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ORIGINAL RESEARCH

Sympathetic Enhancement of Memory T-Cell Homing and Hypertension Sensitization

Liang Xiao, Luciana Simao do Carmo, Jason D. Foss, Wei Chen, David G. Harrison

RATIONALE: Effector memory T lymphocytes (T_{EM} cells) exacerbate hypertension in response to repeated hypertensive stimuli. These cells reside in the bone marrow for prolonged periods and can be reactivated on reexposure to the hypertensive stimulus.

OBJECTIVE: Because hypertension is associated with increased sympathetic outflow to the bone marrow, we hypothesized that sympathetic nerves regulate accumulation and reactivation of bone marrow–residing hypertension-specific T_{EM} cells.

METHODS AND RESULTS: Using unilateral superior cervical ganglionectomy in wild-type C57BL/6 mice, we showed that sympathetic nerves create a bone marrow environment that supports residence of hypertension-specific $CD8^+$ T cells. These cells, defined by their proliferative response on coculture with dendritic cells from Ang (angiotensin) II–infused mice, were reduced in denervated compared with innervated bone of Ang II–infused mice. Adoptively transferred $CD8^+$ T cells from Ang II–infused mice preferentially homed to innervated compared with denervated bone. In contrast, ovalbumin responsive T cells from OT-I mice did not exhibit this preferential homing. Increasing superior cervical ganglion activity by activating Gq-coupled designer receptor exclusively activated by designer drug augmented $CD8^+$ T_{EM} bone marrow accumulation. Adoptive transfer studies using mice lacking β2AR (β2 adrenergic receptors) indicate that β2AR in the bone marrow niche, rather than T-cell β2AR is critical for T_{EM} cell homing. Inhibition of global sympathetic outflow using Gi-coupled DREADD (designer receptor exclusively activated by designer drug) injected into the rostral ventrolateral medulla or treatment with a β2AR antagonist reduced hypertension-specific $CD8^+$ T_{EM} cells in the bone marrow and reduced the hypertensive response to a subsequent response to low dose Ang II.

CONCLUSIONS: Sympathetic nerves contribute to the homing and survival of hypertension-specific T_{EM} cells in the bone marrow after they are formed in hypertension. Inhibition of sympathetic nerve activity and β2AR blockade reduces these cells and prevents the blood pressure elevation and renal inflammation on reexposure to hypertension stimuli.

VISUAL OVERVIEW: An online visual overview is available for this article.

Key Words: angiotensin II ■ dendritic cells ■ ganglionectomy ■ hypertension ■ inflammation

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Accumulating evidence from the past decade indicates that adaptive immunity, and especially T lymphocytes, plays a crucial role in the development of hypertension. Various hypertensive stimuli, such as Ang (angiotensin) II, high salt, catecholamines, and chronic psychological stress, lead accumulation of activated T cells with an effector phenotype in the kidney and vasculature.[1–4] Cytokines released from these cells, including interferon-γ and interleukin-17A, promote both renal and vascular dysfunction and damage, leading to enhanced sodium retention and increased systemic vascular resistance.[5]

The majority of activated T cells ultimately die after antigen withdrawal and resolution of an immune response; however, a few remaining cells become memory T cells that can persist for years in humans. On antigen reexposure,

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Novelty and Significance

What Is Known?

- Adaptive immunity contributes to the cause of hypertension and associated end-organ damage.
- Effector memory T cells (T_{EM}) reside in the bone marrow and can be reactivated by antigen reexposure.
- Bone marrow sympathetic drive is increased in hypertension.

What New Information Does This Article Contribute?

- β2 adrenergic signaling preferentially mediates the accumulation of hypertension-specific T_{EM}
- β2 adrenergic blockade prevents sensatization to repeated hypertensive stimuli by creating a bone marrow environment that is hostile to survival of hypertension-specific memory T cells.

T_{EM} cells play a crucial role in the blood pressure elevation and the renal dysfunction caused by repeated hypertensive stimuli. Formed during an initial immune challenge, T_{EM} cells reside in the bone marrow in a quiescent state for prolonged periods and can be reactivated on reexposure to the hypertensive stimulus. Hypertension is associated with increased sympathetic outflow. We performed sympathetomy and used DREADD (designer receptors exclusively activated by designer drugs) methodology to manipulate local and systemic sympathetic drive, and showed that the bone marrow homing of $CD8^+$ T_{EM} cells is guided by sympathetic innervation. We further found that β2 adrenergic receptors in the bone marrow are critical in mediating this process. Genetic deletion or pharmacological blockade of β2 adrenergic receptors protects mice from repeated hypertensive stimuli. These data define a novel role of sympathetic nerves in regulating memory T-cell trafficking in hypertension. We propose that even a short course of sympatholytics, nonselective β-blockers, or β2 antagonists could create an environment hostile to the survival of T_{EM} cells, and thus protect against future episodes of hypertension and the long-term end-organ damage that accompanies this disease.

Nonstandard Abbreviations and Acronyms

β2AR	β2 adrenergic receptor
AAV	adeno-associated virus
Adrb2$^{-/-}$	beta 2 adrenergic knockout
Ang II	angiotensin II
CCL	ligand chemokine containing cysteine-cysteine motifs
CCR	C-C chemokine receptor
DC	dendritic cell
DREADD	designer receptor exclusively activated by designer drug
ICAM	intracellular adhesion molecule
RVLM	rostral ventrolateral medulla
SCG	superior cervical ganglion
SCGx	superior cervical ganglionectomy
T_{EM}	effector memory T cells

these memory cells can be rapidly reactivated. Memory T cells have been subdivided into ($CD62L^{hi}$/$CD44^{hi}$) central memory cells that predominantly reside in secondary lymphoid organs, ($CD62L^{lo}$/$CD44^{hi}$) effector memory (T_{EM}) cells that remain in the circulation and patrol between peripheral tissues, and resident memory cells that reside and regenerate in peripheral tissues.

The bone marrow plays a central role in the maintenance of long-term T-cell memory. It provides a dedicated niche for memory $CD8^+$ T cells to maintain a nonproliferative quiescent state or self-renewal in the absence of differentiation.[6] After immunization or viral infection, a higher percentage of memory $CD8^+$ T cells proliferate in the bone marrow than in the spleen or lymph nodes.[7,8] Estimates of cell numbers suggest that the bone marrow contributes a large proportion of proliferating memory $CD8^+$ T cells compared with the other secondary lymphoid organs.

Since many hypertensive stimuli are intermittent and reoccurring, including sleep apnea, repeated episodes of dietary indiscretion, or emotional stress, it is likely that memory T cells play a role in hypertension. We recently showed that T_{EM} cells accumulate in the kidney and bone marrow following repeated hypertensive challenges, using either N(ω)-nitro-L-arginine methyl ester hydrochloride followed by high salt or repeated Ang II stimulation.[9] In the kidney, memory T cells are predominant sources of interferon-γ and interleukin-17A.[9] In the N(ω)-nitro-L-arginine methyl ester hydrochloride/high-salt mouse model of hypertension, we found that bone marrow–residing T_{EM} cells proliferate and redistribute to the kidney in response to repeated salt feeding.[9] In this study, we also showed that mice that cannot form memory cells are protected against repeated hypertensive stimuli.

The sympathetic nervous system provides efferent input to the bone marrow and modulates hematopoiesis and the stem-cell niche.[10] Adrenergic nerves play a key role in the circadian recruitment of leukocytes to tissues including the bone marrow.[11] In hypertension, sympathetic tone is elevated, but its circadian rhythmicity is

reduced.[12] In the current study, we tested the hypothesis that sympathetic nerves regulate accumulation and reactivation of hypertension-specific memory T lymphocytes in the bone marrow. Our data suggest new therapeutic interventions to reduce the propensity for homing and survival of hypertension-specific T cells in the bone marrow will protect against blood pressure elevation and end-organ damage in response to repeated hypertensive stimuli.

METHODS

An extended methods section is available in the Online Data Supplement. The authors declare that all supporting data are available within the article and in the Online Data Supplement. All methods have corresponding literature reference. Additional protocol information is available from the corresponding author on reasonable request.

