Supplemental Material

Detailed Methods

Mice and hypertension models

C57BL/6, OT-I (C57BL/6-Tg(TcraTcrb)1100Mjb/J), Nox2$^{-/-}$ mice (B6.129S-Cybb^{tm1Din}/J) and RFP mice (B6.Cg-Tg(CAG-mRFP1)1F1Hadj/J) were purchased from Jackson Laboratories. Enhanced green fluorescent protein (eGFP) mice (B6-TgN(β-act-EGFP)osbY01) were generously supplied by Dr Okabe.[1] The OT-I and Nox2$^{-/-}$ mice were on a C57BL/6 background. The mice used in the experiments were male aged between 8-10 weeks old. Hypertension was induced by s.c. infusion of Ang II (980 ng/kg/min unless otherwise indicated) (Pheonix Pharmaceuticals) via osmotic minipump (Alzet) or by L-NAME treatment (0.5 or 1.5 mg/ml in the drinking water) (Bachem). To induce salt-sensitivity, mice were first exposed to L-NAME (0.5 mg/ml in the drinking water) for 4 weeks followed by a washout period of 1 wk where mice received normal drinking water. Finally, mice were fed a high salt diet (4% NaCl in food) for 3 weeks. All mice were maintained in micro isolator cages, and all experimental protocols were approved by the Institutional Animal Care and Use Committee at Cedars Sinai Medical Center.

Blood pressure measurement

Blood pressure was monitored in conscious mice invasively using radiotelemetry or a computerized non-invasive tailcuff system (Visitech Systems, BP-2000 series II, Apex), as described.[2,3] For the former, mice were anesthetized with isoflurane, and a catheter connected to a radiotelemetry device (model PA-C10; Data Sciences International) was inserted in the left carotid artery. After a 14-day recovery phase, baseline levels were established before Ang II minipump implantation. Data were collected, stored, and analyzed using Dataquest A.R.T. 4.0 software (DSI). For measuring BP by tailcuff, mice were trained for 5 continuous days before data acquisition. BP was determined by averaging 20 measurements, with tracings manually reviewed to verify proper BP determination.

Antibodies and flow Cytometry (FCM)

The following antibody clones were used: M1/70 (anti-CD11b), RB6-8C5 (anti-Gr1), HK1.4 (anti-Ly6C), 30-F11 (anti-CD45), 16-10A1 (anti-CD80), AF6-120.1 (anti-I-A^{b}), mIL4R-M1 (anti-IL-4Rα), 4B12 (anti-CCR7), 2E2 (anti-IFNγR), MR5D3 (anti-mannose receptor), TEK4 (anti-Tie2), 72-1 (anti-CD36), XMG1.2 (anti-IFNγ), TC11-18H10.1 (anti-IL-17A), MP6-XT22 (anti-TNFα), 145-2C11 (anti-CD3), 1M7 (anti-CD44) and BM8 (anti-F4/80). All these antibodies were purchased from either eBioscience, BioLegend or Pharmingen. Polyclonal anti-CCR2 Ab was from Thermo Scientific. 7-amino-actinomycin D (7-AAD) was from BioLegend. For staining intracellular proteins, fixation and permeabilization buffers were purchased from eBioscience. We used rabbit Abs to iNOS, arginase I, C/EBPα, C/EBPβ, C/EBPδ and PU.1. PE-labeled monoclonal anti-iNOS (N-20) was from Santa Cruz Biotechnology, anti-arginase I was from GeneTex, C/EBPβ was from Abcam (clone: E299), and C/EBPα, C/EBPδ and PU.1 were from Cell Signaling Technology. Primary staining using these rabbit Abs was followed by secondary staining with PE-conjugated goat anti-rabbit IgG (SouthernBiotech). The stained samples were analyzed on a Beckman Coulter CyAn ADP and data were analyzed with FlowJo software.

ROS measurement

For measuring oxidative burst, Splenocytes were incubated with 0.1 μg/ml Dihydrorhodamine 123 (Life Technologies) for 60 min at 37°C in HBSS buffer plus 0.1% BSA. The reaction was stopped by moving the cells onto ice. The cells were then stained for CD11b and Gr1 before they were subject to FACS analysis. To measure MDSC ROS production *in vivo*, MDSCs were purified from the spleen of hypertensive RFP-transgenic mice and transferred *i.v.* to naïve

C57BL/6 mice. Twenty-four hours later, 100 μg Dihydrorhodamine 123 were given *i.p.* to the recipients. After another hour, mice were anesthetized and transcardially perfused with 4^0C PBS (50 ml), followed by 4^0C 1% PFA (100 ml). Spleens were immediately removed and embedded in OCT for cryostat cutting at 10 μm longitudinally. After mounting and slip-covering, spleen sections were examined under the confocal microscope (Leica TCS SP5-X).

MDSC surface defining

For examining MDSCs by FCM, we used anti-CD11b vs. 7-AAD to gate the live myeloid cells and then used anti-Gr1 or anti-Gr1 with anti-Ly6C to analyze MDSCs and their subsets. Anti-Gr1 Ab (Clone RB6-8C5) reacts with the structurally related murine Ly6G and Ly6C molecules, but has a higher affinity for Ly6G than Ly6C. It can be used to co-immunostain with Ly6C but not with Ly6G.[4,5] In a preliminary experiment, we co-stained CD11b and Ly6C with either Gr1 or Ly6G for the blood of hypertensive mice. We confirmed that anti-Gr1 and anti-Ly6G antibodies are similar in defining the granulocytic and monocytic MDSCs when co-stained with anti-Ly6C (Online Figure IB). While both populations are $CD11b^+$, granulocytic MDSCs are $Gr1^{high}Ly6C^{low}$ or $Ly6G^{high}Ly6C^{low}$, and monocytic MDSCs are $Gr1^{low}Ly6C^{high}$ or $Ly6G^{-}Ly6C^{high}$. In this paper, we used $CD11b^+Gr1^+$ to label MDSC because it includes both populations.

