

Involvement of Bone Marrow Cells and Neuroinflammation in Hypertension

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Rationale: Microglial activation in autonomic brain regions is a hallmark of neuroinflammation in neurogenic hypertension. Despite evidence that an impaired sympathetic nerve activity supplying the bone marrow (BM) increases inflammatory cells and decreases angiogenic cells, little is known about the reciprocal impact of BM-derived inflammatory cells on neuroinflammation in hypertension.

Objective: To test the hypothesis that proinflammatory BM cells from hypertensive animals contribute to neuroinflammation and hypertension via a brain–BM interaction.

Methods and Results: After BM ablation in spontaneously hypertensive rats, and reconstitution with normotensive Wistar Kyoto rat BM, the resultant chimeric spontaneously hypertensive rats displayed significant reduction in mean arterial pressure associated with attenuation of both central and peripheral inflammation. In contrast, an elevated mean arterial pressure along with increased central and peripheral inflammation was observed in chimeric Wistar-Kyoto rats reconstituted with spontaneously hypertensive rat BM. Oral treatment with minocycline, an inhibitor of microglial activation, attenuated hypertension in both the spontaneously hypertensive rats and the chronic angiotensin II-infused rats. This was accompanied by decreased sympathetic drive and inflammation. Furthermore, in chronic angiotensin II-infused rats, minocycline prevented extravasation of BM-derived cells to the hypothalamic paraventricular nucleus, presumably via a mechanism of decreased C-C chemokine ligand 2 levels in the cerebrospinal fluid.

Conclusions: The BM contributes to hypertension by increasing peripheral inflammatory cells and their extravasation into the brain. Minocycline is an effective therapy to modify neurogenic components of hypertension. These observations support the hypothesis that BM-derived cells are involved in neuroinflammation, and targeting them may be an innovative strategy for neurogenic resistant hypertension therapy. (*Circ Res.* 2015;117:178–191. DOI: 10.1161/CIRCRESAHA.117.305853.)

Key Words: autonomic nervous system ■ bone marrow cells ■ hypertension ■ immune system ■ microglia

Hypertension is the most modifiable risk factor for cardiovascular disease. Despite significant advancement in its control, 20% to 30% of all hypertensive patients remain resistant to available pharmacotherapy. This is primarily because of the involvement of a strong neurogenic component in the establishment of hypertensive state.^{1–3} Mounting evidence implicates a key role for peripheral and neuroinflammation in the pathophysiology of hypertension in both humans and animal models.^{4–7} However, the relationship between the immune system (IS) and the central nervous system (CNS) in hypertension is not well understood. An interesting relationship was

revealed by the indication that increased sympathetic drive can mediate hypertension by norepinephrine-mediated T-cell activation.⁸ As a meeting point for the CNS and IS,^{9–11} and the site of leukocyte and progenitor cell production, the bone marrow (BM) serves as an ideal link between the inflammatory system and hypertension.

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The BM plays an important role in cardiovascular health and disease, leading to the proposal that the autonomic pathways that

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Nonstandard Abbreviations and Acronyms

APC	angiogenic progenitor cell
BM	bone marrow
CCL2	C-C chemokine ligand 2
CCR2	C-C chemokine receptor 2
CNS	central nervous system
CSF	cerebrospinal fluid
IC	inflammatory cell
IS	immune system
MAP	mean arterial pressure
SBP	systolic blood pressure
SHR	spontaneously hypertensive rat
WKY	Wistar Kyoto

control the IS may be dysregulated in hypertension.^{5,7} Indeed, altered inflammatory responses have been associated with impaired autonomic input to the BM in the spontaneously hypertensive rats (SHRs), an established animal model for human hypertension.⁹ Also, early studies have indicated that suppressing the IS by pharmacological treatment or thymectomy could blunt the development and maintenance of hypertension.^{6,12,13} It was later shown that T cells are the critical immune players responsible for the genesis of hypertension.¹⁴ In addition to peripheral inflammatory responses, central neuroinflammation and oxidative stress have been described in several hypertensive animal models.^{15–17} Of particular interest is the activation of microglial cells, which act as the resident immune cells of the CNS.¹⁸ Recent studies have implicated the activation of microglial cell in the autonomic brain regions, particularly the hypothalamic paraventricular nucleus, plays an important role in hypertension.^{19–23} This view is further supported by clinical evidence, wherein chronic treatment of hypertensive patients with minocycline, an anti-inflammatory, small molecule antibiotic that freely passes the blood brain barrier and inhibits microglial activation, produced profound blood pressure-lowering effects.²⁴ Similarly, activated microglia and neuroinflammation have been shown to be associated with cognitive impairment and Parkinson disease.^{25,26} These studies have suggested that BM cells, particularly proinflammatory progenitors, are mobilized from BM to enter the brain parenchymal space in a C-C chemokine ligand 2 (CCL2) and its receptor (C-C chemokine receptor 2 [CCR2]) axis-dependent manner, thus contributing to chronic neuroinflammation.^{27–29}

Although inflammatory cells (ICs) have long been described to infiltrate the vasculature and organs such as the kidney and heart in hypertension,^{30–32} the evidence supporting IC infiltration and accumulation in the brain parenchyma is fewer.^{24,33–35} All these observations have led us to hypothesize that the BM exhibits a proinflammatory state in hypertension, characterized by increased ICs and cytokines. This results in increased peripheral inflammation, extravasation of inflammatory progenitors into crucial cardioregulatory brain centers, where they differentiate into microglia/macrophages and contribute to hypertension. Our study was designed to address this hypothesis.

Methods

All animal procedures were approved by the University of Florida Institute Animal Care and Use Committee. Full details of all

experimental protocols are presented in the Methods section in the Online Data Supplement.

Results**Elevated Proinflammatory Markers in the BM of Hypertensive Animals**

Two distinct rat models of hypertension with neurogenic components (the SHR and chronic angiotensin II [Ang II]-infused rat) were used to evaluate the proinflammatory profile of the BM cells. We observed increased mRNA levels of interleukin-1 β (40%), interferon- γ (80%), and colony-stimulating factor 2 (50%) in the BM-derived mononuclear

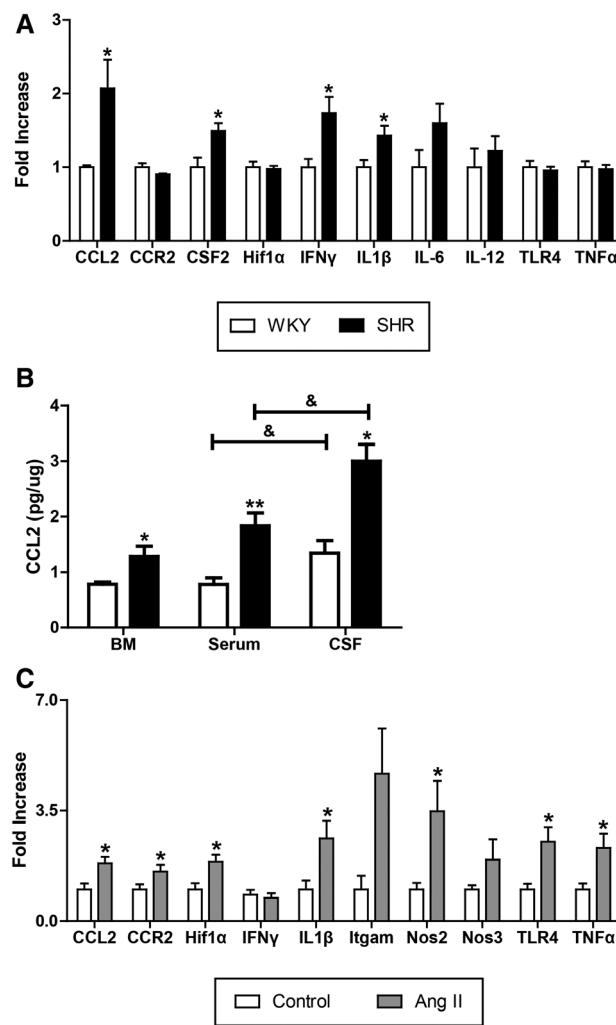


Figure 1. Proinflammatory markers are elevated in the bone marrow (BM) of 2 rat models of hypertension. A, BM mononuclear cells (MNCs) from the spontaneously hypertensive rats (SHR) show increased mRNAs for C-C chemokine ligand 2 (CCL2), colony stimulating factor 2 (CSF2), interferon γ (IFN γ), and interleukin-1 β (IL-1 β) compared with Wistar-Kyoto (WKY; n=4 per group). *P<0.05 vs WKY. **B,** CCL2 levels are elevated in SHR BM supernatant, serum, and cerebrospinal fluid (CSF; n=4 per group). *P<0.05, **P<0.01 vs WKY; &P<0.05 vs serum. **C,** BM MNCs from chronic angiotensin II (Ang II) infusion in Sprague Dawley (SD) rats have increased mRNA of CCL2, C-C chemokine receptor 2 (CCR2), hypoxia-inducible factor-1 α (Hif1 α), IL-1 β , NO synthase (Nos2), toll-like receptor 4 (TLR4), and tumor necrosis factor- α (TNF α) compared with saline-infused SD control rats (n=6 per group). *P<0.05 vs control.