Animals Studied

Wild-type male C57BL/6 mice, B6 Cd45.1, Adrb2$^{-/-}$, and OT-I mice on a C57Bl/6 background were originally obtained from Jackson Laboratories and were studied at 3 months of age. Hypertension was induced by subcutaneous infusion of Ang II (490 ng/kg per minute) via mini-osmotic pumps for 2 weeks unless otherwise indicated. For unilateral superior cervical ganglionectomy (SCGx), mice were anesthetized by intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg). The left superior cervical ganglion (SCG) was identified underneath the left carotid bifurcation and was removed. For unilateral DREADD (designer receptor exclusively activated by designer drug) gene transduction in the SCG, an adeno-associated viral (AAV) vector (6×10^7particles in 50 nL) was injected into the SCG on one side with a 34-gauge needle attached to a 2.5-µL micro syringe. The expression of Gq-coupled hM3D DREADD fused with mCherry was under the control of neuron-specific human synapsin promoter. One week after SCGx or DREADD gene transduction, osmotic minipumps were implanted subcutaneously for infusion of Ang II or vehicle for 2 weeks. To perform T-cell adoptive transfer, splenic pan-T cells were obtained from donor mice by magnetic separation with a negative selection kit. Ten million cells were suspended in 200 µL PBS and adoptively transferred to naïve mice by tail vein injection. For OT-I immunization, mice were injected intraperitoneally with the ovalbumin peptide SIINFEKL (0.5 µg/µL in 200 µL alum adjuvant). For DREADD gene transduction in the rostral ventrolateral medulla (RVLM), mice were anesthetized and mounted in a stereotaxic frame as previously described.[2] An AAV vector encoding Gi-coupled hM4D DREADD fused with mCherry under the control of human synapsin promoter was used, and 12×10^7 particles in 100 nL were injected into the RVLM bilaterally with a 30-gauge needle attached to a 2.5-µL micro syringe. Stereotaxic coordinates were –6.64 mm posterior to bregma, 1.15 mm left and right of the midline, and 5.80 mm ventral to the superior surface of the skull. At study termination, mice were euthanized by exposure to CO_2. The Institutional Animal Care and Use Committee approved all experimental protocols.

Data Presentation and Analysis

Data are expressed as mean±SEM. When local bone marrow sympathetic nervous activity was unilaterally manipulated by SCGx or DREADD, the effects were compared with the contralateral control limb by paired *t* tests as indicated. For other comparisons of 2 variables, unpaired *t* tests were employed. Data normality was confirmed using Anderson-Darling, D'Agostino-Pearson omnibus, Shapiro-Wilk, and Kolmogorov-Smirnov tests before *t* test was applied. To determine the effect of SCGx and β2AR (β2 adrenergic receptor) deficiency, 2-way ANOVA was used as indicated. For telemetry blood pressure measurements over time, 2-way ANOVA with repeated-measures was employed, followed with a Bonferroni post hoc test when significance was indicated. *P* values (or Bonferroni-adjusted *P* values if applicable) are reported in the figures, and a value <0.05 was considered statistically significant. Data were analyzed using GraphPad Prism 8 for Windows 64-bit (San Diego, CA)

RESULTS

Sympathetic Innervation in Bone Marrow

The bone marrow is a highly innervated organ, and sympathetic nerves modulate hematopoiesis and the stem-cell niche.[13] In initial experiments, expression of tyrosine hydroxylase in the bone marrow, a marker of sympathetic innervation, was analyzed by Western blot. In mice with Ang II–induced hypertension, tyrosine hydroxylase was increased in the bone marrow (Figure 1A). To ablate local sympathetic nerves in the bone marrow of forelimbs, we performed unilateral SCGx. The SCG innervates one side of the head and the front limb in mice. Successful removal of SCG resulted in ptosis on the surgical side of mice at conscious state (Figure 1B). Denervation of bone marrow was confirmed by a significant decrease of tyrosine hydroxylase expression in the ipsilateral forelimb by Western blot (Figure 1C). The calvaria of SCGx animals were also collected for confocal fluorescence microscopy, and the geometry of sympathetic nerve fibers in these flat bones could be visualized by tyrosine hydroxylase staining. We observed that sympathetic nerves travels along blood vessels as identified by endothelial marker CD31, and tyrosine hydroxylase staining diminished with SCGx (Figure 1D). These data indicate SCGx as an effective model for studying the effect of local sympathetic nerves in bone marrow.

Effect of Unilateral SCGx on Hypertension-Specific T Cells in the Bone Marrow

Memory T cells formed in hypertension comprise only a small minority of the total T cells population in the bone marrow. Our data indicate about 1% of cells are $CD8^+$ T cells and about 0.5% are $CD4^+$ T cells in the bone marrow, and about one-tenth of the T cells are T_{EM} cells. To detect the response of memory T cells that accumulated in response to hypertension, we developed an assay in

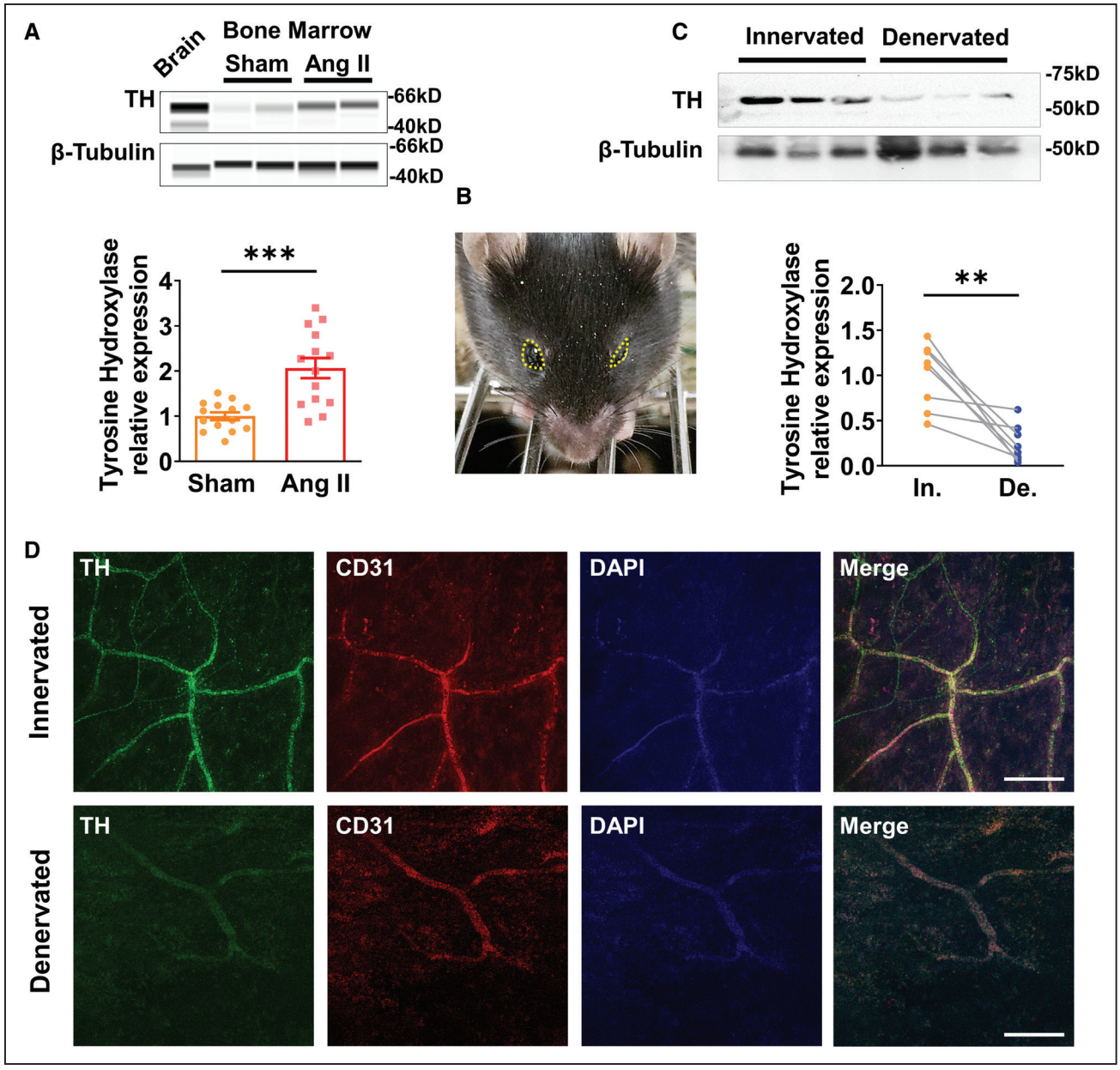

Figure 1. Sympathetic innervation of the bone marrow and the effects of superior cervical ganglionectomy (SCGx). **A**, Western blot analysis of TH (tyrosine hydroxylase) expression in the forelimb bone marrow in mice with 2 wk of sham or Ang (angiotensin) II infusion. Data are expressed as mean±SEM (Sham: 1.000±0.086 vs Ang II: 2.065±0.224), n=14 in each group, P=0.0004 for the effect of Ang II was calculated by unpaired t test with Welch correction. **B**, Example of ptosis resulting from unilateral SCGx. **C**, Western blot analysis of TH expression in the innervated (In.) and denervated (De.) bone marrow. Protein samples were extracted from forelimb bone marrow of humerus, ulna and radius, and β-tubulin was probed as a loading control. Each set of connected symbols represent paired bone marrow samples from the same animal, n=8, P=0.0013 for effect of denervation analyzed by paired t test. **D**, Expression of TH and its colocalization with the vascular marker CD31 were detected in the calvaria from mice that had received unilateral SCGx by confocal fluorescence microscopy. White bars indicate 100 micrometers. **P<0.01 and ***P<0.001

which we cocultured the bone marrow cells with dendritic cells (DCs) isolated from the spleen of another mouse that received sham or Ang II infusion at a 1:10 ratio (Figure 2A). DCs from hypertensive mice present antigens formed in hypertension and can drive proliferation of hypertension-specific T cells.[14] After 7 days of culture, the $CD3^+$ T lymphocytes and specifically the $CD8^+$ T cells amplified by DCs from the hypertensive mice were less when obtained from the denervated compared with the innervated bone marrow (Figure 2B through 2D). Moreover, carboxyfluorescein succinimidyl ester dilution indicated that fewer $CD8^+$ T cells proliferated from the denervated as compared with innervated bone marrow in response to hypertension-specific antigens (Figure 2E and Online Figure I). Dilution pattern modeling indicated that the denervated bone marrow contained

