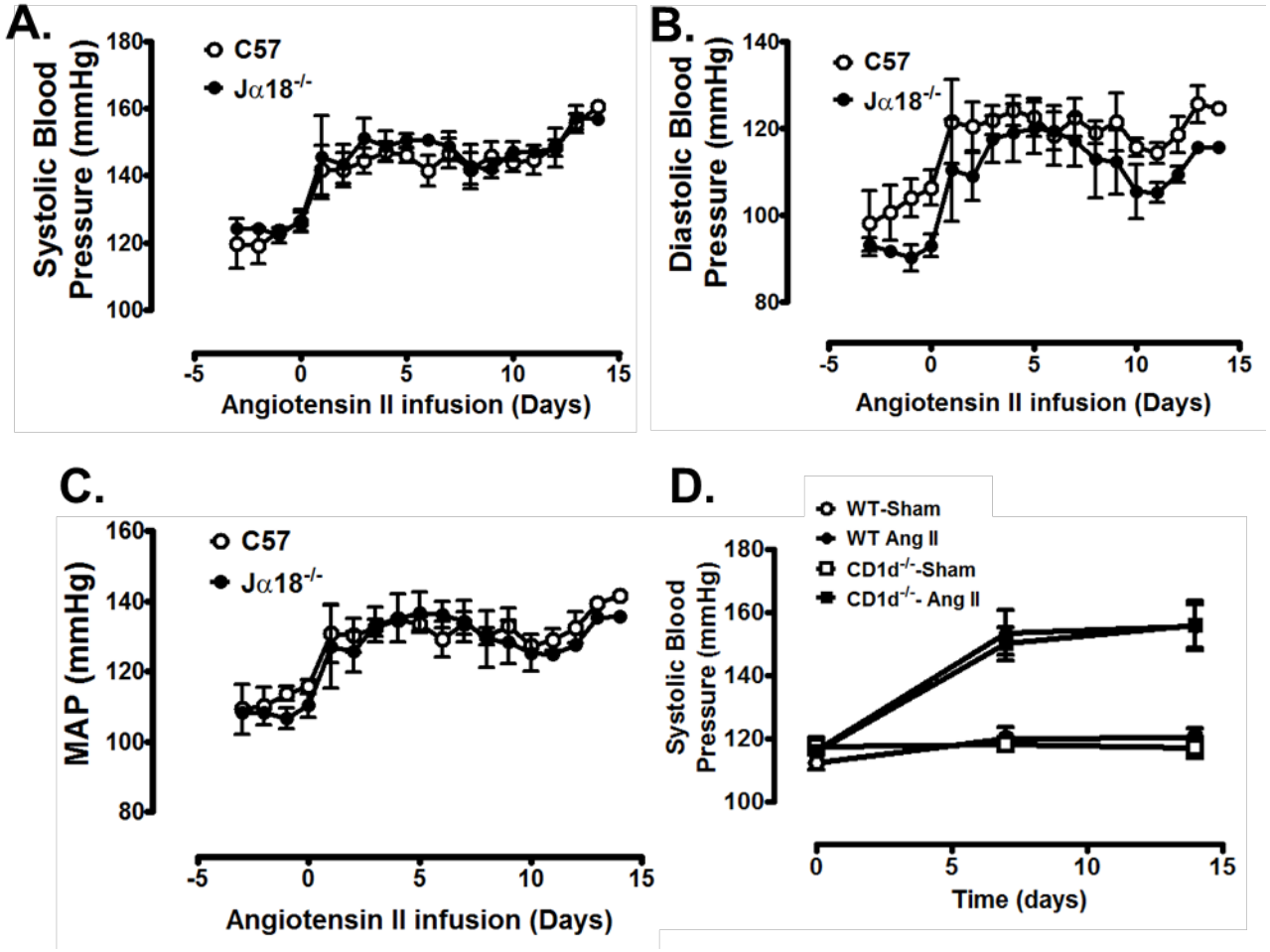
(MS) operating in multiple reaction monitoring mode. Gradient HPLC was performed using a Phenomenex Kinetex 2.6µ C18 100A column at a flow rate of 250 µl/min starting at 95% Solvent A (0.1% acetic acid in water) for 1 min, then a gradient ramp to 50% Solvent B (1 mM ammonium acetate in methanol) at 3 min and then further gradient ramp to 80% Solvent B at 9 min. The HPLC eluant was directly connected to a ThermoFinnigan Quantum electrospray ionization triple quadrapole mass spectrometer operating in positive ion multiple reaction monitoring (MRM) mode for *m/z* 479.3 → 84.1@-35eV for isoketal–lysine-lactam and @-35eV for [$^{13}C_6{}^{15}N_2$] isoketal -lysine-lactam.