cells of SHR as compared with Wistar Kyoto (WKY; Figure 1A). The greatest increase was observed in the CCL2 mRNA (100%). In addition, CCL2 protein levels in the BM supernatant, serum, and cerebrospinal fluid (CSF) of the SHR were increased by 63%, 136%, and 124%, respectively (BM, 0.78 ± 0.04 versus 1.28 ± 0.19 pg/ μ g; serum, 0.78 ± 0.12 versus 1.84 ± 0.23 pg/ μ g; and CSF, 1.34 ± 0.23 versus 3.00 ± 0.30 pg/ μ g; Figure 1B). Importantly, there seems to be a significant gradient in CCL2 concentration from BM<serum<CSF.

Next, we investigated the effect of chronic Ang II infusion on the inflammatory profile of BM cells in this model of hypertension. Similar to the SHR, we found significant increases in the mRNA levels of CCL2 (83%) and interleukin-1 β (162%) after 8 weeks of Ang II infusion (Figure 1C). In addition, increases of 57% in CCR2, 89% in hypoxia-inducible factor 1- α , 248% in inducible NO synthase, 152% in toll-like receptor 4, and 132% in tumor necrosis factor- α mRNA levels were detected. These data indicate that although the individual cytokine profile of the BM cells is different in diverse hypertension animal models, they share a common proinflammatory nature.

Modulation of Blood Pressure and Hemodynamics in Both SHR and WKY by BM Reconstitution

Because the hypertension BM cells were found to have increased expression of proinflammatory cytokines, we tested the hypothesis that reconstitution of WKY rats with SHR BM would increase their mean arterial pressure (MAP). Details on the design of this experiment are presented in the Online Data Supplement and Online Figure I. The validity of reconstitution in this model was simultaneously confirmed in female rats receiving male BM cells, thereby allowing tracking of reconstitution success by Y-FISH (Y-chromosome fluorescence in situ hybridization; Online Figure II), as previously described.³⁶ Briefly, simultaneous BM reconstitution experiments were performed in the adult female SHR, and reconstitution with male WKY and SHR whole BM cells was confirmed by detecting the Y-chromosome in BM-derived mononuclear cells. Successful reconstitution was adjudged by >90% Y chromosome-stained mononuclear cells isolated from the blood. In all subsequent experiments, adult age-matched male WKY and SHR were used to investigate the role of BM in hypertension. Data presented from here forth are exclusively from male rats receiving male cells.

MAP was found to be elevated in WKY rats reconstituted with SHR cells as compared with those that were reconstituted with WKY cells (WKY-SHR, 147 ± 16 mmHg; WKY-WKY 114 ± 2 mmHg; Figure 2A). Conversely, reconstitution of SHR with WKY BM resulted in lowering of MAP in comparison to those reconstituted with SHR cells (SHR-WKY, 138 ± 11 mmHg and SHR-SHR, 188 ± 6 mmHg). A parallel experiment was performed where MAP was measured by noninvasive tail-cuff plethysmography. The data obtained from the tail-cuff method were consistent and comparable with that obtained by radiotelemetry (Online Figure III).

Next, we evaluated cardiac hypertrophy by quantifying heart weight:tibia length ratio. Similar trend as that

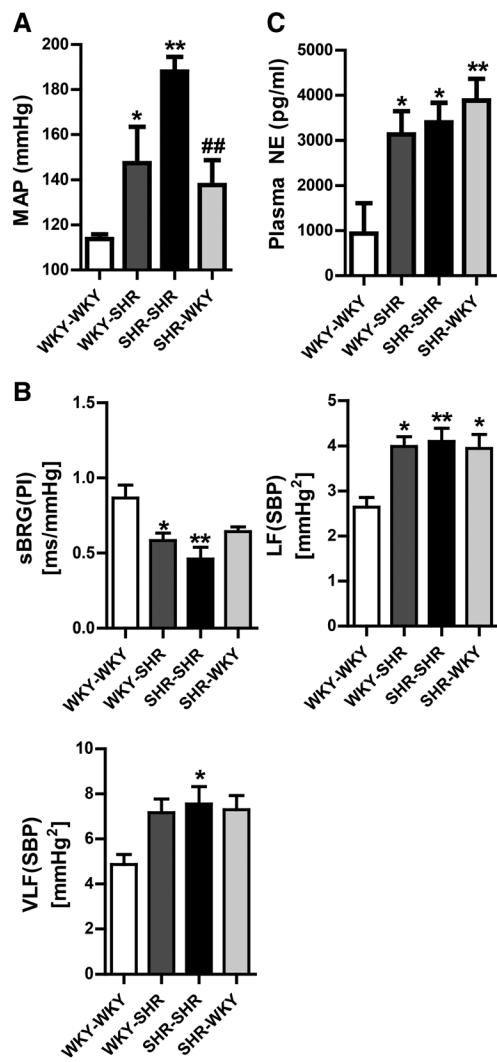


Figure 2. Bone marrow reconstitution modulates blood pressure and autonomic function in spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. **A**, Mean arterial pressure (MAP) was measured directly by radiotelemetry ($n=5$ per group). Reconstitution of the WKY rat with SHR bone marrow increases MAP. Conversely, reconstitution of the SHR with WKY bone marrow lowers MAP. **B**, Spectral analysis of the systolic blood pressure (SBP) and pulse interval (PI) waveforms of telemetry ($n=5$ per group). **C**, Plasma norepinephrine (NE) is elevated in the WKY-SHR vs WKY-WKY. There is no change in the SHR rats with WKY bone marrow ($n=5$ per group). * $P<0.05$, ** $P<0.01$ vs WKY-WKY; # $P<0.05$, ## $P<0.01$ vs SHR-SHR. LF indicates low frequency; SBRG, spontaneous baroreflex gain; and VLF, very low frequency.

seen in MAP data was observed for cardiac remodeling (WKY-WKY, 27.9 ± 1.3 ; WKY-SHR, 32.6 ± 1.0 ; SHR-SHR, 36.1 ± 1.1 ; and SHR-WKY, 30.1 ± 2.1 mg/mm; Online Figure IV). These findings were further confirmed by measuring the cardiomyocyte diameter from the left ventricular free wall. The results were consistent with the heart weight:tibia length ratio findings.

Plasma norepinephrine levels were found to be elevated in the WKY-SHR as compared with the WKY-WKY controls (3131 ± 521 versus 937 ± 671 pg/mL; Figure 2C). However, no significant difference in plasma norepinephrine was observed

between SHR–WKY and SHR–SHR groups (3888 ± 484 versus 3405 ± 432 pg/mL). This was also confirmed by spectral analysis of the telemetry waveform of systolic blood pressure (SBP) and pulse interval (PI), as previously described.¹⁰ This analysis revealed that cardiac spontaneous baroreflex gain was attenuated in the WKY–SHR but not in WKY–WKY (0.58 ± 0.05 versus 0.87 ± 0.09 ms/mmHg; Figure 2B) and was further accompanied by an increase in overall vasomotor sympathetic tone in these 2 groups (low frequency [SBP], 4.0 ± 0.2 versus 2.6 ± 0.2 mmHg² and very low frequency [SBP], 7.2 ± 0.6 versus 4.9 ± 0.4 mmHg²), although the latter failed to reach significance. However, none of these autonomic variables were attenuated in the SHR–WKY as compared with the SHR–SHR.