MDSC collection

To collect $CD11b^+Gr1^+$ cells generated in vivo, spleens were minced with scissors and were digested with collagenase IV (Worthington) and DNase I (Sigma-Aldrich) for 40 min at 37ºC. Cells were then passed through 70 μm strainer (BD) and were purified by immunomagnetic separation using biotinylated anti-Gr1 antibody and streptavidin-conjugated MicroBeads with MiniMACS columns (Miltenyi Biotec). The isolated cells were >98% $CD11b^+$. Viability was checked by Trypan blue dye exclusion (>95%). For examining the morphology of MDSCs, $Gr1^{low}Ly6C^{high}$ and $Gr1^{high}Ly6C^{low}$ cells were purified through anti-Ly6C selection followed by anti-Gr1 selection according to the manufacturer's instructions (Miltenyi Biotec #130-094-538). Cells were cytospined and stained with Wright-Giemsa. Alternatively, we generated MDSCs from BM culture in vitro. Mice were sacrificed and BM cells were obtained from the femurs and tibias. BM cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 25 ng/ml GM-CSF (Peprotech) and 40 ng/ml IL-6 (Peprotech).[6] The cultures were maintained at 37°C in a 5% CO2-humidified atmosphere and, after 4 days, floating cells were harvested. These were predominantly MDSCs.

Immunosuppressive assay

These cells were added in various amounts to CFSE-labeled 2×10^5 naïve OT-I splenocytes in flat-bottomed 96-well plates. The plates were pre-coated with 2.5 μg/ml anti-CD3 (Clone 145-2C11 from SouthernBiotech), and when cells were seeded, 1 μg/ml anti-CD28 was added. In some experiments, the co-cultures were promptly stimulated with 0.4 μM SIINFEKL peptide (AnaSpec) without anti-CD3 pre-coating. Three days later, cells were harvested and T cells were identified by APC-labeled anti-CD4 and anti-CD8 Abs (in the wells following anti-CD3/anti-CD28 stimulation) or APC-labeled anti-CD8 Ab alone (in the wells following SIINFEKL stimulation) with co-staining using 7-AAD and APC-Cy7-labeled anti-CD11b to exclude dead cells and myeloid cells. Cells were then studied by FACS analysis of CFSE profiles. In some experiments, $CD11b^+Gr1^+$ cells were pre-treated with 10 μM 1400W (Cayman Chemical), 50 μM nor-NOHA (Cayman Chemical), 100 U/ml catalase (Worthington), 100 nM Ebselen (Enzo Life Sciences) or 1 nM mitoEbselen (Enzo Life Sciences) for 30 min before they were co-incubated with CFSE-labeled splenocytes in the presence of these reagents for 3 d. Proliferation index (PI) was calculated as $PI=Log[FI_{nd}/MFI_{all}]/Log[2]$, where MFI_{al} is the median fluorescence intensity of all viable T cells and FI_{nd} is the peak fluorescence intensity of the viable non-divided cells.[7] The PI is a mathematic approximation to the median number of divisions.

In vitro MDSC culture and differentiation
$CD11b^+Gr1^+$ cells were isolated from the spleens of normotensive mice or mice treated with 3 wk of Ang II. 2×10^5 cells were cultured with 10 ng/ml M-CSF (Peprotech), 200 ng/ml Flt3l (Peprotech) or 20 ng/ml GM-CSF in RPMI 1640 supplemented with 10% FBS and 10 mM HEPES. After 3 d, the live cells were counted by Trypan blue dye exclusion and were analyzed by FACS.

MDSC depletion
Ten days after the initiation of a hypertensive stimulus, mice were injected *i.p.* with 75 µg/g of gemcitabine (APP Pharmaceuticals) in 100 µl of saline twice per week for the first week and once per week thereafter. Control mice were hypertensive animals injected with 100 µl of saline alone. Alternatively, hypertensive mice were treated with anti-Gr1 Ab (Clone RB6-8C5, Bio X Cell) *i.p.* or the control isotype Ab (Clone LTF-2, Bio X Cell) at a dose of 0.25 mg/mouse in 200 µl PBS every other day. The numbers of circulating MDSC were 0.14 ± 0.03 k/µl (ie 1000s per µl) in gemcitabine-treated mice and 0.09 ± 0.02 k/µl in anti-Gr1 treated mice vs. 1.44 ± 0.11 k/µl in vehicle-treated mice ($P<0.001$). The percentage of MDSC in the spleen were 0.51 ± 0.09% in gemcitabine-treated mice and 0.4 ± 0.04% in anti-Gr1 treated mice vs. 6.25 ± 0.31% in vehicle-treated mice ($P<0.001$). These data indicate that MDSC depletion was efficient.

Kidney inflammatory cell dissociation and cytokine analysis
Following transcardiac perfusion with sterile heparinized PBS (100 ml, 4°C), kidneys were harvested and minced, followed by enzymatic digestion with 1.6 mg/ml collagenase IV (Worthington) and 15 units of DNase I (Sigma) at 37°C for 45 min in RPMI1640 containing 10% fetal calf serum. Digested tissues were passed through a 70 µm strainer (BD) and inflammatory cells were enriched using a 36% - 72% step Percoll (GE Healthcare) gradient. The cells at the interface of the 36% and 72% Percoll were collected and counted. Cells were then cultured in a 96-well plate for 6 h with brefeldin A (eBioscience). Some cells were stimulated with 100 nM PMA (Sigma) and 2 µM ionomycin (Santa Cruz Biotechnology). After culture, cells were studied by FACS staining.

MDSC transfer
MDSCs were either isolated from spleens of mice treated with Ang II (980 ng/kg/min) for 3 wk or derived from *in vitro* BM culture with GM-CSF and IL-6 for 4 d. The cells were washed and 2×10^6 of splenic MDSCs or 5×10^6 BM-derived MDSCs were injected *i.v.* in 100 µl or 200 µl saline, respectively. The controls were mice injected with either the same number of $CD11b^+Gr1^+$ cells derived from normotensive spleens or the same volume of vehicle. For some experiments, 2×10^6 CFSE-labeled OT-I $CD8^+$ T cells were co-transferred *i.v.* with 2×10^6 MDSCs derived from normotensive or hypertensive mice. One day later, the recipients were *i.p.* immunized with 400 µg ovalbumin. After another 2 days, mice were sacrificed and the activation profile of splenic OT-I cells were examined.