In additional experiments, we sought to determine if 2-HOBA could scavenge either superoxide or peroxynitrite using electron spin resonance as previously described (8).

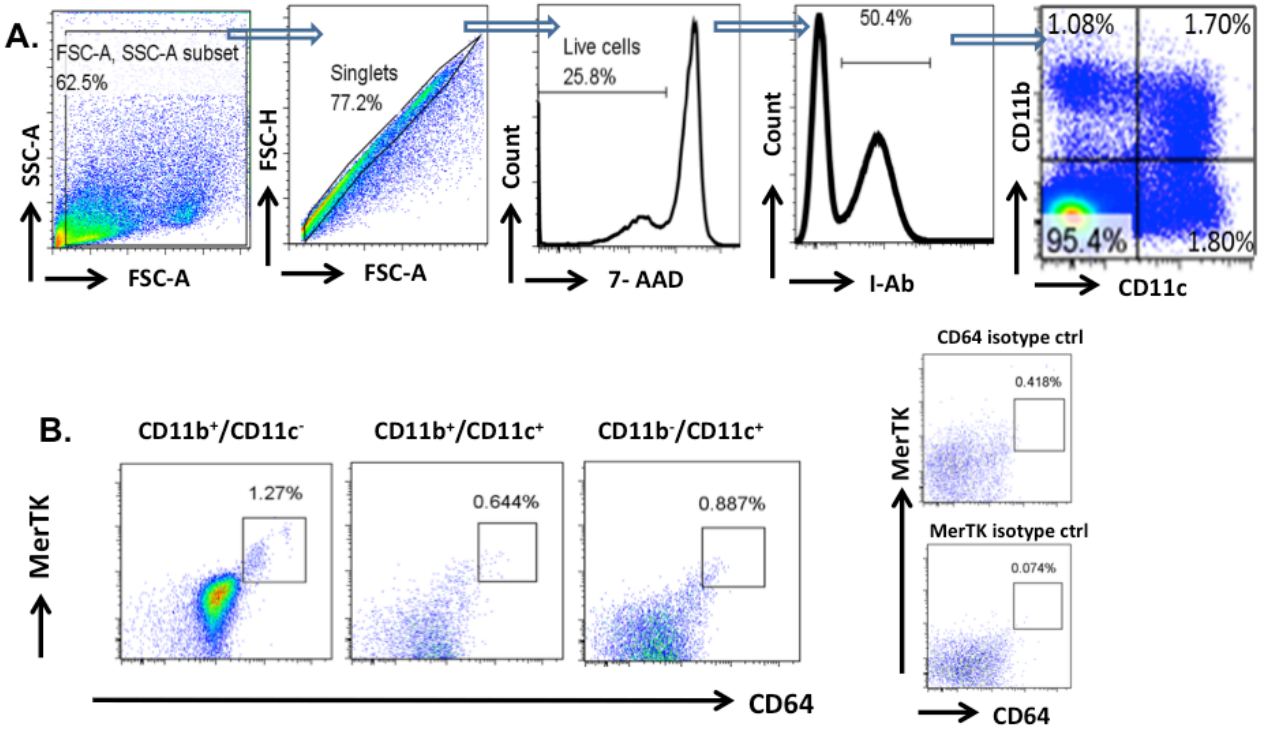
SUPPLEMENTAL FIGURES AND LEGENDS



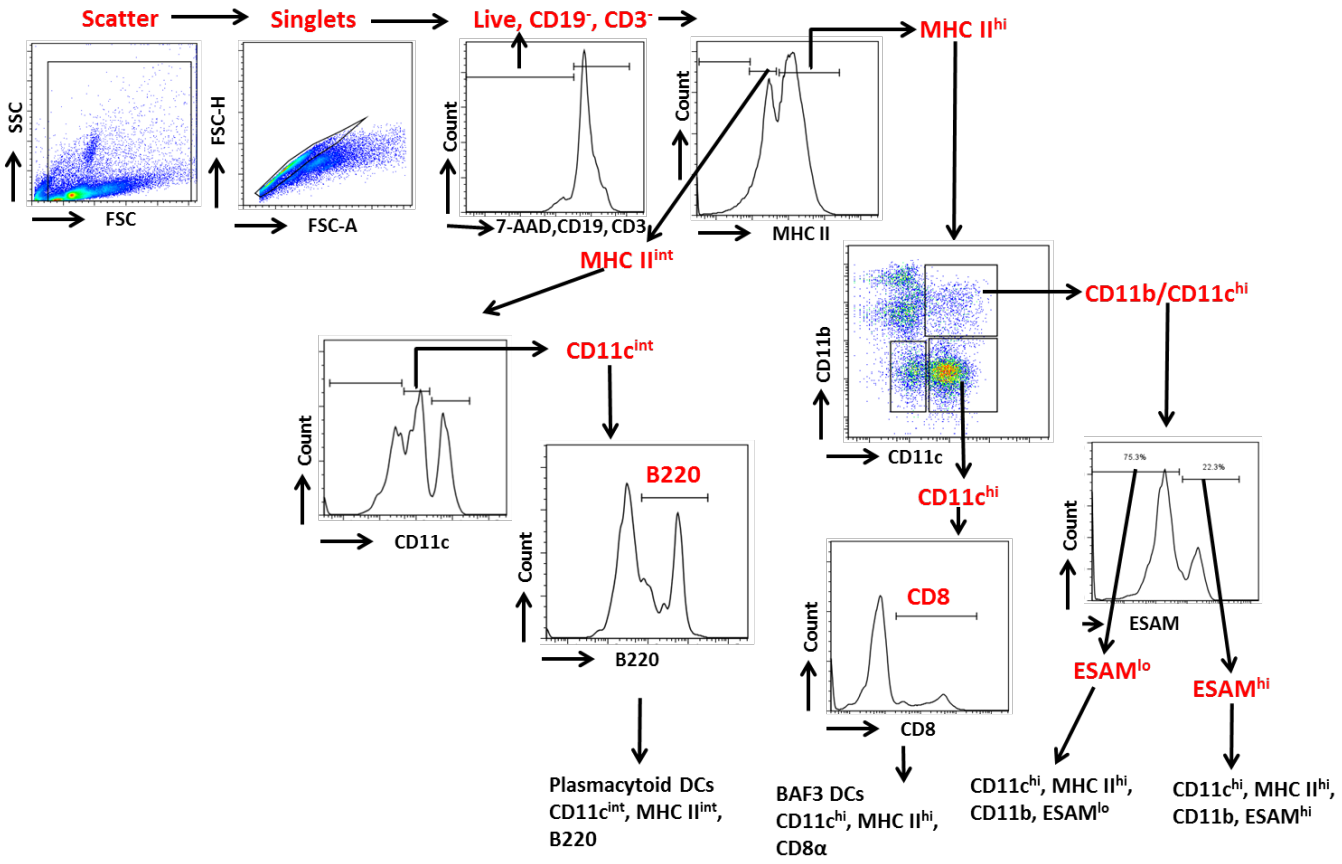
Supplemental figure 1: Failure of 2-HOBA to scavenge superoxide or peroxynitrite. Oxidation of the cyclic hydroxylamine 1-Hydroxy-2,2,6,6-tetramethylpiperidin-4-yl-trimethylammonium chloride (CAT1H) by superoxide (panel A) or 1-Hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine (CPH) by peroxynitrite (panel B) was measured using electron spin resonance. Superoxide was generated by a reaction of xanthine (0.5 mM) and xanthine oxidase (5 mU/ml). Peroxynitrite was generated by the spontaneous decomposition of SIN-1 (1mM). As positive controls, superoxide was scavenged with superoxide dismutase (SOD, 100 U/ml) and peroxynitrite with tetrahydrobiopterin (BH_4, 500 μM). Mean values for 3 experiments each are shown in the panels below.