Next, we evaluated the blood perfusion to the hindlimbs of these chimeric rats using a laser speckle contrast imager (Figure 3A–3D). Two analyses were performed: (1) the rate of blood flow change (slope) during the first 1.5 minutes and (2) stabilized perfusion during the last 1.5 minutes. Isoflurane anesthesia induces a slow vasodilatory response,³⁷ which is observed in the first 2 minutes of recording. This rate of change in blood perfusion was decreased in the WKY–SHR when compared with the WKY–WKY (19 ± 7 versus 45 ± 6 arbitrary unit blood perfusion/min; Figure 3B). In addition, the stabilized blood perfusion was lower in the WKY–SHR than in the WKY–WKY (164 ± 16 versus 219 ± 12 arbitrary unit of intensity). No differences were observed between the SHR–WKY and the SHR–SHR.

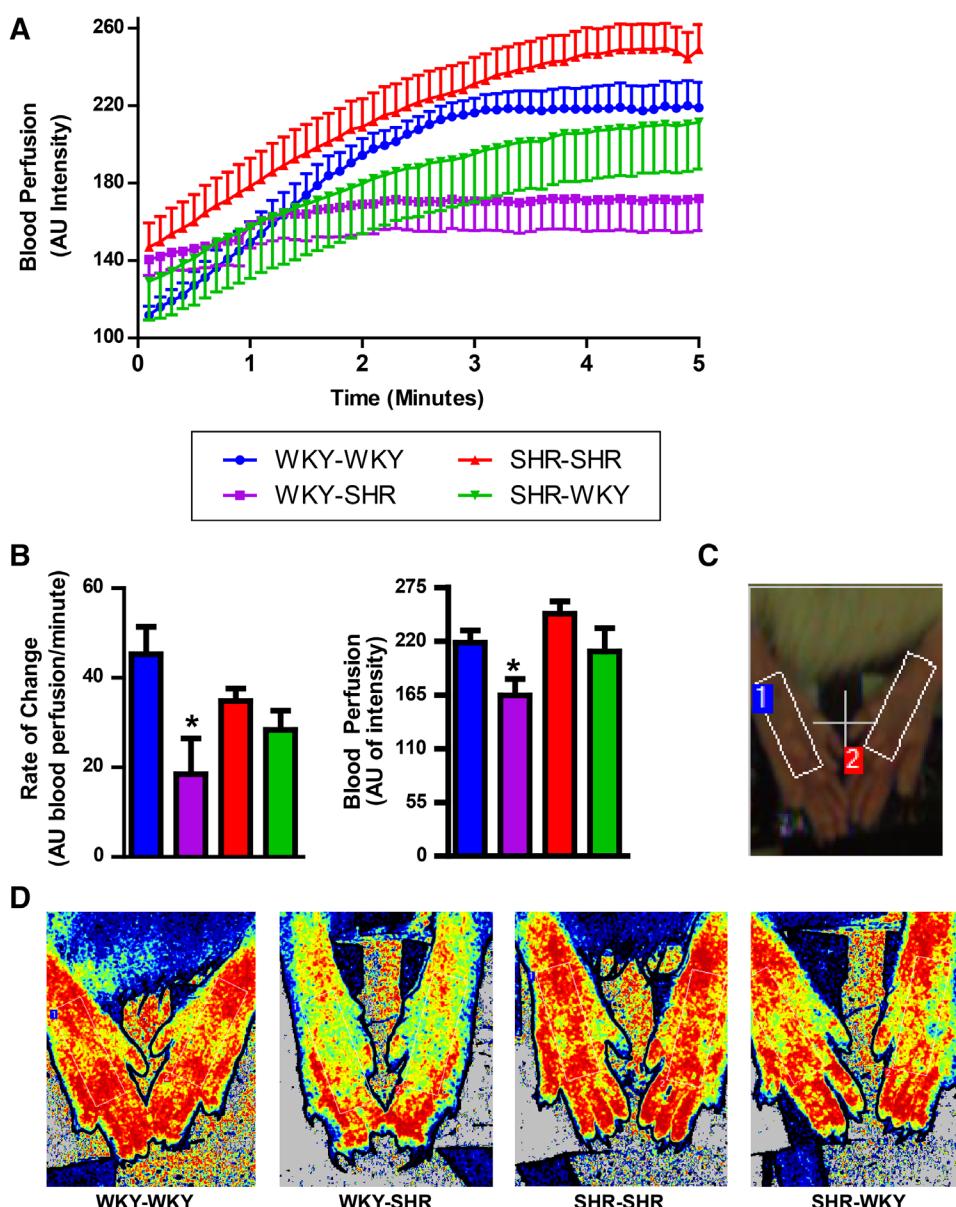


Figure 3. Bone marrow reconstitution with spontaneously hypertensive rats (SHR) cells alters blood perfusion of the hind limbs in the Wistar-Kyoto (WKY). A, Hind paw perfusion in arbitrary units (AU) for 5 minutes of recording time. B, Rate of blood perfusion change during the first 1.5 minutes and stabilized blood perfusion of the hind paw are both decreased in WKY–SHR vs WKY–WKY. C, Photograph indicating region of interest on both hind paws. D, Representative blood flow intensity maps during the last minute of the sample period. The color scale is from blue to red, lower to higher flow (n=3–4 per group). *P<0.05 vs WKY–WKY.