Proteinuria
Mice were individually housed in metabolic cages for urine sampling. To avoid urine contamination with food, mice were fed a gelled diet containing all necessary nutrients (Nutra-gel; Bio-Serv; Frenchtown, NJ; Cat: S4798). Animals had free access to food and water at all times. Urinary protein excretion was measured using the micro BCA method.

Statistics
All data are expressed as the arithmetic means ± SEM. Two-way ANOVA with Bonferroni's post-test was used when analyzing changes in data collected over time. One-way ANOVA and

2-tailed unpaired Student's t test were used to analyze group comparisons. All statistical tests were calculated using GraphPad Prism 5.00 (GraphPad Software). A value of $P<0.05$ was regarded as significant.

Supplemental references

1. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 'Green mice' as a source of ubiquitous green cells. FEBS Lett. 1997;407:313-19.
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7. Wallace PK, Tario JD Jr, Fisher JL, Wallace SS, Ernstoff MS, Muirhead KA. Tracking antigen-driven responses by flow cytometry: monitoring proliferation by dye dilution. *Cytometry A*. 2008;73:1019-34.

Supplemental Figures and Figure Legends

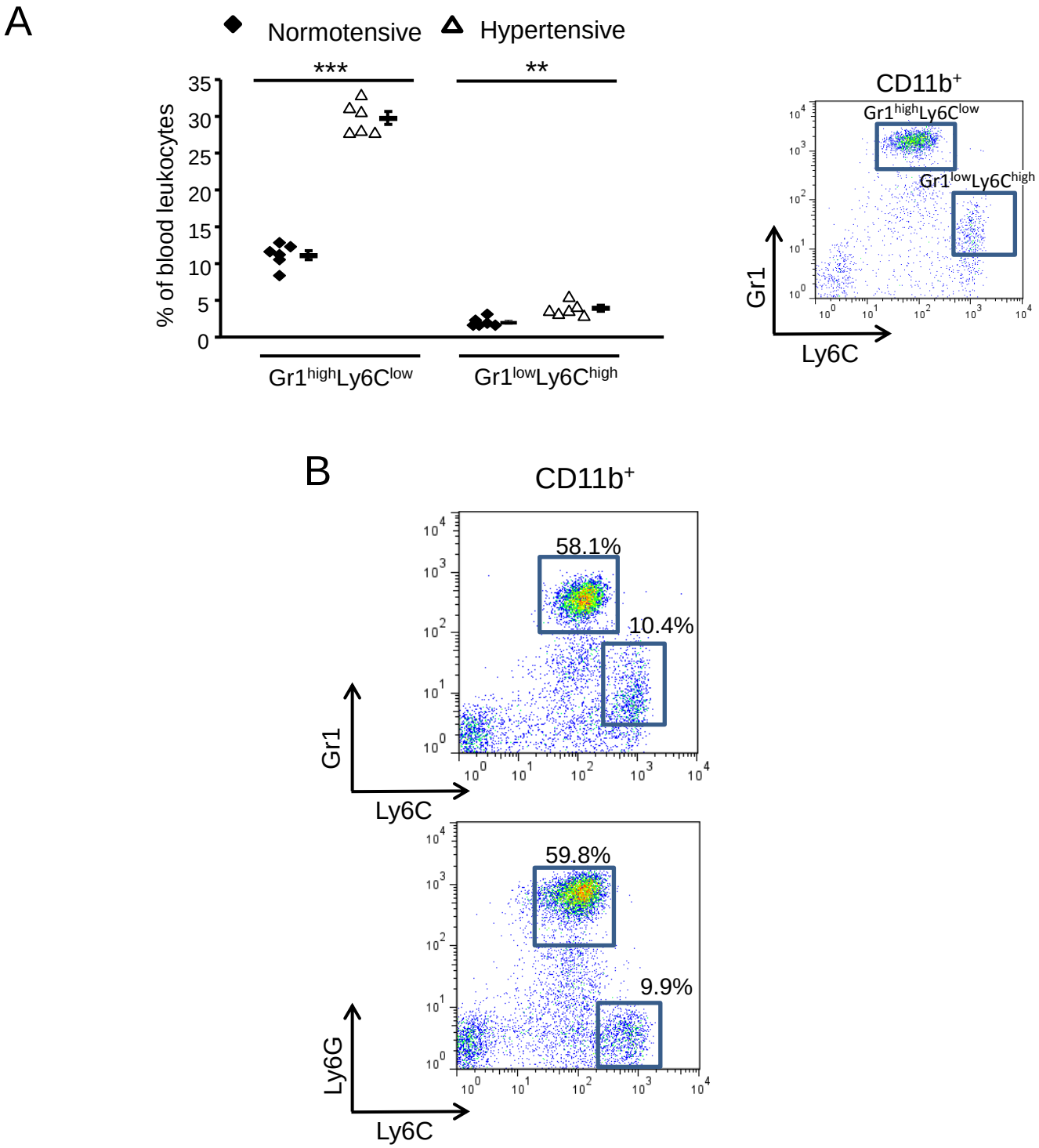

Figure I Myeloid cellularity in the hypertensive mice. **A**, $CD11b^{+}Gr1^{+}$ cells were composed of $Gr1^{high}Ly6C^{low}$ and $Gr1^{low}Ly6C^{high}$ cells. The percentages of these cell subtypes in the blood of normotensive mice and mice treated with Ang II for 3 weeks are indicated. The gating strategy is on the right. **B**, Blood from hypertensive mice were co-stained with anti-CD11b, anti-Ly6C and anti-Gr1 or anti-Ly6G antibodies. It shows that the subpopulations of $CD11b^{+}Gr1^{high}Ly6C^{low}$ and $CD11b^{+}Gr1^{low}Ly6C^{high}$ correspond to $CD11b^{+}Ly6G^{high}Ly6C^{low}$ and $CD11b^{+}Ly6G^{-}Ly6C^{high}$ subpopulations, respectively.