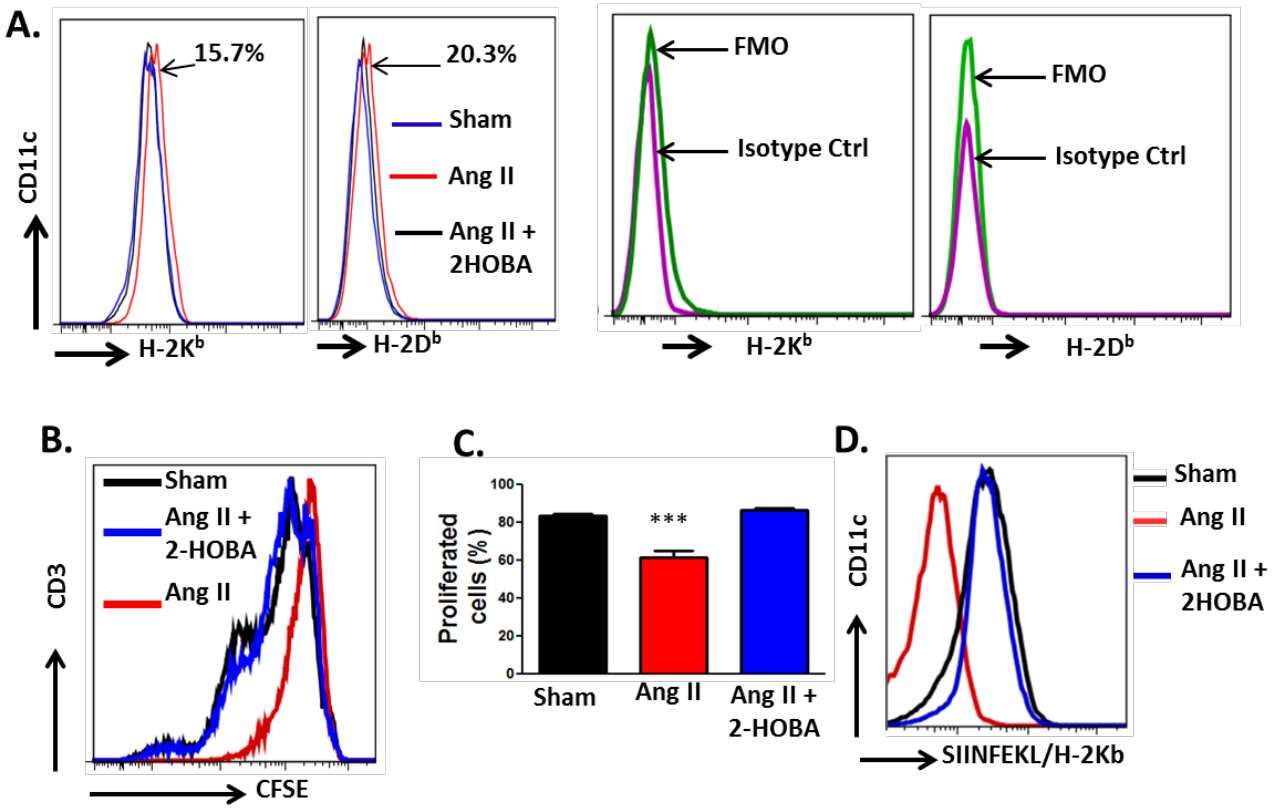
Supplemental Figure 2: Effect of natural killer T cells on angiotensin II-induced hypertension. Telemetry recordings of systolic (A), diastolic (B) and mean arterial blood pressure (C) of wild type C57 and *Jalpha18*$^{-/-}$ mice. (D) Hypertensive response to angiotensin II in *CD1*$^{-/-}$ mice. (n=9-12).