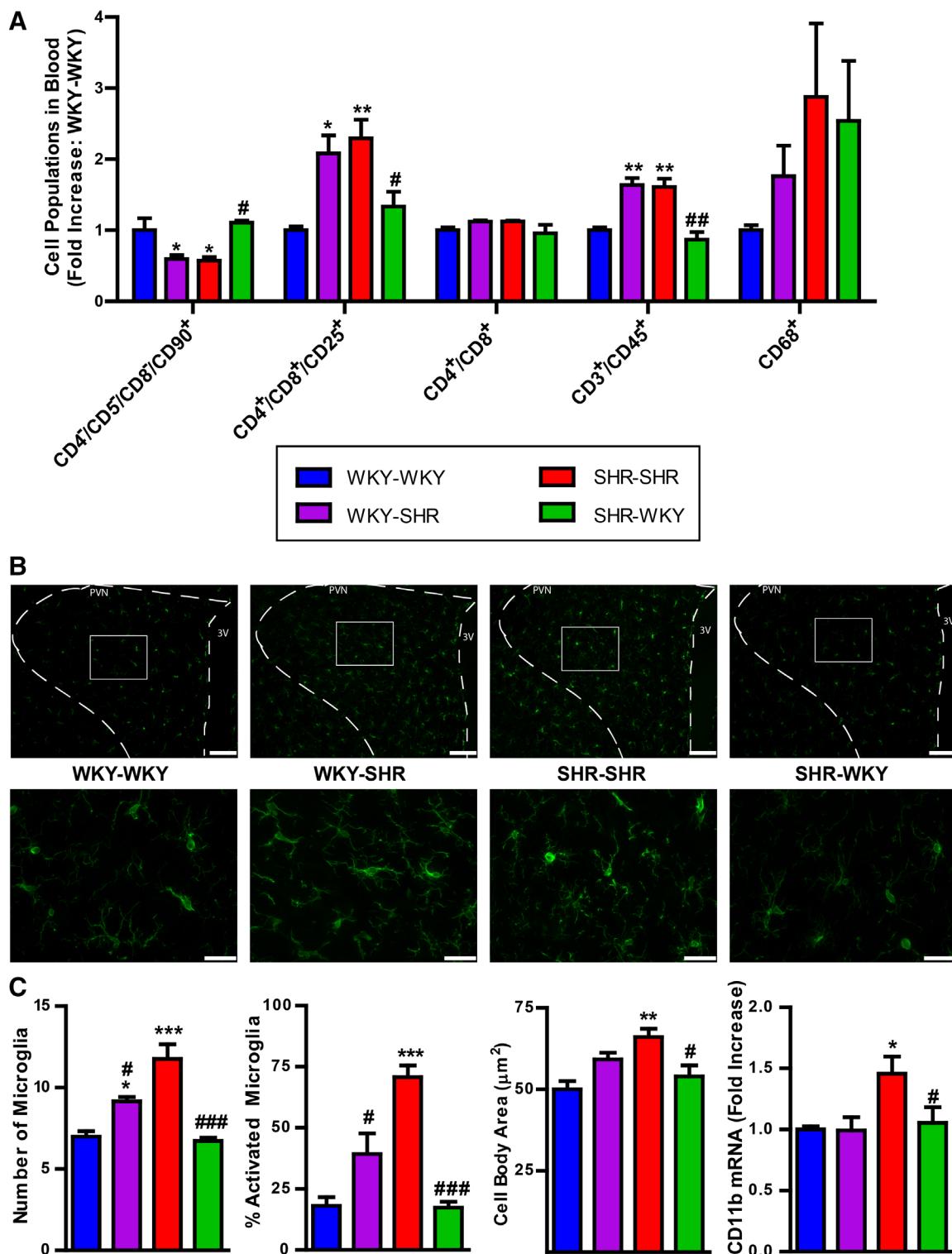


Figure 4. Peripheral inflammatory cells and activated microglia in the hypothalamic paraventricular nucleus (PVN) are decreased in the spontaneously hypertensive rats (SHRs) after reconstitution with Wistar-Kyoto (WKY) bone marrow. **A,** Specific inflammatory cell populations are increased in the circulation of WKY-SHR, including CD4⁺/CD8⁺/CD25⁺ and CD3⁺/CD45⁺ T cells; these were decreased in the SHR-WKY. In addition, CD4⁺/CD5⁺/CD8⁺/CD90⁺ were decreased in the WKY-SHR and increased in the SHR-WKY (n=5–8 per group). **B,** Representative images at $\times 10$ and $\times 40$ magnification of Iba1⁺ microglia in the PVN. Scale bar, 100 μm in $\times 10$ and 30 μm in $\times 40$. **C,** Quantification of activated microglia in the PVN: number of microglia and percentage of activated microglia per 40 000 μm^2 , cell body area, are increased in WKY-SHR; these values are decreased in SHR-WKY (n=5 per group). CD11b mRNA was higher in the SHR-SHR than in WKY-WKY control and restored in the SHR-WKY (n=5 per group). However, no changes were detected in WKY-SHR vs WKY-WKY. *P<0.05, **P<0.01 vs WKY-WKY; #P<0.05, ##P<0.01 vs SHR-SHR.

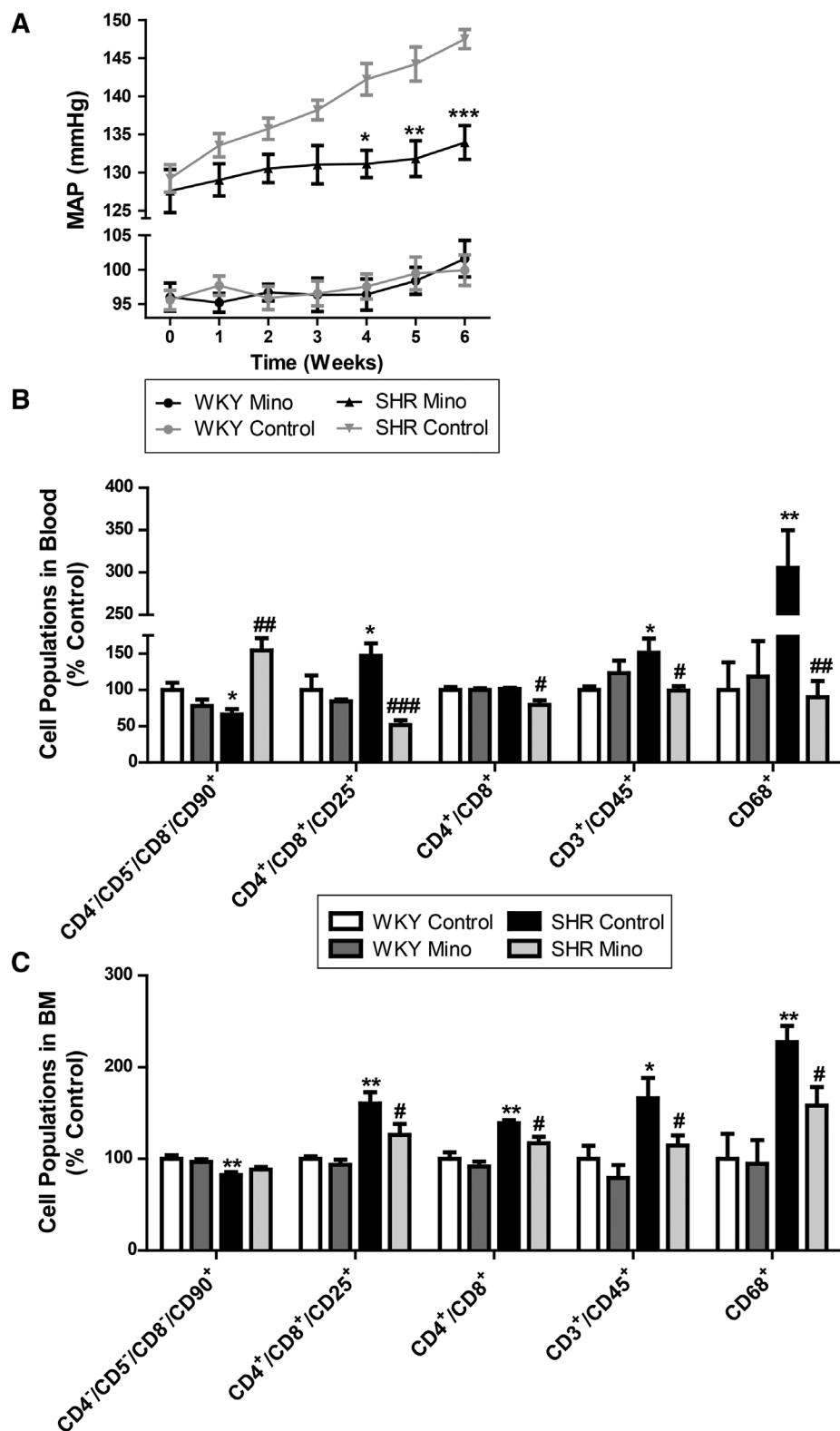


Figure 5. Oral minocycline (mino) attenuates mean arterial pressure (MAP) and peripheral inflammation in spontaneously hypertensive rats (SHRs). **A,** MAP measure by telemetry indicates that mino attenuates the development of hypertension in the SHR (n=5–6 per group). **B,** Specific inflammatory cell populations were increased in the blood in SHRs, including CD4⁺/CD8⁺/CD25⁺, CD4⁺/CD8⁺, and CD68⁺. Mino treatment lowers these ratios back to control (n=5–6 per group). **C,** After a similar trend, CD4⁺/CD5⁺/CD8⁺/CD90⁺ cells (angiogenic progenitor cells) were lower in SHRs than in WKY and restored by oral mino (n=4–8 per group). *P<0.05, **P<0.01, ***P<0.001 vs WKY control; #P<0.05, ##P<0.01, ###P<0.001 vs SHR control.

Decreased Peripheral Inflammation and Activated Microglia in the PVN of SHR Reconstituted With WKY BM

Our previous studies have established that hypertension is associated with increases in pro-ICs and decreases in angiogenic progenitor cells (APCs).^{9,10} Thus, we decided to investigate the circulatory levels of these cells in chimeric rats. We measured circulating CD4⁻/CD5⁻/CD8⁻/CD90⁺ (representing APCs) and CD4⁺/CD8⁺/CD25⁺ and CD3⁺/CD45⁺ cells (representing subpopulations of T cells previously shown to be elevated in hypertension^{9,10}) in WKY–SHR animals, to determine whether the increase in MAP in these animals is associated with changes in these cell populations. We observed a 43% reduction in circulating CD4⁻/CD5⁻/CD8⁻/CD90⁺ cells, a 61% increase in circulating CD4⁺/CD8⁺/CD25⁺ cells, and a 40% increase in CD3⁺/CD45⁺ cells in the WKY–SHR as compared with WKY–WKY (Figure 4A). In contrast, the SHR–WKY group presented with a 92% increase in APCs, a 45% reduction in CD4⁺/CD8⁺/CD25⁺ cells, and a 37% reduction in CD3⁺/CD45⁺ cells as compared with SHR–SHR. No changes were observed in the CD4⁺/CD8⁺ or CD68⁺ populations.