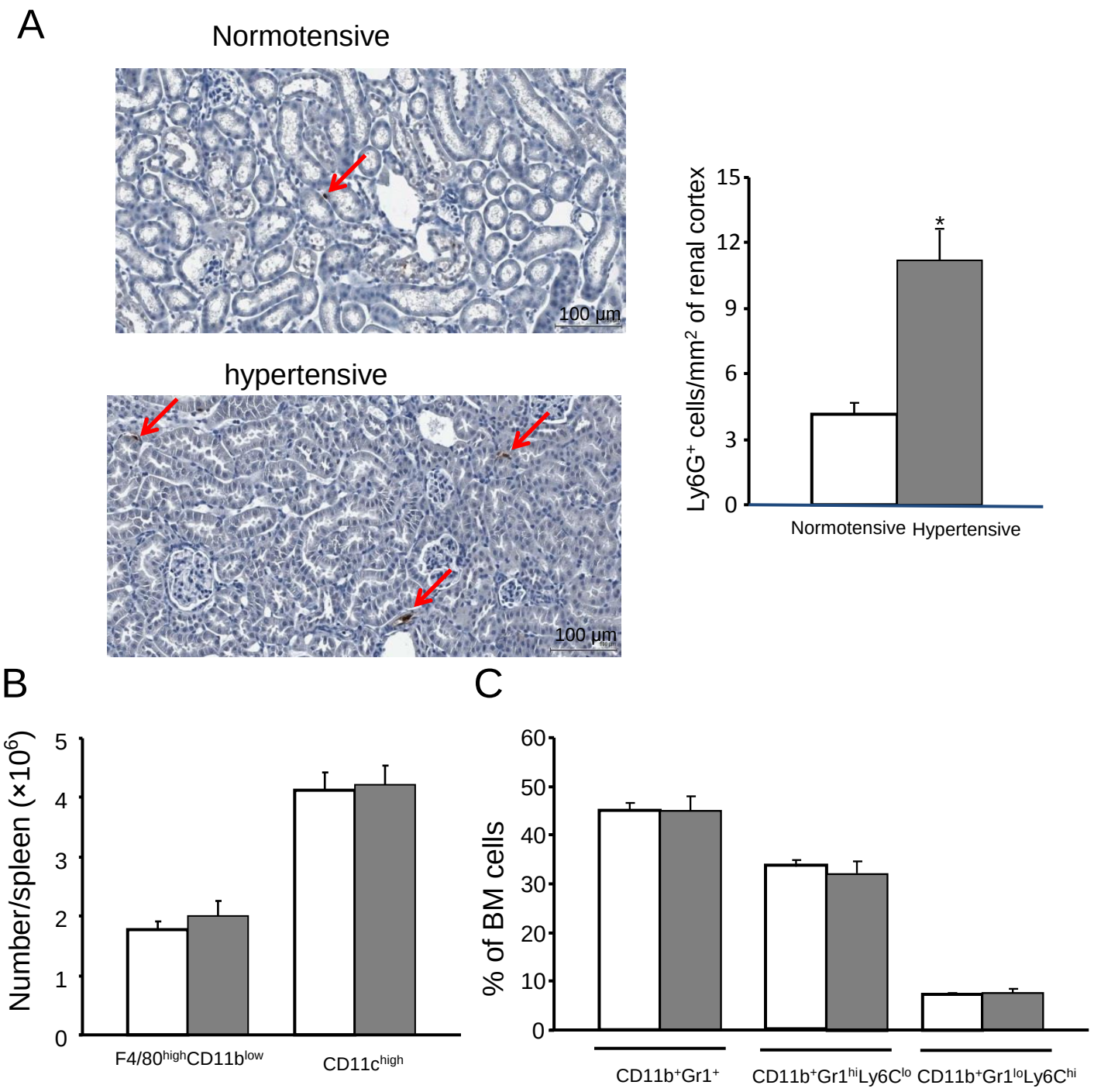

Figure II. A, Renal immunohistochemistry of Gr1 shows increased $Gr1^+$ cell aggregation after 3 weeks of Ang II treatment. Arrow heads point at $Ly6G^+$ cells. A total of 20 random fields from each renal cortex were analyzed with ImageJ (version 1.46r; National Institutes of Health, Bethesda, MD). *$P < 0.05$, n=3. **B**, Numbers of $F4/80^{high}CD11b^{low}$ red pulp macrophages and $CD11c^{high}$ conventional dendritic cells in the spleens of normotensive mice (white) and mice treated with Ang II for 3 weeks (grey). **C**, Percentages of $CD11b^+Gr1^+$ cells, as well as their $Gr1^{high}Ly6C^{low}$ and $Gr1^{low}Ly6C^{high}$ subpopulations in the BM. n=6 mice per group.