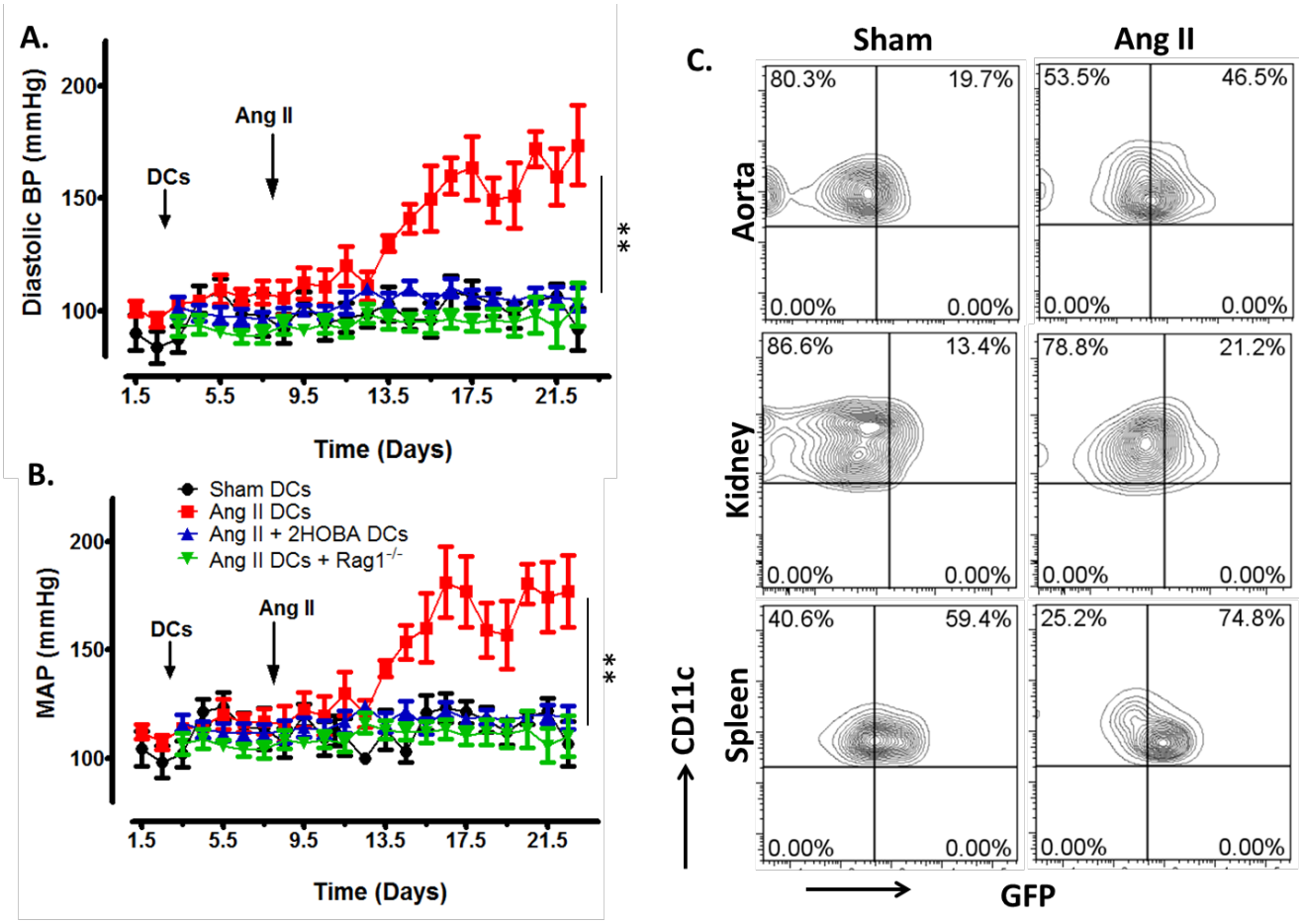
Supplemental Figure 3: Selection of myeloid cells from splenocytes. Panel A shows sequential gating for single and live cells, followed by selection of MHCII positive cells (I-Ab+). Cells were then gated for the presence of CD11b and CD11c. Panel B further analyzes these subtypes for the macrophage markers MerTK and CD64.