Next, we compared the levels of activated microglia in different experimental groups to determine whether the change in MAP was associated with changes in microglial activation in the PVN. We found a significant decrease in activated microglia in SHR–WKY rats versus SHR–SHR rats when measured as total number of microglia per 40 000 μm^2 (43%), percent activated microglia (76%), and cell body area (18%; Figure 4B and 4C). This was confirmed by a 27% decrease in the mRNA levels of CD11b in the SHR–WKY group (Figure 4C). However, total microglia per 40 000 μm^2 was increased by 31% in the PVN of WKY–SHR versus WKY–WKY; however, changes in the percent activated microglia and cell body area did not reach significance.

These observations demonstrate transplantation of SHR BM into the WKY rats increases MAP, elevates circulating pro-ICs, and promotes microglial cells in the PVN. On the contrary, transplantation of WKY cells into SHR decreases MAP that is associated with increased APCs, decreased pro-ICs, and reduced PVN microglial activation.

Oral Delivery of Minocycline Attenuates MAP, Decreases Inflammation, and Restores Autonomic Balance in the Chronic Ang II Infusion Rat Model of Hypertension

Next, we examined the effects of oral minocycline treatment on hypertension and associated pathophysiology, including the BM. Our previous studies have shown that intracerebroventricular delivery of minocycline prevents the development of hypertension in the chronic Ang II infusion model.¹⁹ Minocycline is an anti-inflammatory, tetracycline-derived antibiotic, which crosses the blood brain barrier and inhibits activation of microglial cells in the CNS. Minocycline is currently being evaluated for many clinical applications, including stroke, because of its inhibitory effects on microglial cell activation.

Oral delivery of minocycline to adult SHR rats attenuated the increase in MAP >6 weeks of treatment (Figure 5A). The heart weight:tibia length ratio, an index of cardiac

hypertrophy, increased in vehicle-treated SHRs as compared with WKY rats, but was lowered by minocycline treatment (Online Figure V). These effects were associated with reduction in the levels of circulating CD4⁺/CD8⁺ (~22%), CD4⁺/CD8⁺/CD25⁺ (~65%), CD3⁺/CD45⁺ (~35%), and CD68⁺ (~70%) cells in minocycline-treated SHR as compared with SHR control. In addition, CD4⁻/CD5⁻/CD8⁻/CD90⁺ cells were increased by ~134%. This was coupled with similar reductions in CD4⁺/CD8⁺ (~12%), CD4⁺/CD8⁺/CD25⁺ (~21%), CD3⁺/CD45⁺ (~31%), and CD68⁺ (~31%) cells in minocycline-treated SHR. No significant effect of minocycline treatment was observed on the BM or blood cell populations of WKY rats.

Spectral analysis of the SBP signal from transmitter-implanted rats revealed dampening of the spontaneous baroreflex gain (ΔsBRG) in SHR versus WKY rats (0.06 ± 0.01 versus 0.24 ± 0.05 ms/mm Hg; Online Figure VI). However, this effect was attenuated by minocycline treatment (-0.24 ± 0.04 ms/mm Hg). In addition, low frequency (SBP), very low frequency (SBP), and vasovagal balance (Δ low frequency[SBP]:high frequency[PI]) values were increased in SHRs when compared with those in WKY (0.5 ± 0.3 , 1.4 ± 0.4 , and 0.1 ± 0.03 mm Hg²/ms², respectively), which were normalized by minocycline treatment (-1.1 ± 0.2 , -1.1 ± 0.4 , and -0.7 ± 0.03 mm Hg²/ms², respectively). No significant changes were observed in WKY rats treated with minocycline.

Oral Delivery of Minocycline Attenuates MAP, Decreases Inflammation, and Restores Autonomic Balance in the Chronic Ang II Infusion Rat Model of Hypertension

Oral delivery of minocycline to Sprague Dawley rats attenuated the increase in MAP induced by chronic Ang II infusion (129 ± 3 versus 161 ± 10 mm Hg; Figure 6A). The telemetry data on MAP were comparable with the tail-cuff plethysmography measurements (Online Figure VII). The heart weight:tibia length ratio was found to be increased by chronic Ang II infusion (Online Figure VII), which was significantly decreased by oral minocycline treatment (control, 28.5 ± 1.5 ; Ang II, 37.2 ± 1.8 ; Ang II+minocycline, 31.4 ± 1.0 ; minocycline, 30.4 ± 1.5 mg/mm). In addition, chronic Ang II infusion was associated with increased levels of circulating CD4⁺/CD8⁺ (30%), CD4⁺/CD8⁺/CD25⁺ (130%), and CD68⁺ (100%) cells in comparison with the control group (Figure 6B). This was coupled with similar increases in CD4⁺/CD8⁺ and CD4⁺/CD8⁺/CD25⁺ cells in the BM and a 20% decrease in CD4⁻/CD5⁻/CD8⁻/CD90⁺ APCs (Figure 6C). However, these cell populations were restored back to control levels on treatment with minocycline. Small differences are present between the blood and BM cell populations; however, they follow the same trends in both and support the same conclusions.

Spectral analysis of the SBP signal from transmitter-implanted rats revealed that spontaneous baroreflex gain was dampened in Ang II-infused rats versus control (-0.52 ± 0.07 versus 0.21 ± 0.23 ms/mm Hg; Online Figure VIII). However, this effect was attenuated by minocycline treatment (-0.11 ± 0.06 ms/mm Hg). Low frequency (SBP), very low frequency (SBP), and Δ low frequency(SBP):HF(PI) were all increased in Ang II-infused rats (1.08 ± 0.34 , 3.5 ± 0.3 , and