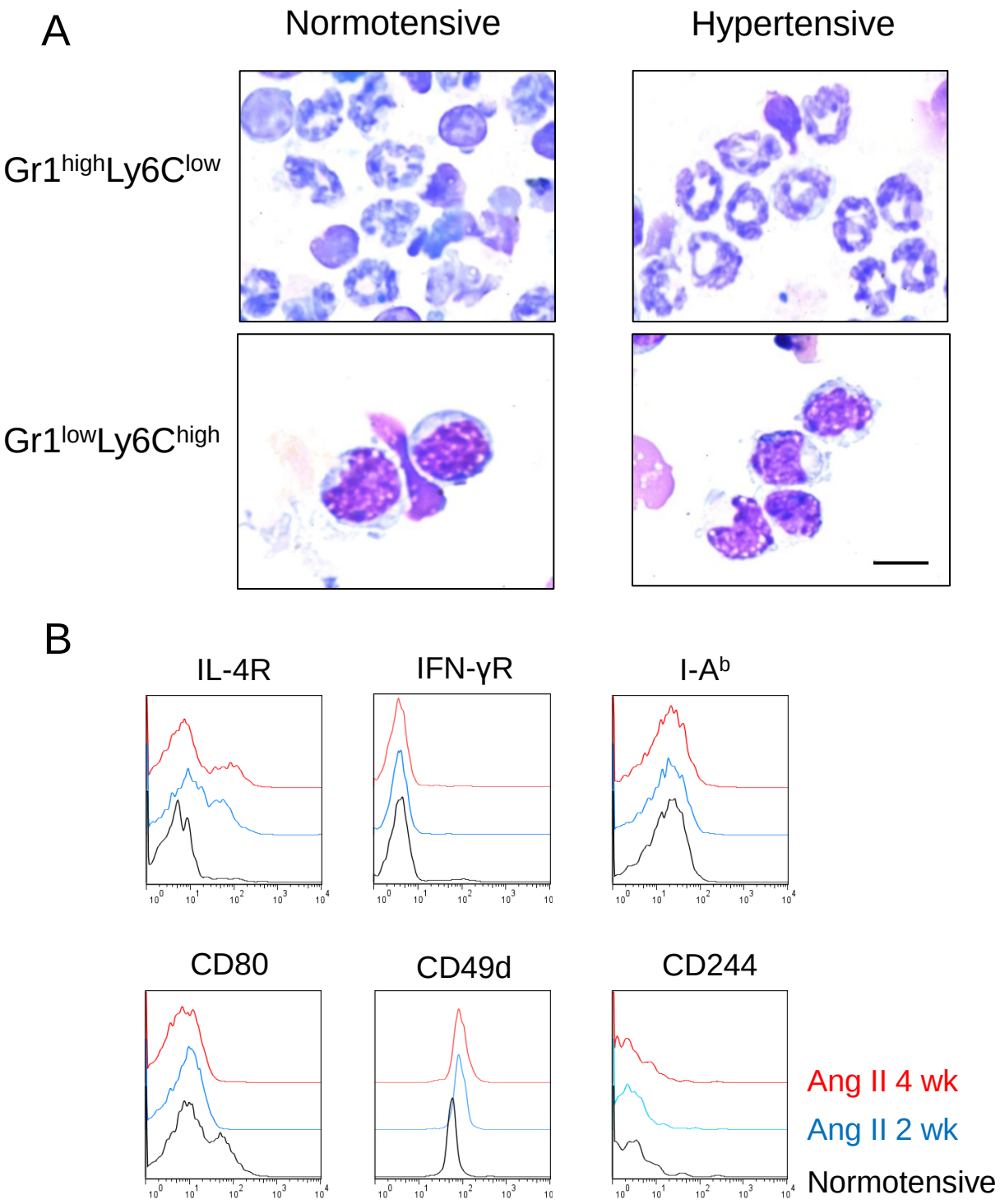

Figure III. The phenotype of CD11b^{+}Gr1^{+} cells. **A**, Wright-Giemsa staining of CD11b^{+}Gr1^{high}Ly6C^{low} and CD11b^{+}Gr1^{low}Ly6C^{high} cells isolated from spleens of normotensive mice and mice treated with Ang II for 3 weeks. Representative pictures from 3 pairs of mice. **B**, The surface expression of functional and maturation markers. The blood was collected from normotensive mice and the mice induced with hypertension for 2 or 4 weeks by Ang II. CD11b^{+}Gr1^{+} cells were examined for their surface expression of functional and maturation markers. Representative histograms from 8 mice per groups.

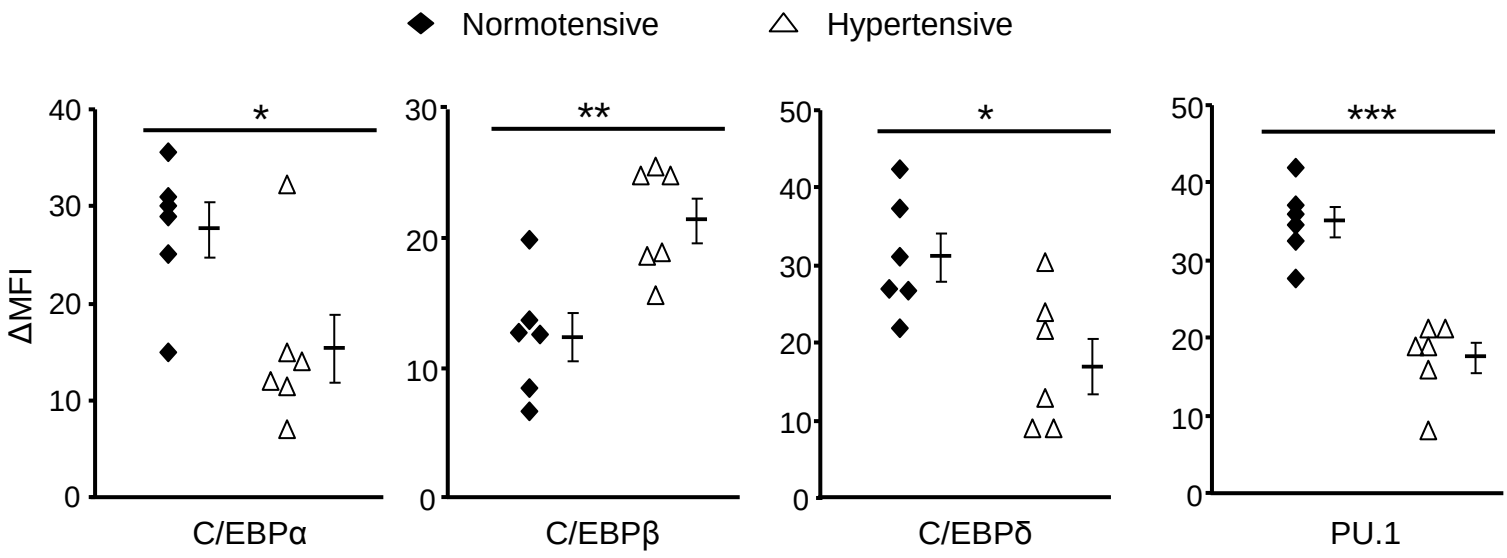

Figure IV. The transcription phenotype of hypertensive MDSCs. Splenic $CD11b^{+}Gr1^{+}$ cells were isolated from normotensive or hypertensive mice (Ang II treatment for 3 weeks). The expression of myeloid transcription factors was assessed by FCM. **P* < 0.05; ***P* < 0.01; ****P* < 0.005.