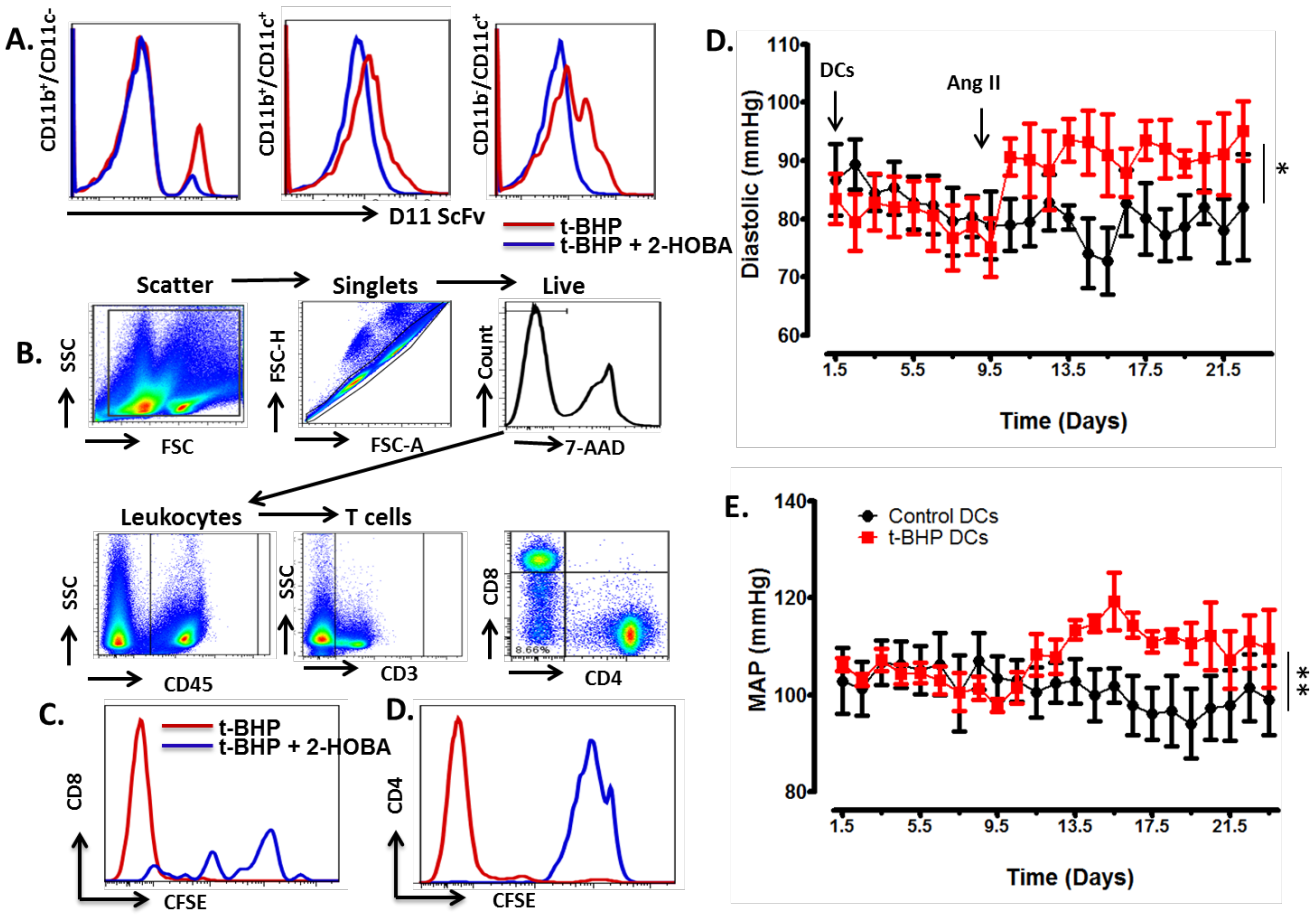
Supplemental Figure 4: Flow cytometry gating strategy to identify $CD8^+$, $B220^+$, $MHCII^{hi}$ and $MHCII^{int}$, and $ESAM^{hi}$ and $ESAM^{lo}$ $CD11c^+/CD11b^+$ cells.