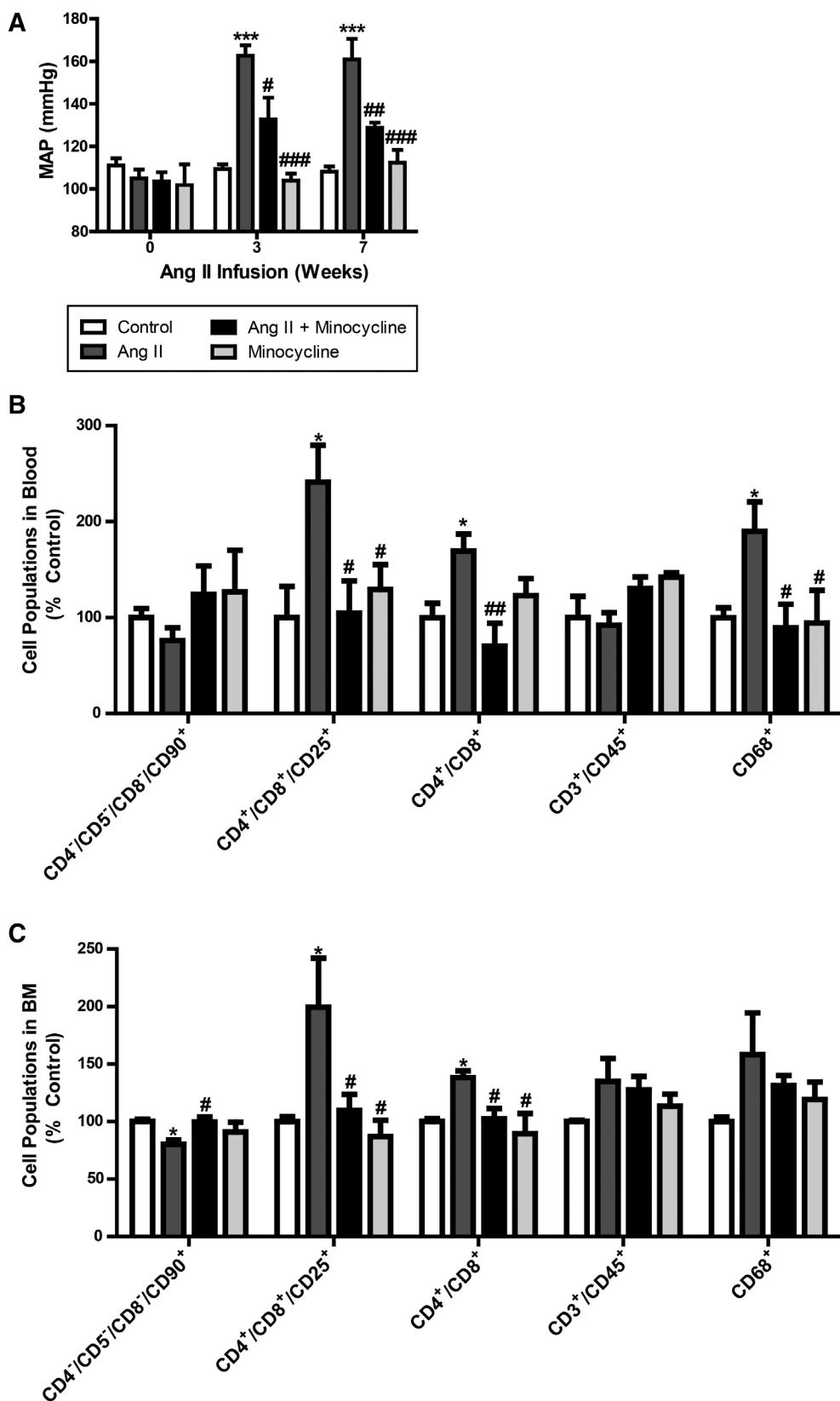


Figure 6. Oral minocycline (mino) attenuates mean arterial pressure (MAP) and peripheral inflammation in chronic angiotensin II (Ang II) infusion. **A,** Mino attenuates the development of hypertension in the chronic Ang II infusion model (n=4 per group). This effect was consistent for 7 weeks of treatment. **B,** Specific inflammatory cell populations were increased in the blood in chronic Ang II infusion, including CD4⁺/CD8⁺/CD25⁺, CD4⁺/CD8⁺, and CD68⁺. Mino treatment lowers these ratios back to control (n=4–8 per group). **C,** After a similar trend, CD4⁺/CD8⁺/CD25⁺ and CD4⁺/CD8⁺ cells were increased in the bone marrow and decreased by mino treatment. CD4⁺/CD5⁺/CD8⁺/CD90⁺ cells (angiogenic progenitor cells) were lower in chronic Ang II infusion and restored by oral mino (n=4–8 per group). *P<0.05, **P<0.01, ***P<0.001 vs control; #P<0.05, ##P<0.01, ###P<0.001 vs Ang II.

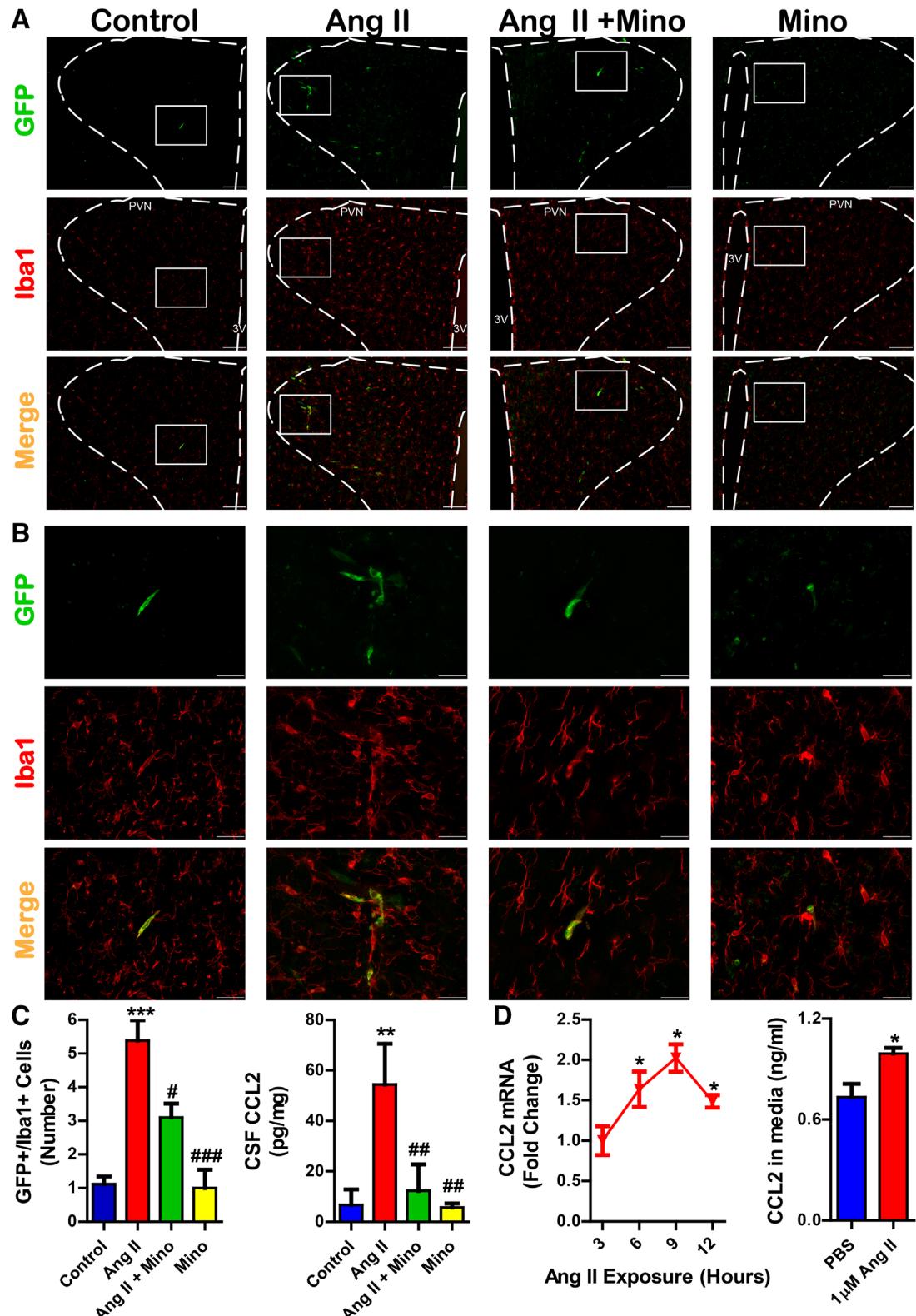


Figure 7. Chronic angiotensin II (Ang II) infusion increases bone marrow-derived microglia/macrophages in the hypothalamic paraventricular nucleus (PVN). **A**, Representative images at $\times 10$ magnification from the PVN of experimental groups. Green fluorescent protein (GFP)+ cells are bone marrow derived, and Iba1+ cells indicate microglia/macrophages. Scale bar, 100 μ m; images taken at Bregma -1.80 mm; PVN and third ventricle (3 V) are labeled for orientation. **B**, Higher magnification ($\times 40$) images of GFP+/Iba1+ cells in the PVN. Scale bar, 30 μ m. **C**, Quantification of GFP+/Iba1+ cells in the PVN reveals an increase in the number of cells in chronic Ang II infusion group, which is decreased by minocycline (mino) treatment. C-C chemokine ligand 2 (CCL2) content in the cerebrospinal fluid (CSF) was also attenuated by mino (n=5–8 per group). **D**, Ang II treatment (1 μ mol/L) of primary hypothalamic neurons induces an increase in CCL2 mRNA and CCL2 protein in the cell culture media. *P<0.05, **P<0.01, ***P<0.001 vs control; #P<0.05, ##P<0.001 vs Ang II.

0.12 ± 0.05 mm Hg $^2/\text{ms}^2$, respectively), but were normalized by minocycline treatment (-0.66 ± 0.28 , 0.6 ± 0.8 , and -0.11 ± 0.05 mm Hg $^2/\text{ms}^2$, respectively). In contrast, no significant changes were observed in cardiac parasympathetic ($\Delta\text{HF [PI]}$) and sympathetic tone (Δ low frequency[PI]:HF[PI]; data not shown).

In addition, plasma norepinephrine levels were increased by chronic Ang II infusion (885 \pm 62 versus 1610 \pm 165 pg/mL; Online Figure VIII), whereas minocycline treatment resulted in a significant decrease (733 \pm 92 pg/mL). These findings are consistent with BM supernatant norepinephrine contents (control, 54 \pm 17; Ang II, 280 \pm 66; Ang II+minocycline, 120 \pm 22; and minocycline, 165 \pm 22 pg/ μg).