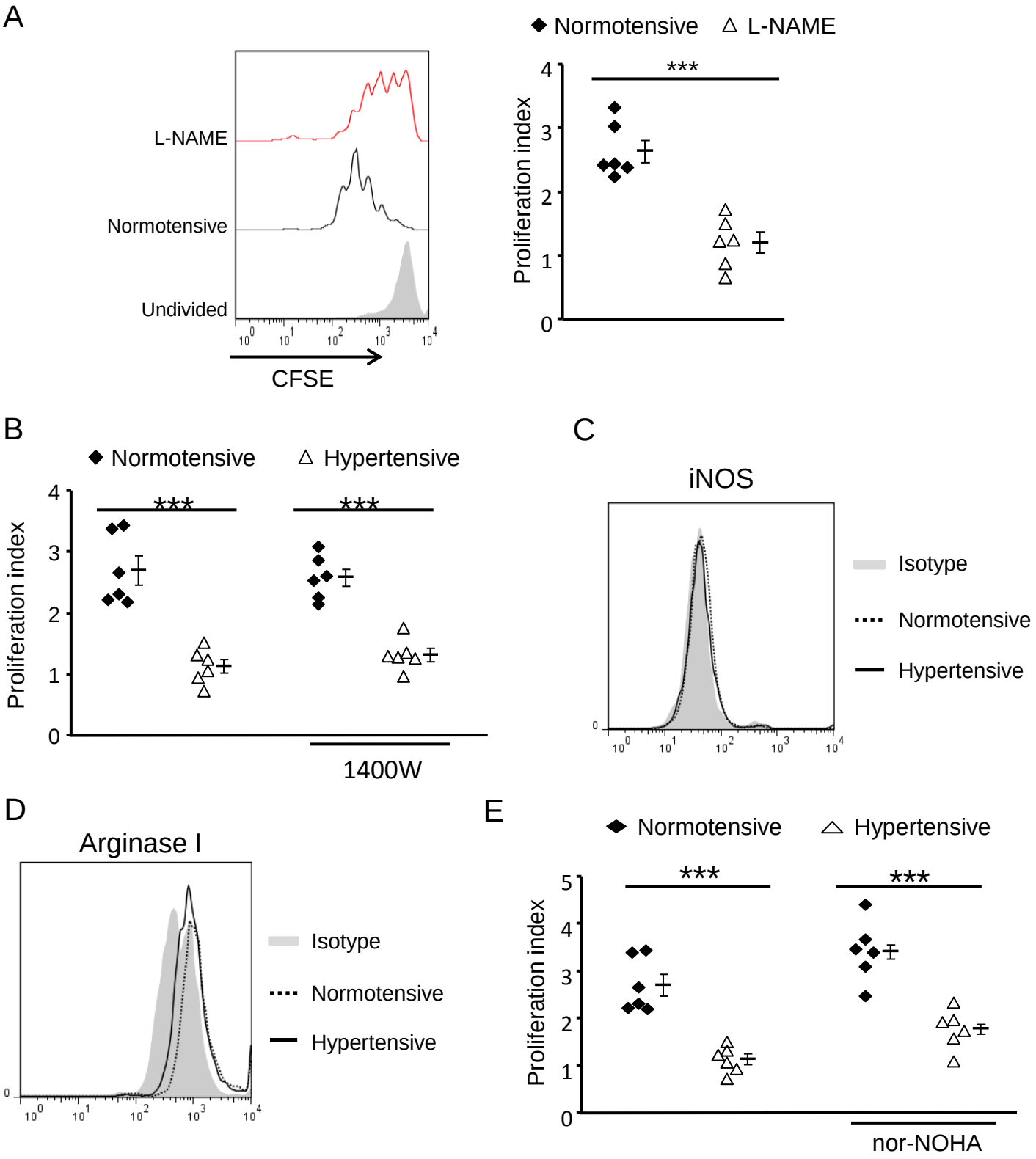

Figure V. Neither iNOS nor arginase I is the major effector mediating MDSC function. **A**, An immunosuppressive assay was performed by incubating splenocytes from C57BL/6 mice with CD11b^{+}Gr1^{+} cells from normotensive mice or mice treated with L-NAME for 4 weeks. T cells were stimulated with anti-CD3/anti-CD28 antibodies. **B**, The immunosuppressive assay was performed similarly to **A**, except that the hypertensive MDSCs were from Ang II-treated mice. In some groups, 10 μM 1400W was used in the 3-day assay. **C** iNOS and **D** arginase I expression in CD11b^{+}Gr1^{+} cells from normotensive mice and Ang II-treated hypertensive mice was evaluated by FCM. Representative histograms are shown from 4 different experiments. **E**, The immunosuppressive assay was performed similar to **B** except that in some groups 50 μM nor-NOHA was used in the 3-day assay. ***$P < 0.005$.