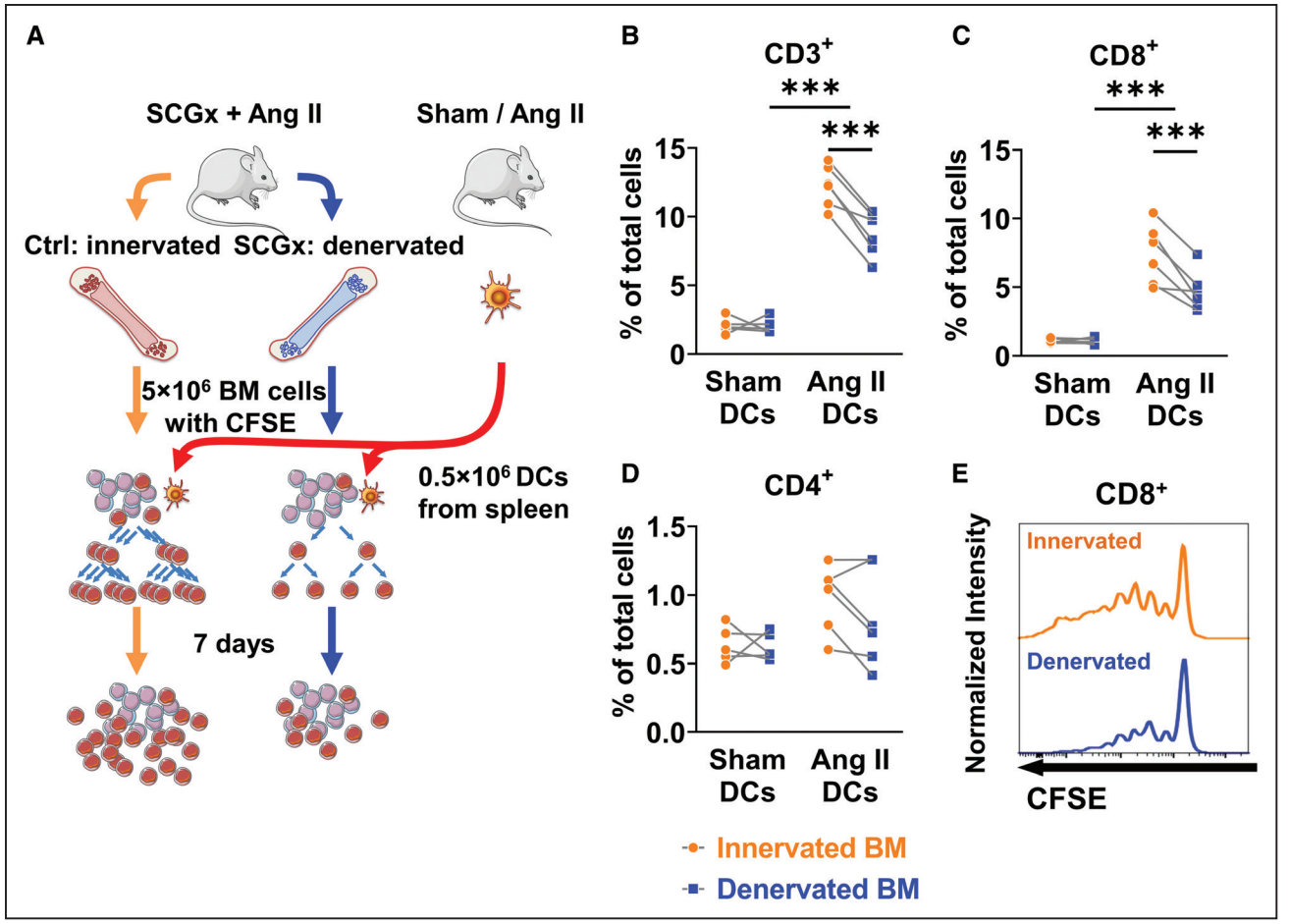

Figure 2. Effects of sympathetic nerves on the accumulation of hypertension-specific T cells in the bone marrow.
A, Dendritic cells (DCs) isolated from sham or Ang (angiotensin) II–infused mice were cultured with bone marrow (BM) cells obtained from either denervated or innervated forelimbs of other hypertensive mice. Bone marrow cells were prelabeled with carboxyfluorescein succinimidyl ester (CFSE). After 7 d in culture, $CD3^+$, $CD4^+$, $CD8^+$ T cells were quantified by flow cytometry (**B**–**D**). Differences in the proliferation of $CD8^+$ T cells from control and denervated bone marrow were determined by CFSE dilution and flow cytometry (**E**). Each set of connected symbols represent paired bone marrow samples from the same animal, n=5 to 6 in each group, $P<0.0001$ for the effect of denervation calculated by paired *t* test is shown. SCGx indicates superior cervical ganglionectomy.

fewer precursor $CD8^+$ T cells (Online Figure IB), but these precursor $CD8^+$ T cells underwent similar numbers of divisions regardless of whether the bone marrow was denervated or not (Online Figure IC). These results indicate that sympathetic innervation promotes residence of memory $CD8^+$ T cells in the bone marrow after they are formed in hypertension. The data also suggest that these memory T cells can be reactivated and proliferate on reexposure to antigens formed in hypertension.

Effect of Local Sympathetic Nerves on T Cells Homing in the Bone Marrow After Hypertension

To test the hypothesis that sympathetic nerves contribute to bone marrow homing of memory T cells after hypertension, we performed adoptive transfer of T cells as shown in Figure 3A. We tracked the bone marrow homing of donor $CD45.2^+$ T cells into either innervated or denervated bone marrow of recipient B6 Cd45.1 mice (Figure 3A and 3B). We found the numbers of adoptively transferred $CD8^+$ T_{EM} cells were consistently lower in the denervated bone marrow as compared with the innervated marrow. This pattern was not observed for total $CD3^+$, $CD4^+$, or naive $CD8^+$ lymphocytes from the donors or for central memory T cells (Online Figure II). As the recipient B6 Cd45.1 mice were not hypertensive, these results indicate that sympathetic nerves regulate $CD8^+$ T_{EM} homing in the bone marrow even in the absence of hypertension.

In additional experiments, we increased local sympathetic activity by injecting an AAV vector encoding Gq-DREADD into SCG unilaterally (Figure 3A). Successful induction of the AAV gene product was confirmed by coexpression of an mCherry-fused transgene in postganglionic fibers of the bone marrow. These colocalized with tyrosine hydroxylase (Figure 3E). After T-cell adoptive transfer, the DREADD specific ligand clozapine-N oxide was given in the drinking water to