Chronic Ang II Infusion Increases BM-Derived Microglia/Macrophages in the PVN

Finally, we investigated the link between the BM and brain microglial activation. Chronic Ang II infusion in the enhanced green fluorescent protein (eGFP) chimeric Sprague Dawley rats was used to track mobilization of the BM-derived cells. Online Figure IX shows that the Ang II-induced increase in MAP is comparable in chimeric eGFP and naïve Sprague Dawley rats (149 \pm 3 mm Hg in chimera versus 151 \pm 8 mm Hg in naïve). We observed a 4.3-fold increase in GFP+/Iba1+ cells in the PVN of Ang II-infused chimeric animals (Figure 7A–7C). Minocycline treatment was associated with a 37% decrease in GFP+/Iba1+ cells in the PVN. No distinct changes were observed in other autonomic brain centers, including the subfornical organ and the solitary tract nucleus (Online Figure X).

Because the mechanism of BM cell extravasation into the CNS has been suggested to be associated with the CCL2/CCR2 chemokine axis,^{27–29} we further investigated this possibility. We observed that chronic Ang II infusion increased the protein concentration of CCL2 in the CSF (Figure 7C; 47.4 \pm 10.6 versus 6.7 \pm 3.5 pg/ μg protein). This increase was blocked with minocycline treatment (12.3 \pm 6.1 pg/ μg). In addition, incubation of primary neuronal cultures with Ang II (1 $\mu\text{mol/L}$) significantly increased the mRNA expression of CCL2 (1.6, 2.0, and 1.5 fold increase at 6, 9, and 12 hours, respectively), whereas increased levels of CCL2 protein were observed in the culture media after 9 hours of Ang II exposure (\approx 35% increase; Figure 7D).

Discussion

The major findings of this study are as follows: (1) the SHR BM is characterized by increased ICs and cytokines in hypertension and plays a key role in blood pressure regulation, (2) proinflammatory BM cells migrate to the PVN and enhance neuroinflammation, and (3) oral minocycline produces antihypertensive effects by attenuating both peripheral and neuroinflammation. These findings greatly enhance our understanding of the communication that exists between the autonomic nervous system and IS, which contributes to the development and maintenance of hypertension. Furthermore, our findings suggest that minocycline could be a potential therapeutic approach for combating drug-resistant hypertension in patients exhibiting high levels of inflammation.

Evidence supporting the proinflammatory status of BM in hypertension is presented not only in this study but also in

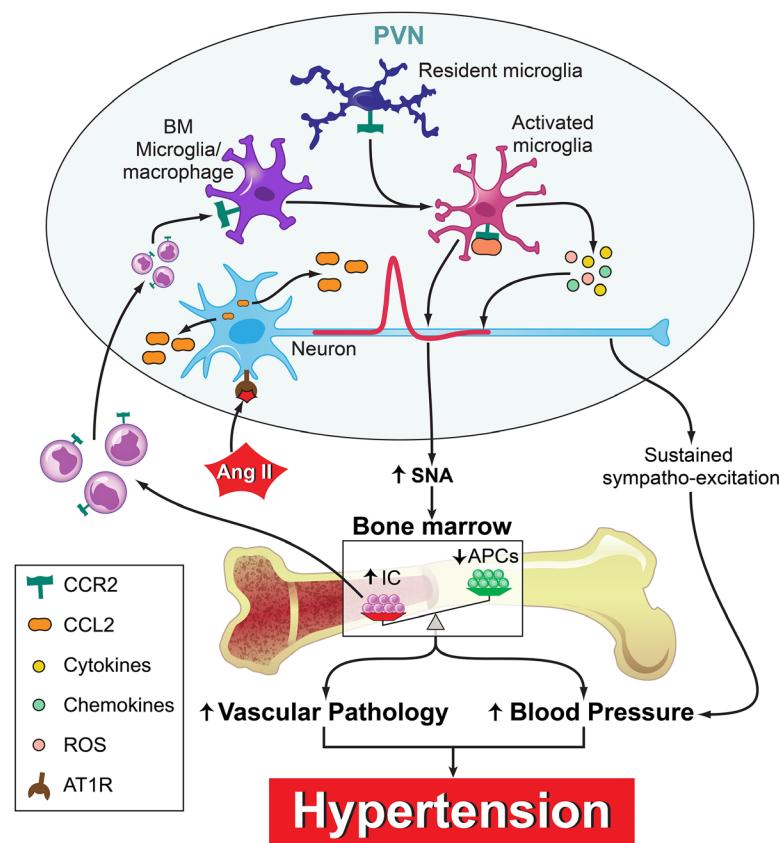


Figure 8. Proposed hypothesis for the extravasation of bone marrow cells to the hypothalamic paraventricular nucleus (PVN) and the involvement of neuroinflammation in hypertension (HTN). Prohypertensive signals such as angiotensin II (Ang II) activate PVN preautonomic neurons to increase in sympathetic nerve activity (SNA) and cause release of C-C chemokine ligand 2 (CCL2). The increased SNA affects the bone marrow (BM) resulting in an increase in inflammatory cells (IC) and decrease in angiogenic progenitor cells (APCs). This imbalance is associated with vascular pathology and increase in blood pressure. In addition, some of these inflammatory progenitors migrate to the PVN as a result of an increased neuronal release of CCL2 where they differentiate into BM-derived microglia/macrophages. Both resting microglia and BM-derived microglia/macrophages are activated to release an array of cytokines, chemokines, and reactive oxygen species (ROS), which will further increase preautonomic neuronal activity. This leads to a state of sustained sympathoexcitation, which will result in a perpetuation of high blood pressure and ultimately established hypertension.

published literature. (1) Our previous data have demonstrated that changes in the direct BM innervation through the femoral sympathetic nerve are associated with changes in the BM activity^{9,11}; (2) the SHR BM is characterized by elevated IC counts when compared with WKY rats⁹; (3) similar increases in BM ICs have also been demonstrated in the chronic Ang II infusion model of hypertension¹⁰; and (4) neurohormonal modulation of the SHR IS is proinflammatory.³⁸ Thus, accumulating data from experimental models of hypertension and other pathophysiological conditions describe a neural control of the IS.⁵⁻⁷ The novelty of the present study lies in the finding that the prohypertensive, proinflammatory BM may also affect the brain, that is, that the overactive IS may reciprocally modulate the autonomic nervous system and further contribute to its dysfunction by exacerbating neuroinflammation.

Our study is the first to demonstrate the extravasation of BM cells into the autonomic CNS areas in hypertension. More specifically, increased infiltration of the proinflammatory BM cells into the PVN of hypertensive rats was associated with increased CCL2 levels in the CSF, which was considerably decreased by oral minocycline treatment. These observations, although novel in the field of hypertension, align with those reported in chronic psychological stress,²⁷ amyotrophic lateral sclerosis,^{39,40} experimental autoimmune encephalomyelitis,^{41,42} and Alzheimer disease.^{43,44} This concept has been further validated by *in vivo* imaging of turnover of microglia and infiltration of BM-derived cells into the murine retina.⁴⁵ Although the total number of GFP+/Iba1+ macrophage/microglia cells in the PVN may seem low, it is nevertheless significant and can have a drastic effect on perpetuation of neuroinflammation because of release of reactive oxygen species and proinflammatory cytokines, especially as the 5-fold increase in GFP+/Iba1+ microglia/macrophages observed in the present study is similar to that previously reported in the PVN of chronically stressed mice.²⁷ Our study, showing an increase in CCL2 in the CSF and a significant gradient from BM<serum<CSF, suggests the involvement of CCL2/CCR2 signaling system in extravasation of BM cells in hypertension and neuroinflammation. The ability of minocycline to attenuate MAP and to decrease CCL2 levels in the CSF further supports this contention. It is pertinent to note that this chemokine axis is shown to be an important player in the migration of T lymphocytes, monocytes, and natural killer cells in type 2 diabetes mellitus and rheumatoid arthritis.⁴⁶ There is evidence that Ang II directly stimulates the production of CCL2 in monocytes⁴⁷ and vascular smooth muscle cells⁴⁸ in an AT1 receptor-mediated pathway.⁴⁹⁻⁵¹ In addition to these sources of CCL2, we have presented data that indicate that Ang II can also directly stimulate the production and release of CCL2 in hypothalamic neurons. Thus, we think that the beneficial effects of minocycline involve multiple mechanisms that include inhibiting the activation of microglia and lowering CCL2 concentration in the CSF.