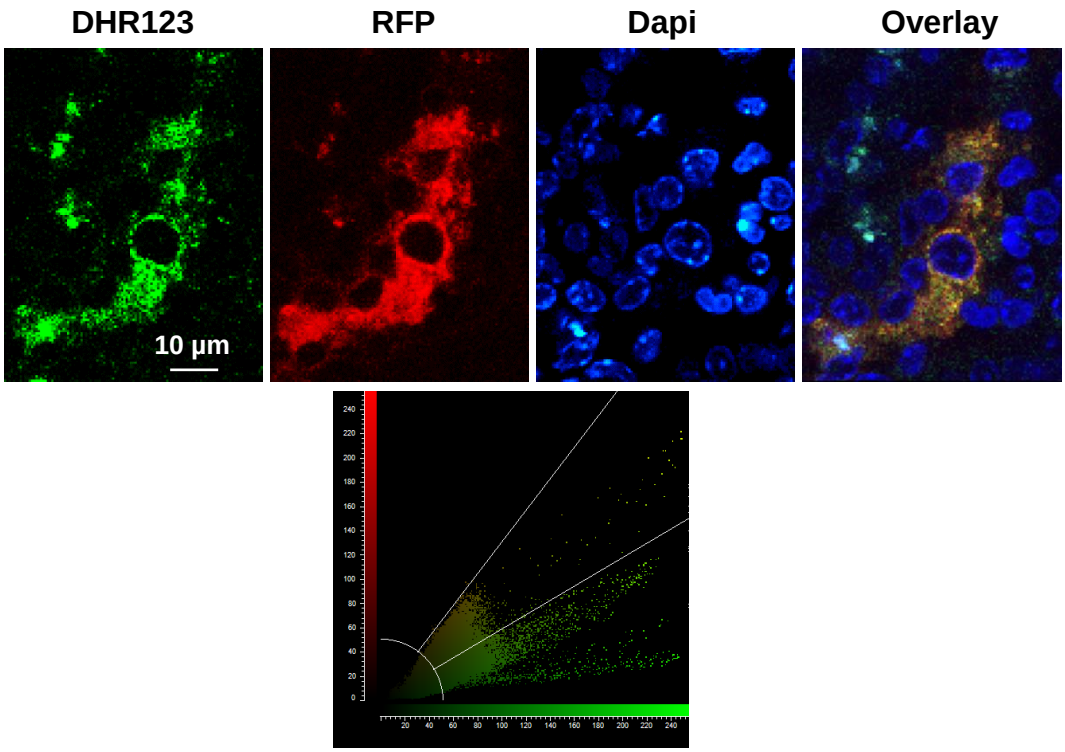

Figure VI. Hypertensive MDSCs express ROS *in vivo*. MDSCs were collected from red fluorescent protein (RFP) transgenic mice 2 weeks after Ang II infusion, and transferred i.v. into normotensive WT mice. Twenty-four hours later, the recipients were given i.p. dehydrorhodamine 123 (DHR 123) ; after another hour, mice were harvested and their spleen were snap frozen and prepared for confocal microscopy. The cytofluorogram below shows that almost all RFP^+ MDSCs are producing ROS.

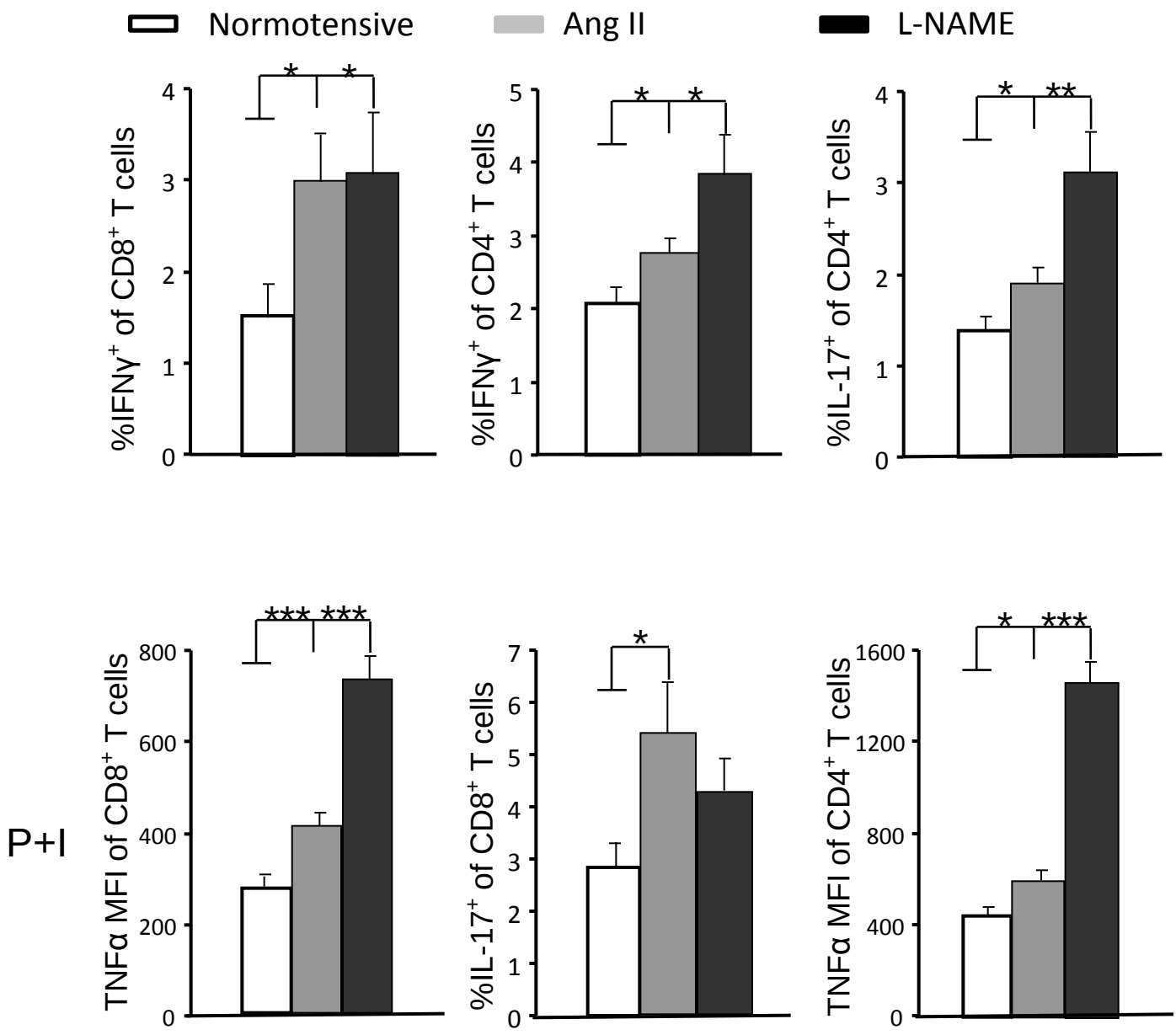

Figure VII. T cells are activated in hypertension. Mice were induced with hypertension by infusion with Ang II or L-NAME in the drinking water. Three weeks later, the spleens were harvested and the splenocytes were incubated with brefeldin A for 6 hours. Some of the cells were stimulated with PMA and ionomycin (P+I) during the 6 hour *ex vivo* incubation. Cells were then intracellularly stained for IFNγ, TNFα and IL-17 together with surface staining with CD3, CD4 and CD8 and studied by FCM. n=6-10. **P* < 0.05; ***P* < 0.01; ****P* < 0.005.