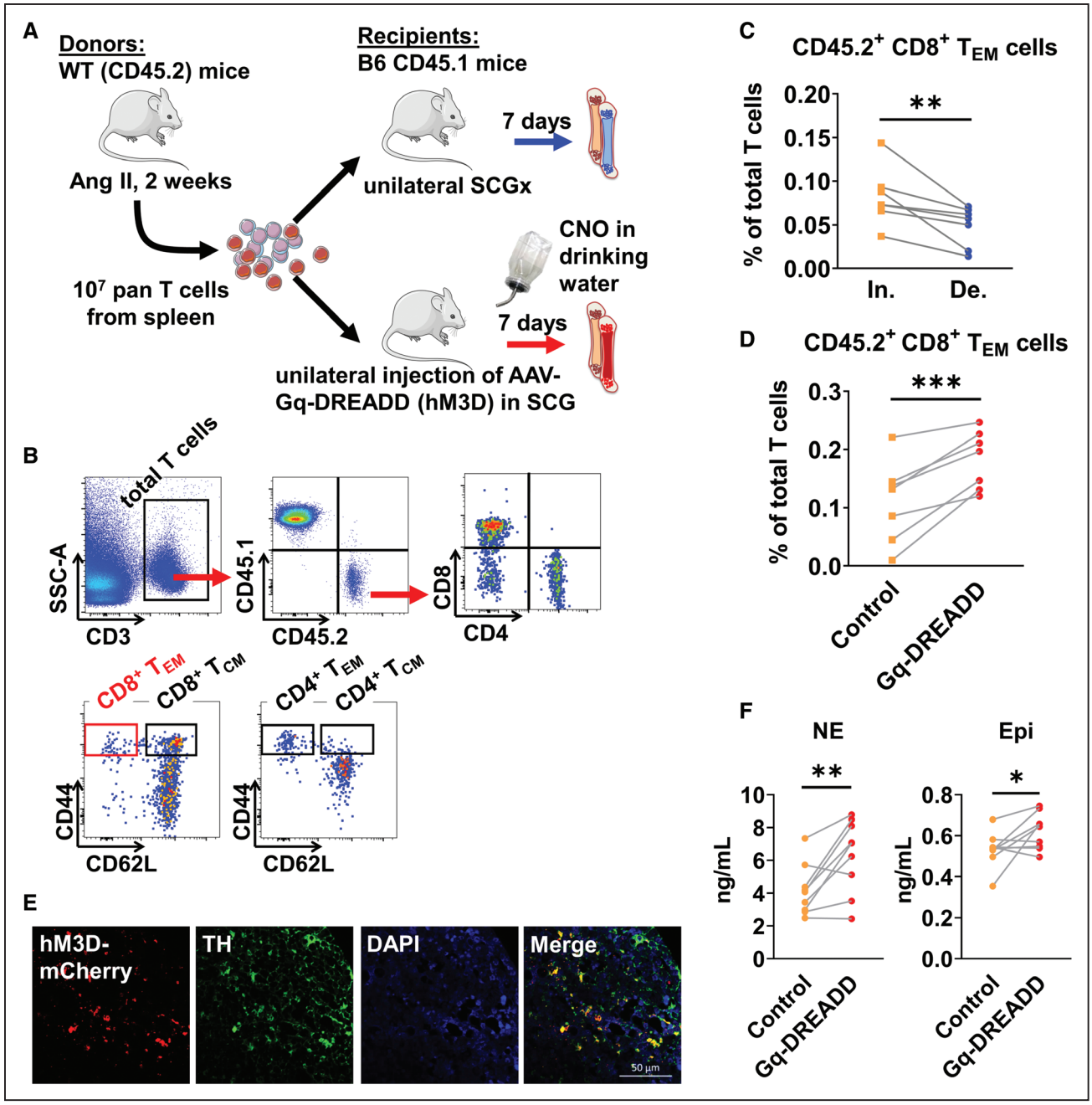

Figure 3. Effects of sympathetic nerves on the accumulation of CD8⁺ effector memory T cells in the bone marrow after hypertension.

A, Splenic pan-T cells were isolated from hypertensive wild-type CD45.2 donor and adoptively transferred to CD45.1 recipient that either had unilateral superior cervical ganglionectomy (SCGx) or adeno-associated virus (AAV) expressing either a control or Gq-DREADD (designer receptor exclusively activated by designer drug) injected into the superior cervical ganglion (SCG). In the case of the Gq-DREADD experiments, clozapine-N-oxide (CNO) was administered in the drinking water for a week after adoptive transfer. One week later, recipient forelimb bone marrow was analyzed by flow cytometry. **B**, A representative sample showing the gating strategy of central memory T cells (T_{CM}) and effector memory T cells (T_{EM}) in both CD4⁺ and CD8⁺ population from donor mice. CD8⁺ T_{EM} cells are emphasized in red. After adoptive transfer, CD45.2⁺/CD8⁺ T_{EM} cells were detected in the recipient mice with unilateral SCGx or those that had unilateral Gq-DREADD activation in SCG. The cells were quantified respectively in (**C**; innervated vs denervated, P=0.0073) and (**D**; control vs Gq-DREADD, P=0.0009), n=7 in each experiment. Expression of mCherry tagged Gq-DREADD was detected by confocal fluorescence microscopy in the SCG-innervated bone marrow and was colocalized with sympathetic nerve marker tyrosine hydroxylase (TH; **E**, white bars indicate 50 micrometers). Levels of norepinephrine (NE) and epinephrine (Epi) in the bone marrow were measured by high performance liquid chromatography (**F**), n=9 in both groups. Each set of connected symbols represent paired bone marrow samples from the same animal, P=0.0040 in norepinephrine and P=0.0255 in epinephrine for the effect of Gq-DREADD as calculated by paired t test. WT indicates wild-type. *P<0.05 and **P<0.01.

augment local sympathetic nerve activity. Clozapine-N oxide treatment was accompanied by an increase in tissue norepinephrine levels as measured by high performance liquid chromatography (Figure 3F). In contrast to our results with denervation, augmenting sympathetic nerve activity in bone marrow promoted $CD8^+$ T_{EM} homing (Figure 3D and Online Figure III). Thus, by manipulating local sympathetic outflow to the bone marrow, we found that sympathetic tone modulates $CD8^+$ T_{EM} cell homing to the bone marrow even under baseline conditions.

Role of β2 Adrenergic Receptors in T-Cell Bone Marrow Homing

β-adrenergic signaling, and especially β2 adrenergic receptors, has been previously shown to regulate multiple cellular processes that contribute to the physiological function of bone and bone marrow.[11,15] To address a role of β adrenergic receptors in T-cell homing, bone marrow cells from either sham or Ang II–infused B6 Cd45.1 mice were placed in the lower chamber of a transwell device and pan-T cells isolated from wild-type (CD45.2) mice were placed in the upper chamber (Figure 4A). In initial experiments, we observed that a significantly higher number of $CD8^+$ T_{EM} cells migrated to bone marrow derived from Ang II–infused compared with sham-infused mice (Figure 4B and Online Figure IV). We performed additional transmigration assays in which we added either norepinephrine (1 μmol/L), norepinephrine, and the β2AR antagonist ICI118551 (10 nmol/L) or the β2AR agonist salbutamol (1 μmol/L) to the medium. Norepinephrine enhanced $CD8^+$ T_{EM} migration to the bone marrow cells, and this was blocked by ICI118551 (Figure 4C and Online Figure V). The β2AR agonist salbutamol potently enhanced $CD8^+$ T_{EM} cell transmigration to the bone marrow cells. These effects of norepinephrine and salbutamol were identical for bone marrow obtained from either innervated or denervated bones (data not shown).

To further determine if β2 adrenergic receptors promote memory T-cell homing in vivo, we performed T-cell adoptive transfer between B6 Cd45.1 mice and mice that were deficient of $Adrb2^{-/-}$ (β2 adrenergic receptors) as shown in Figure 4D. Similar to the experiments in Figure 3, the T-cell donors received Ang II infusion for 2 weeks, and the recipients underwent unilateral SCGx before adoptive transfer. When T cells were isolated from $Adrb2^{-/-}$ donors and adoptively transferred to B6 Cd45.1 recipients, we observed a pattern identical to that observed in mice with intact β2 adrenergic receptors; that is, $CD8^+$ T_{EM} homed to both the innervated and denervated bone marrow, albeit to a lesser extent to the denervated limb. Interestingly, if the T cells from B6 Cd45.1 donors were transferred to $Adrb2^{-/-}$ mice, $CD8^+$ T_{EM} homing was virtually eliminated, whether the bone marrow was denervated or not (Figure 4E). Of note, this phenomenon was only seen for $CD8^+$ T_{EM} cells but not in other T-cell populations (Online Figure VI). These studies indicate that β2ARs in the bone marrow niche, but not in T cells, mediate the effects of sympathetic tone on $CD8^+$ T_{EM}-cell migration.

Specificity of Sympathetic Regulation of T Cells Homing in the Bone Marrow

T-cell migration is a multistep process initiated by selectin-mediated rolling on the endothelium. Subsequently, CCR7 (C-C chemokine receptor 7) binding CCL (C-C motif ligand) chemokines CCL19 and CCL-21a leads to the activation of cell-surface integrin adhesion molecules in T cells, which binds to its ligands ICAM (intracellular adhesion molecule)-1 and VCAM (vascular cell adhesion molecule)-1. We, therefore, examined bone marrow expression of CCL19 and CCL-21 and found that denervation reduced mRNA expression of both of these ligands. In contrast, ICAM-1 and VCAM-1 expression were not affected by denervation (Figure 5).