As a therapeutic agent, minocycline was also able to decrease total sympathetic nerve activity, as measured by plasma norepinephrine concentration and spectral analysis in 2 different models of hypertension. Therefore, minocycline may provide a novel avenue to target the neurogenic component of resistant hypertension. The neural effects of oral minocycline stem from its ability to cross the blood-brain barrier, where

once inside the brain parenchyma, minocycline can act as an anti-inflammatory inhibitor of microglial activation, as supported by our data. Our previous study has indicated that decreased microglia activation by either intracerebroventricular minocycline or intracerebroventricular mitoTEMPO (mitochondrial-targeted antioxidant) is associated with decreased MAP.^{10,19} Thus, the present study further supports the involvement of microglial activation in neurogenic hypertension. However, we incorporated an additional novel aspect to this hypothesis, as we report an increase in BM-derived microglia/macrophages in the brain parenchyma of the PVN in hypertension. This extravasation of the proinflammatory BM cells to the PVN could also be blocked by oral minocycline treatment, suggesting a dual role for minocycline: as a central and peripheral anti-inflammatory agent. Considering the present evidence for reciprocal autonomic nervous system-IS communication in hypertension, this apparent characteristic of minocycline to act peripherally and centrally may be vital in breaking the vicious cycle of drug-resistant hypertension.

It has been previously shown that transplanting the thymus from a normotensive WKY can decrease hypertension in the SHR.^{6,52} Here, we generated SHR and WKY chimeric animals to establish a direct relationship between the BM and blood pressure regulation. We did not observe any acute or chronic graft rejection or adverse graft-versus-host effects because (1) all groups had a >80% survival and did not display sickness behaviors, (2) the results from WKY to SHR reconstitution and SHR to WKY reconstitution are different and opposite, and (3) a set of control animals survived for 6 months without complications. Therefore, our results represent true effects of BM cells and long-term compatibility of WKY and SHR tissues is not an issue.

The levels of ICs were dependent on the type of BM cells in that particular animal, that is, rats receiving SHR BM cells consistently presented with higher percentages of the T-cell subpopulations CD4⁺/CD8⁺/CD25⁺ and CD3⁺/CD45⁺ in the blood than those receiving WKY BM cells. This finding is particularly important as hypertension development has been shown to be heavily dependent on T cells.¹⁴ In addition, the SHR BM cells contributed to an elevation in sympathetic drive in the WKY recipients, which was associated with increased activated microglia, suggesting that the extravasation of the proinflammatory BM cells to the PVN contributed to neuroinflammation and hypertension in WKY animals. However, the WKY BM cells were unable to lower the overactive sympathetic drive in the SHR, despite somewhat reversing the neuroinflammation. This is supported by the hemodynamic measurements that indicate a difference in blood perfusion between the WKY-SHR and WKY-WKY, but no change between the SHR-WKY and SHR-SHR groups. It seems that reducing both systemic and neuroinflammation by transplanting the WKY BM in the SHR is not sufficient to correct/reverse the autonomic dysfunction, despite significantly lowering the blood pressure. Therefore, this may indicate that BM ICs, under already established hypertension in the SHR, may not play a major role in neurogenic regulation of blood pressure, and that the effect may predominantly be peripheral, at least under these specific experimental conditions. Considering that by transplanting the WKY BM in the SHR we also see a significant improvement in the BM

APCs, previously shown to have both angiogenic and reparative abilities, it is possible that the WKY BM transplant has also improved the vascular endothelial repair in the SHR, thereby contributing to blood pressure lowering in these rats.

In summary, we propose the following hypothesis (Figure 8). prohypertensive signals such as Ang II activate PVN preautonomic neurons to increase in sympathetic nerve activity and cause release of CCL2. The increased sympathetic nerve activity affects the BM resulting in an increase in IC and decrease in APCs. This imbalance is associated with vascular pathology and increase in blood pressure. Therefore, the BM of hypertensive animals (both SHR and chronic Ang II infusion model) is characterized by an increase in ICs and factors and is in turn able to contribute to the blood pressure regulation. In addition, some of these inflammatory progenitors migrate to the PVN as a result of an increased neuronal release of CCL2 where they differentiate into BM-derived microglia/macrophages and exacerbate neuroinflammation, thereby perpetuating the CNS-BM dysfunctional pathway in the establishment of resistant hypertension. Both resting microglia and BM-derived microglia/macrophages are activated to release an array of cytokines, chemokines, and reactive oxygen species, which will further increase preautonomic neuronal activity. This leads to a state of sustained sympathoexcitation which will result in a perpetuation of high blood pressure and ultimately established hypertension. Extensive evidence exists for the role of neuroinflammation in hypertension, which are thoroughly outlined in our previous review.⁵³ Increased renin–angiotensin system activity in hypertensive models has a role in driving proinflammatory responses in the brain.^{54,55} Neuroinflammation in key cardiovascular centers of the brain is associated with increased sympathetic activity and hypertension and inhibition of inflammation in these brain regions attenuates the hypertension.^{10,15–17,19} In addition, these cardioregulatory brain centers seem to control the peripheral IS through autonomic output.^{8,9}

This proposal is supported by a preliminary report where minocycline was able to produce impressive antihypertensive effects in obese, drug-resistant hypertensive patients.²⁴ However, considering its antibiotic nature and peripheral anti-inflammatory role, the contribution of potential changes in gut microbiota in the overall beneficial effects of minocycline cannot be ruled out at the present time. This is particularly relevant in view of the evidence that minocycline has been shown to influence inflammatory responses in depression, protect the gut mucosal damage induced by chemotherapy, enhance morphine's effectiveness in diabetic neuropathy, and improve intestinal damage and prevent reactivation of colitis.^{56,57} Clinical trials are underway (ClinicalTrials.gov Identifiers: NCT02133872 and NCT02188381) to evaluate the role of neuroinflammation and gut microbiota in the treatment of resistant hypertension with minocycline.

Perspectives

In this study, we present direct evidence for the involvement of whole BM cells in hypertension. This is evidenced by the following: (1) whole BM reconstitution between SHR and WKY animals is able to modulate blood pressure in the parent

rat, (2) BM status contributes to peripheral inflammation, and (3) central inflammation by modulation of microglia activation in the PVN. We found evidence that BM cells extravasated into the brain and contributed to central inflammation via the CCL2/CCR2 chemokine axis. Of clinical relevance, we found that oral delivery of minocycline was able to attenuate hypertension in 2 different rat models of hypertension, lower central and peripheral inflammation, as well as decrease total sympathetic activity. This study supports further investigation of minocycline in resistant hypertension patients with neurogenic components.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Increased sympathetic nerve activity and inflammation are hallmarks of human hypertension, particularly drug-resistant hypertension.
- The central nervous system to immune system communication seems to be altered in hypertension and cardiovascular disease in humans, although exact mechanisms remain to be elucidated.

What New Information Does This Article Contribute?

- Oral minocycline delivery is a novel therapy for drug-resistant hypertension with neurogenic components and may be used as a therapy for hypertension and can also target neurogenic and inflammatory components of hypertension.
- Generation of chimeric rats from normotensive and hypertensive bone marrow (BM) demonstrates direct involvement of BM cells on blood pressure regulation.
- BM cells migrate and extravasate into the paraventricular nucleus, where they differentiate into microglia/macrophages and contribute to neuroinflammation in hypertension.

Hypertension is associated with increased sympathetic drive to the BM that leads to dysfunctional BM activity, characterized by elevated inflammatory cells and decreased reparative angiogenic progenitor cells. However, the reciprocal effect of the BM on the central nervous system is unknown. In this study, the spontaneously hypertensive rat BM is characterized by increased inflammatory cells and factors and is in turn able to contribute to the blood pressure regulation. Some of these progenitor cells migrate to the central nervous system via the C-C chemokine ligand 2/C-C chemokine receptor 2 chemokine axis where they exacerbate neuroinflammation, thereby perpetuating the central nervous system-BM dysfunctional pathway in the establishment of resistant hypertension. Finally, minocycline proved to have impressive antihypertensive and anti-inflammatory effects in 2 rat models of hypertension. Therefore, further investigation into the therapeutic potential of minocycline is necessary.