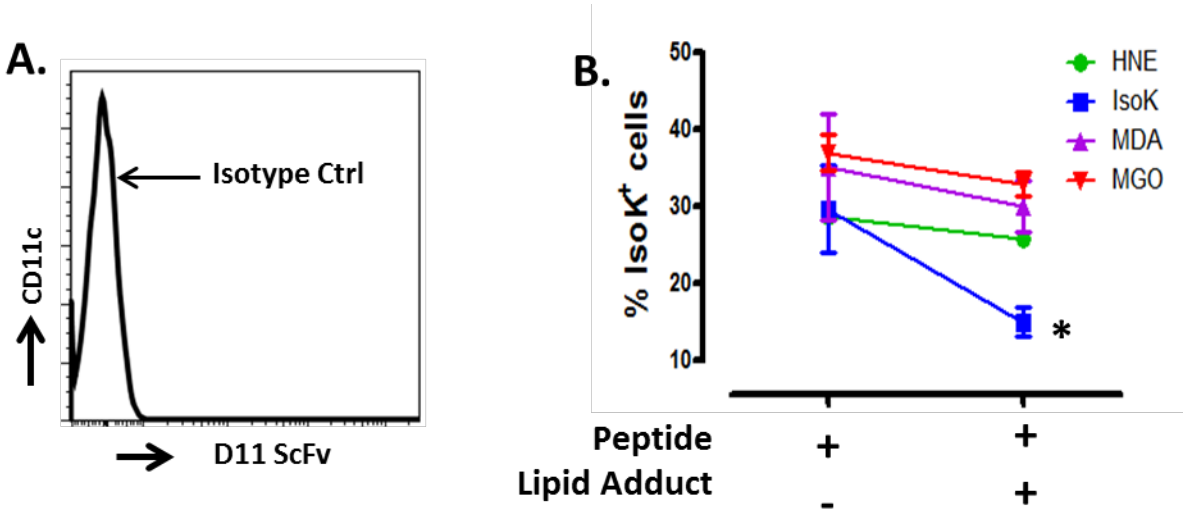
Supplemental Figure 5: (A) Flow cytometry expression of H-2D^b and H-2K^b in dendritic cells. Flow minus one (FMOs) and isotype controls are also shown. (B&C) 3 day CFSE proliferation assay of T cells from OT1 transgenic mice co-cultured with dendritic cells from sham or angiotensin II infused mice with or without 2-HOBA treatment. (D) Dendritic cells were incubated with 10 ug/ml of SIINFEKL for one hour and flow cytometry was used to analyze binding of the anti-mouse SIINFEKL/H-2Kb antibody (n=6, ***p<0.001 vs sham).



Supplemental Figure 6: Transfer of hypertension by dendritic cells. Dendritic cells were obtained from either sham or angiotensin II-infused mice and 1 X 10^6 cells were adoptively transferred to recipient wild-type or $Rag1^{-/-}$ mice and blood pressure was monitored using telemetry. (A) Diastolic and (B), mean arterial blood pressure in response to low-dose angiotensin II infusion (140 ng/kg/min) 10 days after dendritic cell adoptive transfer (n=5-7, **p<0.01). (C) Dendritic cells from mice transgenic for Enhanced Green Fluorescent protein (GFP) treated with either angiotensin II or sham were adoptively transferred into recipient wild type mice. 10 days later, the aorta, kidney and spleen of the recipient mice were analyzed using flow cytometry for presence of $CD11c^+$ and GFP^+ cells.