To examine specificity for homing of $CD8^+$ T_{EM} cells from hypertensive mice, we performed adoptive transfer using T cells from OT-I transgenic mice that express a transgenic T-cell receptor that recognizes the ovalbumin peptide 257 to 264 SIINFEKL in the context of major histocompatibility complex class 1 H2Kb. One week after injection of ovalbumin peptide containing serine, isoleucine, isoleucine, phenylalanine, glutamate, leucine, leucine, we observed robust expansion $CD8^+V_\beta5^+$ T cells observed in the blood and spleen (Online Figure VII). At this time splenic pan-T cells were isolated and injected into the tail vein of a recipient mouse that had undergone unilateral SCGx (Figure 6A). In contrast to $CD8^+$ T_{EM} cells that developed in response to hypertension, bone marrow homing of the OT1 $CD8^+V_\beta5^+$ T cells was not affected by SCGx (Figure 6B through 6D). Thus, sympathetic tone is not required for all memory cell homing to the bone marrow but enhances homing of hypertension-specific T_{EM} cells.

Role of Sympathetic Outflow During T-Cell Homing in Hypertension

The above experiments indicate that sympathetic nerves and β2ARs play a role in T_{EM}-cell migration to the bone marrow. We hypothesized that this sympathetic mediation of T_{EM} cell homing contributes to the development of recurrent hypertension. To address this, we performed bilateral microinjection of an AAV vector encoding inhibitory Gi-DREADD into the RVLM, which contains the presympathetic neurons of the brain stem (Figure 7A and 7B). Global sympathetic tone was temporarily inhibited by adding clozapine-N oxide to the drinking water during T-cell adoptive transfer. This temporary

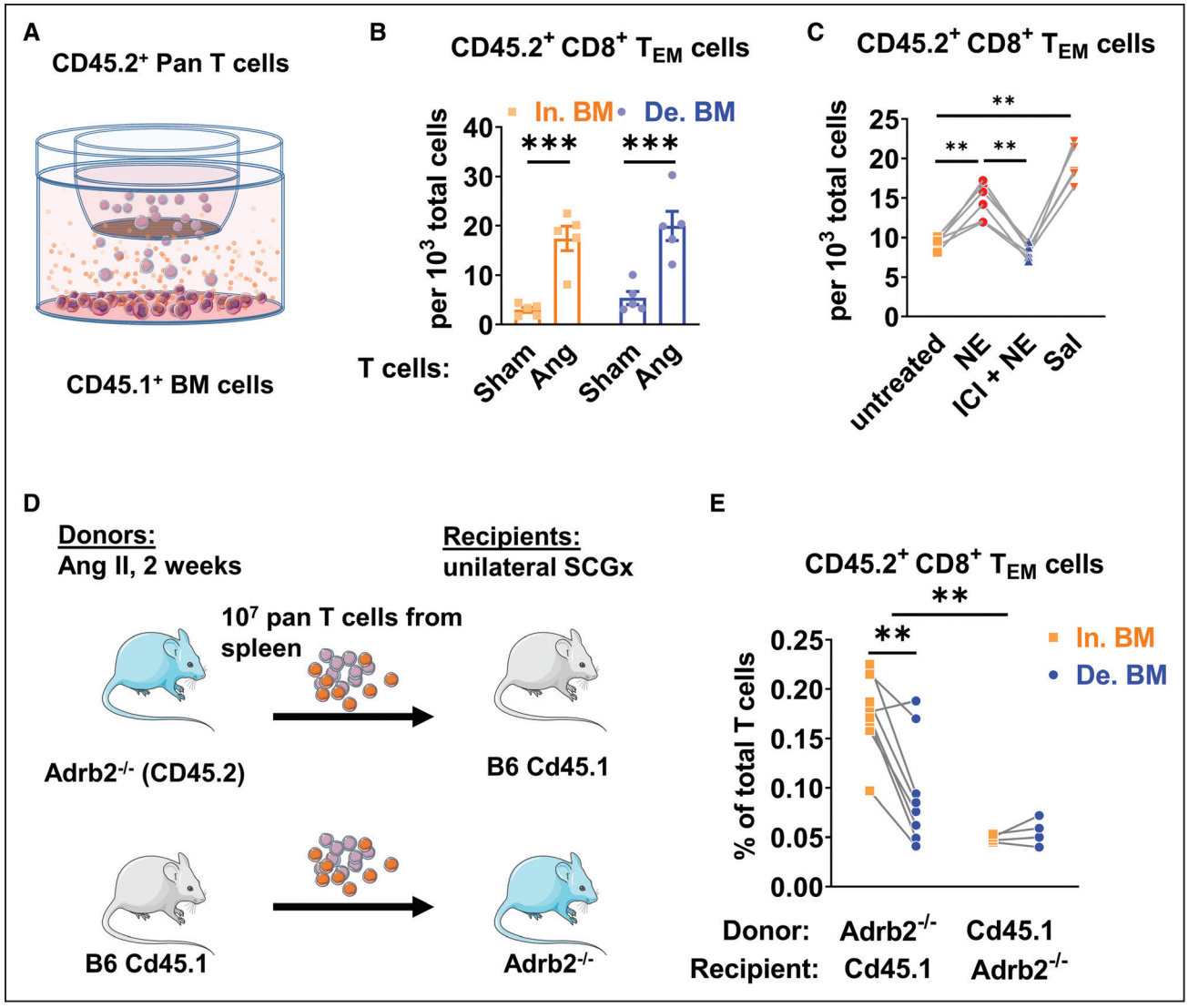

Figure 4. Role of β2 adrenergic signaling in the bone marrow (BM) homing of CD8⁺ effector memory T cells after hypertension. **A**, Pan-T cells were isolated for the spleen of wild-type (CD45.2) mice that had 2 weeks of either sham or Ang (angiotensin) II infusion. Bone marrow cells were isolated from both innervated and denervated forelimbs of B6 Cd45.1 mice that had unilateral superior cervical ganglionectomy (SCGx). **B**, Accumulation of transmigrated T cells isolated from innervated (In.) and denervated (De.) BM. Data are expressed as mean±SEM (Sham In. BM: 3.00±0.53 vs Ang In. BM: 17.46±2.47, *P*=0.0003; Sham De. BM: 5.27±1.31 vs Ang De BM: 20.00±2.96, *P*=0.0002), *P* values for the differences between each treatments was calculated by 2-way ANOVA with repeated measurements followed by a Bonferroni post hoc test, n=5 in each group. **C**, Accumulation of T cells from mice after Ang II infusion with bone marrow cells from naïve B6 Cd45.1 mice in the presence of no treatment, or 1 μmol/L norepinephrine (NE), and the β2 adrenergic receptor antagonist ICI118551 (ICI; 10 nmol/L) added 30 min before NE (ICI+NE), or the selective β2 adrenergic receptor agonist salbutamol (Sal, 1 μmol/L). *P* values for the differences between each 2 groups were calculated by 1-way ANOVA with repeated measurement followed by a Tukey post hoc test, n=6 in each group. **D**, Adoptive transfer of splenic pan-T cells was performed between β2 adrenergic knockout (Adrb2$^{-/-}$) mice and B6 Cd45.1 mice. The donor received 2 weeks of Ang II infusion, and the recipients received unilateral SCGx before the adoptive transfer. **E**, CD8⁺ T_{EM} cells in the innervated and denervated forelimb bone marrow were quantified by flow cytometry. For experiments shown in C and E, each set of connected symbols represent paired bone marrow samples from the same animal, the *P* values for the effect of Adrb2 gene deficiency in T cells vs bone marrow was calculated by 2-way ANOVA followed by a Bonferroni post hoc test, n=4 to 8 in each group. **P<0.01 and ***P<0.001.

sympatho-inhibition was confirmed by decreased blood pressure and heart rate, as well as power spectrum analysis from telemetry recordings (Online Figure VIII). Twenty days after adoptive transfer, we then infused a generally subpressor dose of Ang II and monitored blood pressure with radiotelemetry. As shown in Figure 7C, this dose of Ang II caused hypertension in mice that had received a control vector and adoptive transfer of T cells from hypertensive donors. In contrast, in mice in which sympathetic outflow was inhibited by Gi-DREADD in the RVLM during the time of T-cell adoptive transfer, low dose Ang II infusion had no effect on blood pressure. We have previously shown that bone marrow–residing CD8⁺ T cells can be reactivated to transmigrate to the kidney.[9]