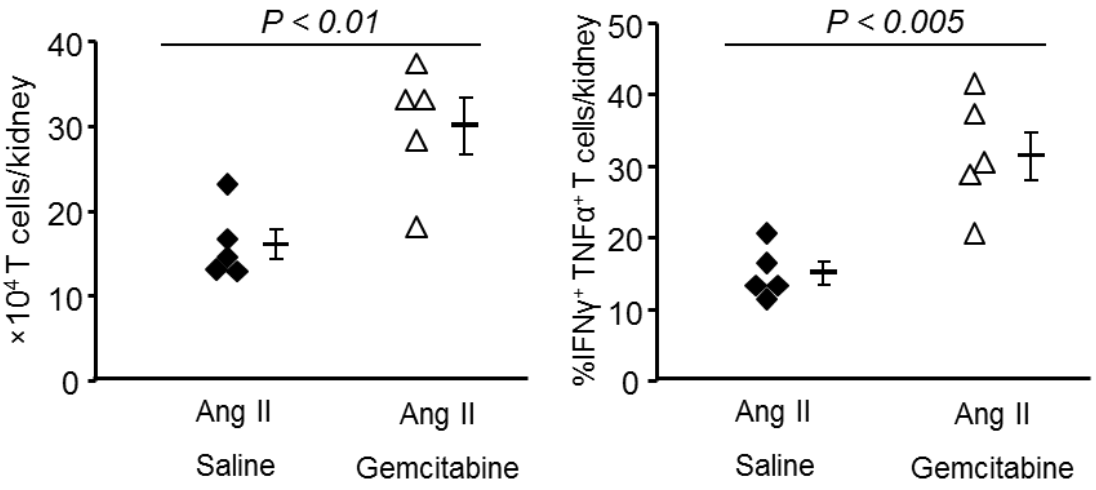

Figure VIII. Depletion of MDSCs by gemcitabine intensified T cell inflammation in the kidney of hypertensive mice. Mice were made hypertensive with Ang II. Starting on day 10, MDSCs were depleted with gemcitabine or saline control. At week 4, kidneys were harvested and T cell numbers were counted. Also, cells were stimulated with PMA and ionomycin and then assayed for the percentage of T cells expressing IFNγ and TNFα.

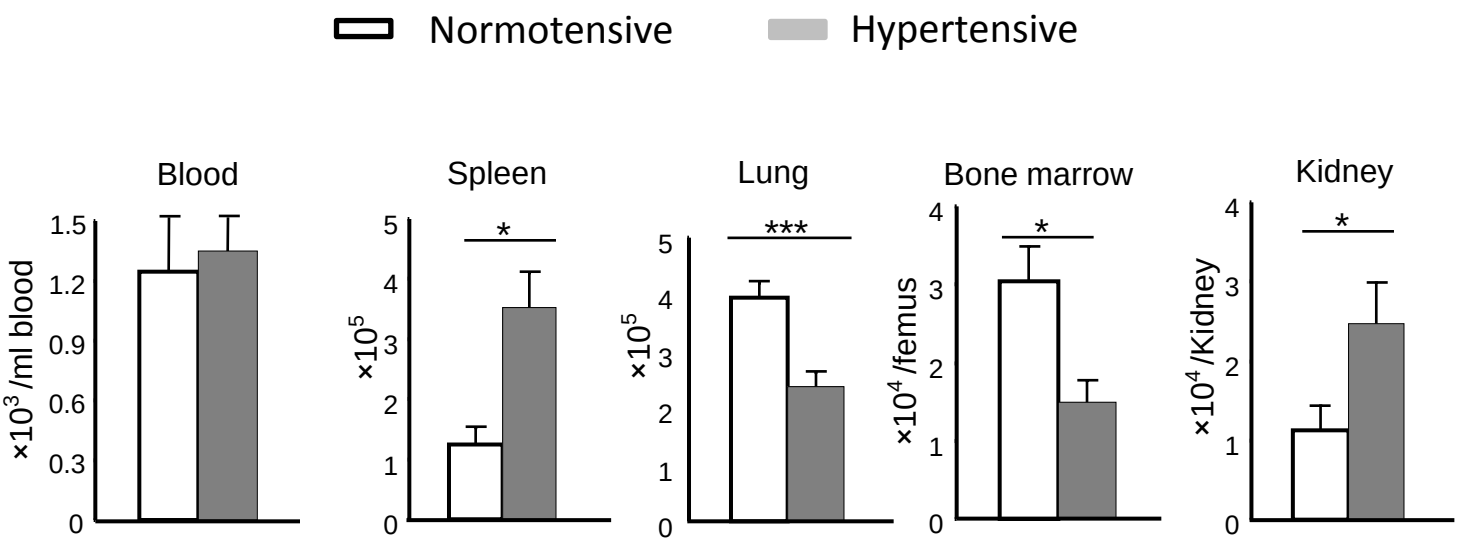

Figure IX. Distribution of MDSCs after transfer. 2×10^6 MDSCs derived from hypertensive GFP transgenic mice were transferred into normotensive and hypertensive mice. Twenty-four hours later, the GFP^+ cells in the blood, spleen, lung, bone marrow and kidney were counted. n=4. $^*P < 0.05$; $^{***}P < 0.005$.