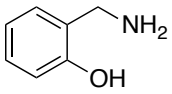
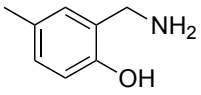
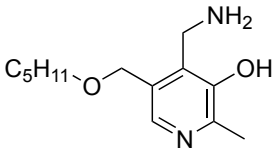
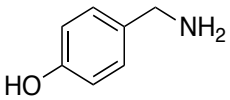
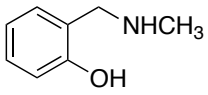


Supplemental Figure 7: (A) Dendritic cells were treated with 1mM tert butyl hypdroperoxide (t-BHP) with or without co-treatment with the isoketal scavenger 2-HOBA, and isoketals were measured by flow cytometry using Alexafluor 488-tagged D11 antibody. t-BHP treated dendritic cells with or without co-treatment with 2-HOBA were cultured with T cells loaded with CFSE and proliferation was monitored. (B) Gating strategy for monitoring proliferated T cells. (C) Proliferation among $CD8^+$ T cells. (D) Proliferation among $CD4^+$ T cells. Effect of adoptive transfer of t-BHP treated dendritic cells on diastolic (D) and mean arterial pressure (E) in mice (n=4-7, *p<0.05, **p<0.01, ***p<0.001).



Supplemental Figure 8: Specificity of the flow cytometric signal was confirmed by using an isotype control (A) and successful competition using an exogenously added isoketal-adducted peptide or peptides adducted by MDA, HNE or MGO (B).

Supplemental Table 1. Second-order rate constants for pyrrole formation between amine and 4-oxopentanal or malondialdehyde.

Amine	Pyrrole formation with 4-oxopentanal $k \times 10^3$ $M^{-1}s^{-1}$	Adduct formation with MDA $k \times 10^3$ $M^{-1}s^{-1}$
 2-HOBA	343.5 ± 12.5	28.7 ± 0.33
 5-Me-2-HOBA	389.6 ± 3.5	18.8 ± 0.10
 PnPM	560.1 ± 21.9	93.9 ± 5.9
 4-HOBA	7.68 ± 0.25	12.8 ± 0.28
 N-Me-2-HOBA	No reaction	11.2 ± 0.16

Supplemental Table 2: Top genes altered by angiotensin II and reversed by absence of superoxide and isoketals

Gene Title	Gene Symbol	p-value (Ang II vs. Sham)	Fold change, Ang II vs. Sham	Fold reversal by *Nox2*$^{-/-}$	Fold reversal by 2HOBA
lipin 1	*Lpin1*	7.66E-05	-1.5	1.3	1.3
tubulin, alpha-like 3	*Tubal3*	5.99E-04	1.5	-1.3	-1.4
DEAD (Asp-Glu-Ala-Asp) box polypeptide 1	*Ddx1*	1.21E-03	-1.5	1.4	1.3
hemicentin 1	*Hmcn1*	2.39E-03	1.6	-1.6	-1.5
3-ketodihydrosphingosine reductase	*Kdsr*	2.48E-03	-1.5	1.3	1.4
heat shock protein 1A /heat shock protein 1B	*Hspa1a*	3.79E-03	1.5	-1.4	-1.3
trypsin 10 / predicted pseudogene 5409	*Try10*	4.90E-03	1.7	-1.6	-1.5
C-type lectin domain family 10, member A	*Clec10a*	6.22E-03	-1.8	1.9	1.4
ATPase, Na+/K+ transporting, beta 1 polypeptide	*Atp1b1*	1.19E-02	-1.5	1.3	1.2
carboxypeptidase A1	*Cpa1*	1.34E-02	2.2	-2.8	-1.4
CD300C antigen	*Cd300c*	1.39E-02	-1.6	1.7	1.7
cathepsin E	*Ctse*	2.20E-02	2.4	-2.0	-3.3
Family with sequence similarity 71, member A	*Fam71a*	2.24E-02	1.6	-1.5	-1.8
chymotrypsinogen B1	*Ctrb1*	2.27E-02	3.1	-4.8	-1.5
amylase 2a5/amylase 2a4	*Amy2a5*	2.47E-02	5.7	-11.9	-1.2
colipase, pancreatic	*Clps*	3.12E-02	1.8	-1.8	-1.2
glycerophosphodiester phosphodiesterase domain containing 2	*Gdpd2*	3.60E-02	1.5	-1.6	-1.2
N-acylsphingosine amidohydrolase 1	*Asah1*	4.23E-02	-1.5	1.2	1.2
phosphatase and tensin homolog	*Pten*	4.57E-02	-1.5	1.7	1.8

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