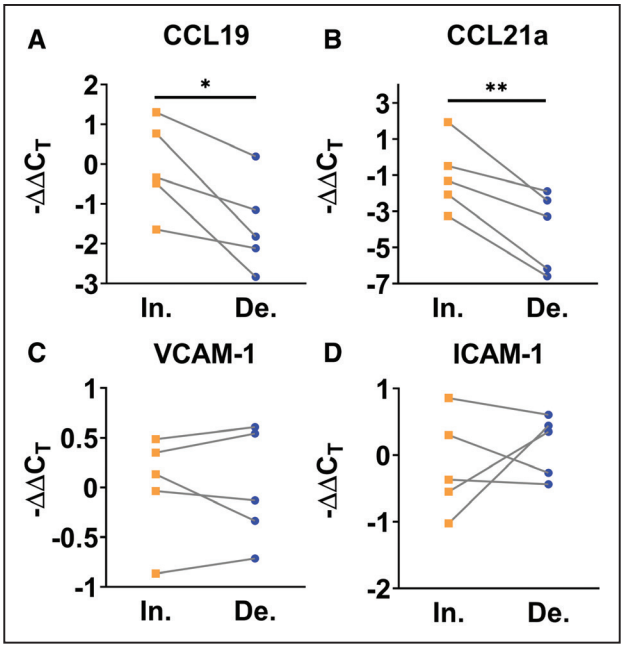

Figure 5. Effects of sympathetic nerves on bone marrow chemokine expression.
Bone marrow samples were collected from both the innervated (In) and denervated (De) bones of mice 1 wk after unilateral superior cervical ganglionectomy (SCGx), and mRNA expression of CCL (C-C chemokine ligands)-19, CCL-21, VCAM-1 (vascular cell adhesion molecule 1) and ICAM-1 (intercellular adhesion molecule 1) were measured by real-time polymerase chain reaction. Each set of connected symbols represent paired bone marrow samples from the same individual animal. The *P* values for the effect of denervation were calculated by paired *t* test, n=5 in each group. **P*=0.0128 in CCL19 and ***P*=0.0032 in CCL-21a.

In keeping with this, flow cytometry analyses of single cell suspensions of kidneys from these mice showed fewer total leukocyte, total T cells, both $CD4^+$ and $CD8^+$ T-cell infiltration in mice that had received Gi-DREADD injection into the RVLM, indicating the role of sympathetic nerves in the potentiation of future hypertension and renal inflammation.

Role of β2 Adrenergic Blockade on T-Cell Homing and Future Hypertension Development

Based on previous results, it is likely that blockade of β2ARs after a blood pressure surge is protective from developing repeated hypertension. To test this hypothesis, wild-type C57BL/6 mice were given pressor dose of Ang II infusion for 2 weeks and then 2-week infusion of either vehicle or β2AR antagonist ICI118551 as shown in Figure 8A. The bone marrow was collected from a subset of mice after euthanasia, and bone marrow cells were cocultured with DCs isolated from mice after 2 weeks of Ang II infusion (Figure 8B). After 7 days of culture, we found that the total T cells ($CD3^+$) proliferated less from mice treated with ICI118551, and this was due to lower numbers of both $CD4^+$ and $CD8^+$ T cells (Figure 8C through 8E). We also confirmed that the differences in T cells were primarily due to the changes in the number of $CD4^+$ and $CD8^+$ T_{EM} cells (Figure 8F and 8G). Another subset of mice received radiotelemetry implant to monitor their response to a 2-week infusion of Ang II infusion at the same subpressor dose at used in Figure 7. Consistent with our earlier study,[9] mice that had previously received high-dose Ang II infusion exhibited potentiated hypertension in response to this generally subpressor dose of Ang II compared with mice receiving only vehicle infusion (Figure 8H). Of note this second hypertensive response was blunted in mice that had ICI118551 infusion between the 2 infusions of Ang II. These results suggest temporary blockade of β2AR may be useful as a potential treatment to improve the prognosis of hypertension and associated end-organ damage.

DISCUSSION

In this study, we show that sympathetic nerves in the bone marrow play a critical role in the homing process of $CD8^+$ T_{EM} after they are formed in hypertension. These $CD8^+$ T_{EM} cells remember a previous surge of blood pressure and can be rapidly activated and divide on reexposure to antigens formed in hypertension. Sympathetic nerves provide a tonic control of the T-cell migration to the bone marrow at baseline condition, and this effect is profoundly enhanced when sympathetic nerve activity is elevated. In addition, experiments with T-cell adoptive transfer further indicate that this effect of sympathetic innervation is mediated by β2 adrenergic receptors in the bone marrow, which lead to upregulation of chemokines such as CCL19 and CCL21. More interestingly, sympathetic innervation of bone marrow does not affect the migration of OT-I memory T cells, indicating a distinct interaction between the sympathetic nerves and $CD8^+$ T_{EM} cells formed in hypertension.

Immunologic memory has been recently identified to play a critical role in repetitive hypertension, but the mechanisms involved in the maintenance and reactivation of memory T cells in the bone marrow were poorly understood. Sympathetic innervation of the bone marrow is well established for several decades, and it plays a crucial role in modulating the circadian rhythm of hematopoietic and immune cell function in the bone marrow.[11,16,17] Consistent with our current findings, spontaneously hypertensive rats have increased sympathetic nerve activity and impaired circadian rhythm and exhibit imbalanced production of endothelial progenitor cells and inflammatory cells in hypertension.[12]

Our adoptive transfer studies and experiments examining transwell transmigration clearly establish a role sympathetic tone and β2 stimulation in directing homing of $CD8^+$ T cells to the bone marrow. Our findings are also compatible with the concept that sympathetic tone provides an environment that maintains hypertension-specific

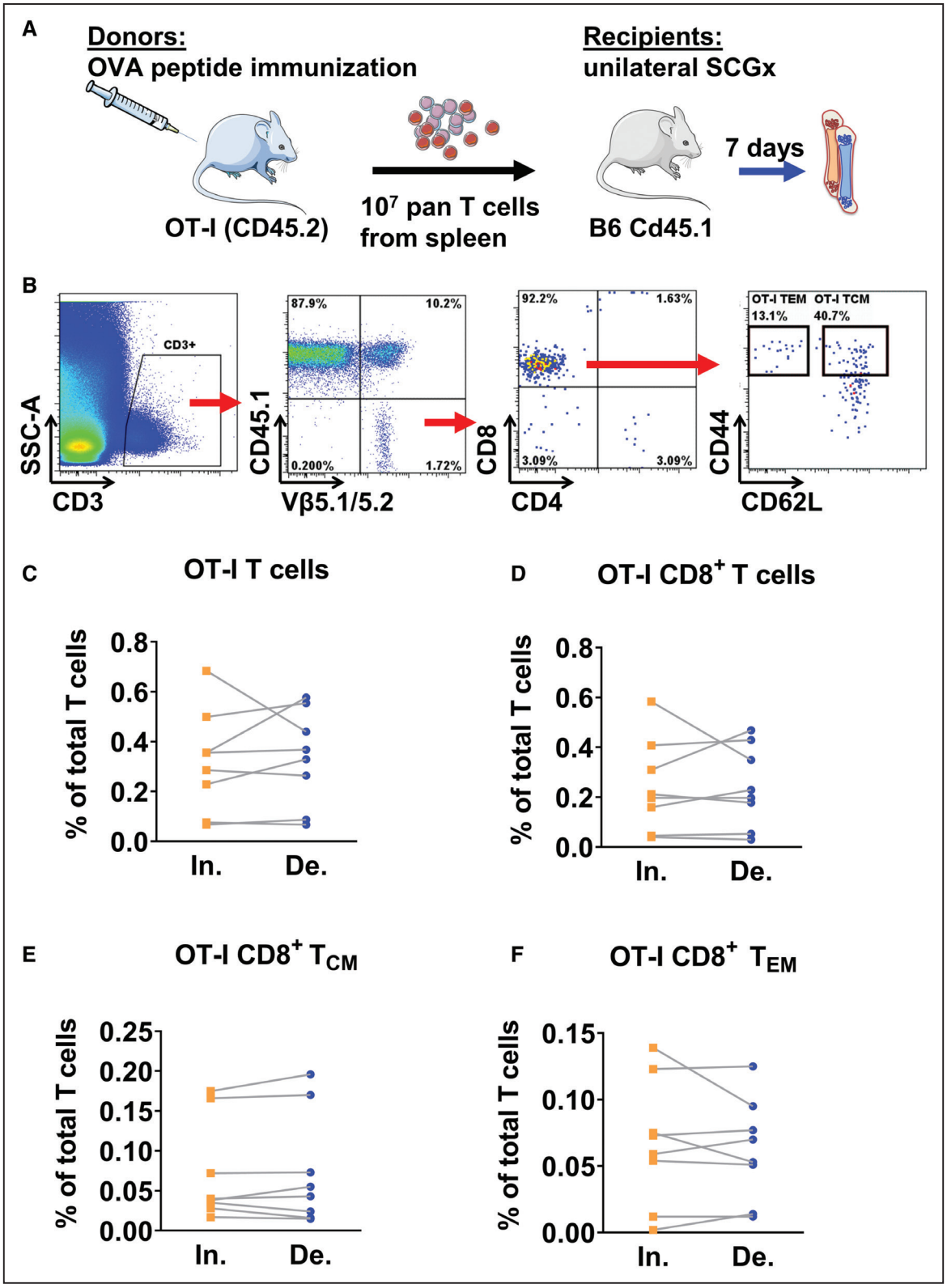

Figure 6. Effects of sympathetic nerves on the accumulation of OT-I memory T cells in the bone marrow after immunization with the OVA257-264 (SIINFEKL) peptide.

Splenic pan-T cells were isolated from immunized OT-I mice and transferred to B6 CD45. One recipient that had unilateral superior cervical ganglionectomy (SCGx) a week earlier. One week later, the recipient bone marrow in was analyzed by flow cytometry. **A**, Representative sample showing the gating strategy. Total T_{CR} (T-cell receptor) Vβ5.1/5.2$^+$ OT-I T cells, Vβ5.1/5.2$^+$/CD8$^+$ T cells as well as subsets of central and effector memory T cells in CD8$^+$ T-cell population were quantified by flow cytometry. Each set of connected symbols represent paired bone marrow samples from the same individual animal. No statistically significant difference was detected by paired *t* test, n=8 in each group.

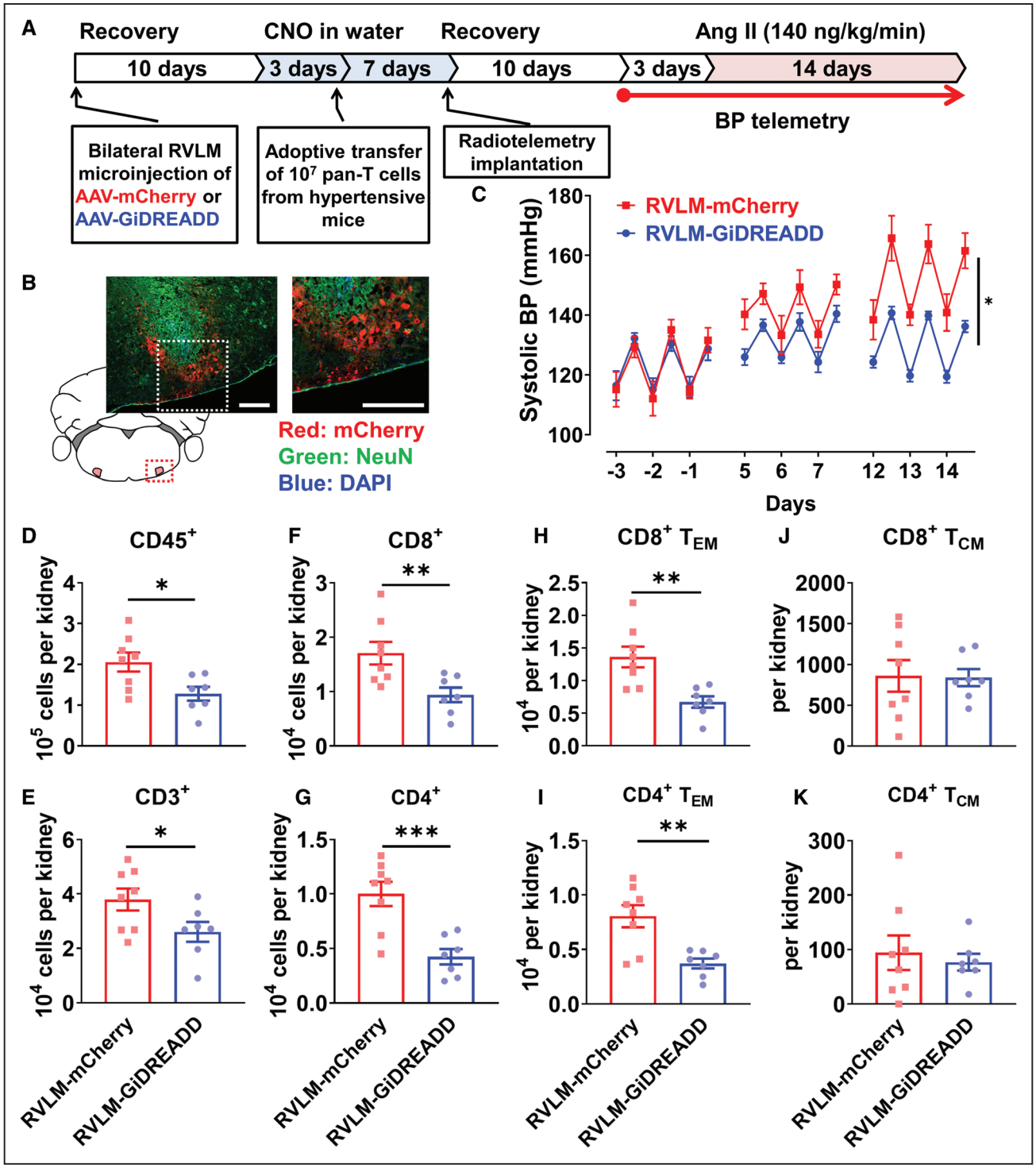

Figure 7. Effects of systemic sympatho-inhibition during memory T-cell homing on future hypertension development in response to low dose Ang (angiotensin) II infusion.

A, Experimental paradigm employed. **B**, Bilateral rostral ventrolateral medulla (RVLM) microinjection targets shown in coronal section of brain stem (marked in red circles). White bars indicate 100 μm. **C**, Three-day measurements of systolic blood pressure (BP) were obtained by radiotelemetry at baseline and during the first and second week of Ang II infusion. After 2 weeks of Ang II infusion, kidneys from mice was harvested, and infiltrating inflammatory cells were quantified by flow cytometry. Mean data for total leukocytes ($CD45^+$), total T cells ($CD3^+$), $CD8^+$, and $CD4^+$ T cells are shown in (**D**) to (**G**). Central and effector memory T-cell subsets in $CD8^+$ and $CD4^+$ cells were further quantified as shown in (**H**) to (**K**). Blood pressure data were analyzed with 2-way ANOVA with repeated measurements, P=0.0124 between the 2 groups during Ang II infusion, n=5 in each group. Flow cytometry data were analyzed by unpaired t tests, and P values between 2 groups were calculated ($CD45^+$: P=0.0102, $CD3^+$: P=0.0248, $CD8^+$: P=0.0051, $CD4^+$: P=0.0005, $CD8^+$ effector memory T cells [T_{EM}]: P=0.0015, $CD4^+$ T_{EM}: P=0.0014, $CD8^+$ central memory T cells [T_{CM}]: P=0.4621, and $CD4^+$ T_{CM}: P=0.3216), n=7 and 8 in each group; *P<0.05, **P<0.01, and ***P<0.001 in the figure. CNO indicates clozapine-N-oxide.

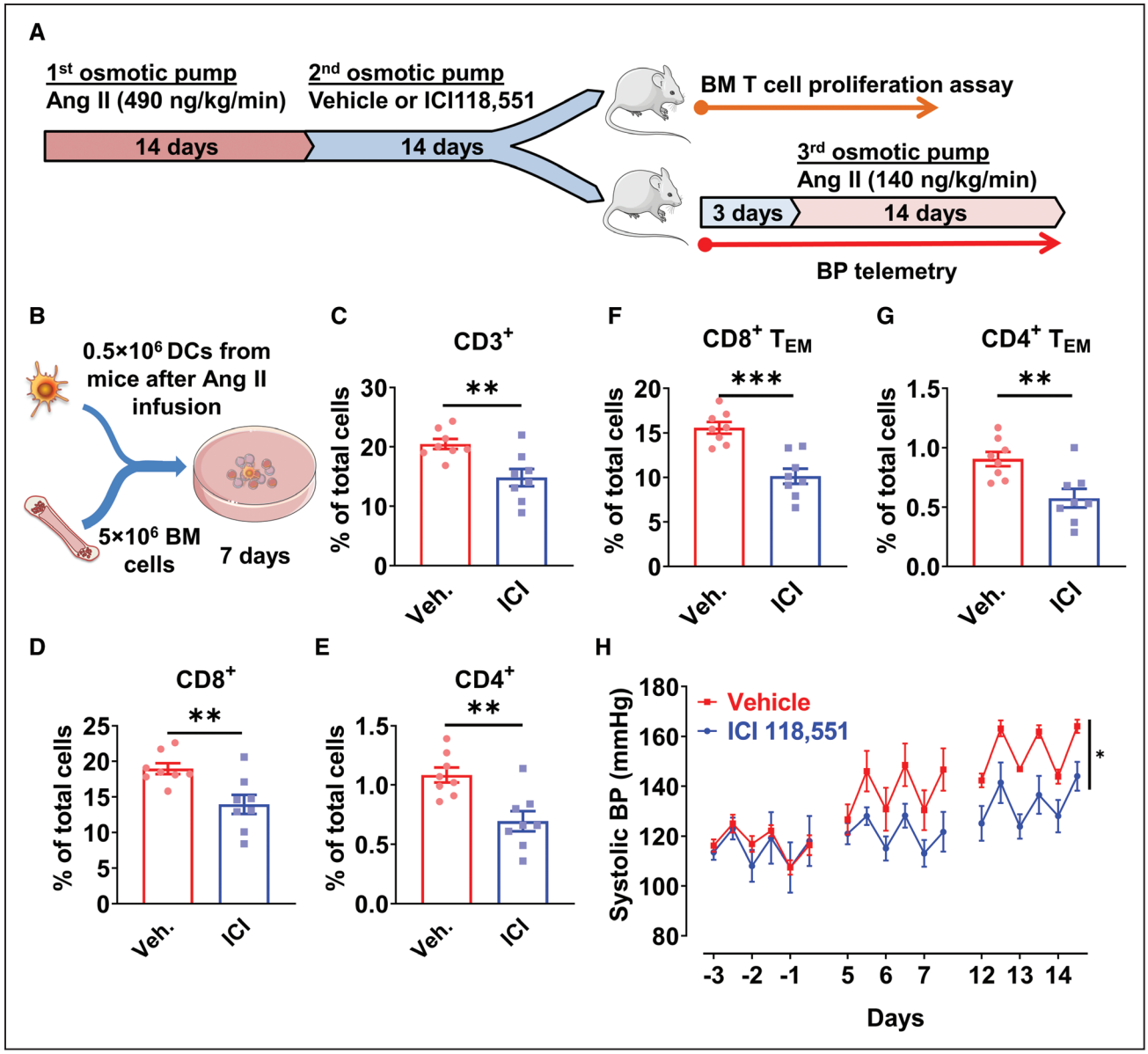

Figure 8. Effects of β2 blockade on bone marrow (BM) memory T cells and future hypertension development.
A, After 2 wk of Ang (angiotensin) II infusion at pressor dose (490 ng/kg per minute), mini-osmotic pumps for Ang II infusion were removed, and the mice received another pump for either vehicle (Veh.) infusion or selective β2 adrenergic receptor antagonist ICI118551 (ICI) infusion (200 ng/kg per minute) for 2 weeks. **B**, In a subset of mice, bone marrow was harvested for T-cell proliferation assay. After 7 d of coculture with dendritic cells (DCs), $CD3^+$, $CD4^+$, $CD8^+$ T cells and 2 T_{EM}-cell subsets in the bone marrow were quantified by flow cytometry as shown respectively from (**C**) to (**G**). $CD3^+$: Veh: 20.5±0.8% vs ICI: 14.8±4.1%, P=0.0024; $CD8^+$: Veh: 19.0±0.8% vs ICI: 14.0±1.4%, P=0.0031; $CD4^+$: Veh: 1.08±0.06% vs ICI: 0.70±0.08%, P=0.0012; $CD8^+T_{EM}$: Veh: 15.6±0.6% vs ICI: 10.1±0.9%, P<0.0001; and $CD8^+T_{EM}$: Veh: 0.91±0.17% vs ICI: 0.58±0.08%, P=0.0023. In another subset of mice, the ICI118551 minipump was removed and a third minipump inserted to administer a subpressor dose of Ang II (140 ng/kg per minute). Blood pressure was measured using radiotelemetry (**H**). Data are expressed as mean±SEM. Flow cytometry data were analyzed by unpaired *t* tests, n=8 in each group. Blood pressure was analyzed using 2-way ANOVA with repeated measurements, n=5 in each group, P=0.030 between the 2 groups during Ang II infusion, n=5 in each group. *P<0.05, **P<0.01, and ***P<0.001.

$CD8^+$ T cells once they have homed to the marrow. The treatment of mice with ICI118551 after a period of pressor dose Ang II exposure reduced the presence of T cells that proliferate in response to DCs from a hypertensive mouse. There is substantial discussion as to whether T_{EM} cells survive in the bone marrow because these cells are truly quiescent, that they are maintained by sustained antigen exposure or that they undergo low-level homeostatic proliferation. Our studies cannot differentiate between these conditions. For $CD8^+$ T cells, current evidence supports the concept that these cells are truly quiescent.[18] In preliminary experiments, we examined the presence of isolevuglandin adducts in DCs within the bone marrow, as these could potentially support a low level of proliferation of hypertension-specific T cells, but we found that these are not altered by denervation. It is possible that

specific antigenic peptides are altered by isolevuglandin, and these might be affected by β2 adrenergic stimulation. Our findings that T cells with adoptive transfer of T cells from OT-I mice suggest that sympathetic innervation within the bone marrow specifically promotes homing and maintenance of T cells related to hypertension and not simply all T_{EM} cells. This further supports the concept that features specific for hypertension, like the presentation of antigen, or perhaps unique conditions of the stroma with which the memory cells interact.

In the current study, we found that sympathetic innervation plays a predominant role in homing of $CD8^+$ T cells to the bone marrow. $CD8^+$ T cells seem to have a particularly important role in hypertension. We and others have previously shown that $CD8^+$ T cells have a particularly important role in hypertension. We found that mice lacking these cells were protected against Ang II–mediated hypertension, whereas mice lacking $CD4^+$ T cells were not.[19] Likewise, Youn et al[20] have shown that activated, immunosenescent-like $CD8^+$ T cells are increased in hypertensive individuals. We have also found that DCs of hypertensive mice present isolevuglandin modified peptides in the class 1 major histocompatibility complexes and that these seem to selectively drive $CD8^+$ T-cell proliferation.[14] Thus, the role of sympathetic nerves in modulating $CD8^+$ T-cell homing and residence in the bone marrow is likely important in the pathophysiology of hypertension. We have previously shown that repeated hypertensive stimuli promote accumulation of both $CD4^+$ and $CD8^+$ T cells in the bone marrow,[9] and in the present study, we demonstrated that ICI118551 administration after a period of hypertension reduced both $CD4^+$ and $CD8^+$ T cells that proliferate in response to DCs from a hypertensive mouse. Thus, $CD4^+$ T cells are likely also influence by sympathetic stimulation of the bone marrow.

With regard to the above considerations, we found that sympathetic innervation modulates expression of the chemokines CCL21 and CCL19, which are ligands for the chemokine receptor CCR7. CCR7 is expressed on both innate and adaptive immune cells, and it is conceivable that this promotes homing of both T cells and innate immune cells to the bone marrow. In contrast, we found no differences in expression of VCAM-1 or ICAM-1, ligands for LFA4 (lymphocyte function-associated antigen 4) and VLA4 (very late antigen-4), in innervated or denervated bone marrow. The precise receptor ligand pairs governing T_{EM} cell homing and residence in the bone marrow remains to be defined; however, the interactions of CCR7 with CCL19 and CCL21 are likely important.

Our findings might have important clinical implications. Historically, β-adrenergic blockade was considered first line therapy for hypertension, dating to the first report of the Joint National Committee on Detection, Evaluation and Treatment of High Blood Pressure in 1977.[21] Later randomized clinical trials, including the ASCOT (Anglo-Scandinavian Cardiac Outcomes Trial) and LIFE (Losartan Intervention For Endpoint Reduction in Hypertension) trials, showed that atenolol is inferior to amlodipine and losartan.[22,23] Several meta-analyses have indicated that atenolol is at best neutral, and in many cases worsens all-cause mortality, compared with inhibitors of the renin-Ang system, calcium channel blockers, and diuretics.[24] This has led to the current American College of Cardiology/American Heart Association recommendation that β-receptor antagonists be used only as add-on therapy except in special populations.[25] Given that the randomized clinical trials have employed the selective β1 antagonist atenolol, and that this drug has been used in over 75% of other studies, the efficacy of nonselective β-blockers has not been adequately studied. Our findings suggest that β2 adrenergic receptors are involved in allowing homing and survival and suggest that either nonselective β antagonists or perhaps β2 blockade might be efficacious in preventing accumulation of hypertension-specific T_{EM} cells in sites like the bone marrow. As shown in our experiments with ICI118551, even a short-term course of β blockade, or perhaps drugs that reduce sympathetic outflow like β-methyldopa or β2 adrenergic agonists might create an environment hostile to survival of such cells, causing their ultimate death and thus alleviating the risk of subsequent blood pressure elevation on repeated hypertensive challenges.

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Disclosures

None.

Supplemental Materials

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