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Chronic sympathetic driven hypertension promotes atherosclerosis by enhancing hematopoiesis

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ABSTRACT

Hypertension is a major, independent risk factor for atherosclerotic cardiovascular disease. However, this pathology can arise through multiple pathways, which could influence vascular disease through distinct mechanisms. An overactive sympathetic nervous system is a dominant pathway that can precipitate in elevated blood pressure. We aimed to determine how the sympathetic nervous system directly promotes atherosclerosis in the setting of hypertension. We used a mouse model of sympathetic nervous system-driven hypertension on the atherosclerotic-prone apolipoprotein E-deficient background. When mice were placed on a western type diet for 16 weeks, we showed the evolution of unstable atherosclerotic lesions. Fortuitously, the changes in lesion composition were independent of endothelial dysfunction, allowing for the discovery of alternative mechanisms. With the use of flow cytometry and bone marrow imaging, we found that sympathetic activation caused deterioration of the hematopoietic stem and progenitor cell niche in the bone marrow, promoting the liberation of these cells into the circulation and extramedullary hematopoiesis in the spleen. Specifically, sympathetic activation reduced the abundance of key hematopoietic stem and progenitor cell niche cells, sinusoidal endothelial cells and osteoblasts. Additionally, sympathetic bone marrow activity prompted neutrophils to secrete proteases to cleave the hematopoietic stem and progenitor cell surface receptor CXCR4. All these effects could be reversed using the β-blocker propranolol during the feeding period. These findings suggest that elevated blood pressure driven by the sympathetic nervous system can influence mechanisms that modulate the hematopoietic system to promote atherosclerosis and contribute to cardiovascular events.

Introduction

Hypertension is a major, independent risk factor for atherosclerotic cardiovascular disease (CVD).[1] As the pathophysiology of hypertension is both complex and multifactorial, the direct mechanism(s) that ultimately contribute to CVD remain

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unclear.[2] The most frequently targeted pathway in reducing blood pressure is the renin-angiotensin system (RAS). The contribution of the RAS to hypertension and atherosclerosis is not exclusive, as angiotensin II (AngII) can also accelerate atherogenesis independent of hypertension.[3,4] Another major determinant of hypertension is an overactive sympathetic nervous system (SNS).[5,6] There are indications that autonomic input into the bone marrow (BM) may be altered in the setting of hypertension.[7-10] However, the mechanisms promoting atherogenesis with SNS activation associated hypertension are not completely elucidated. While there is an overlap in some atherosclerosis promoting mechanisms between the RAS and SNS, a distinct subset of events is also likely to be evoked by the SNS, which requires further investigation.

Atherosclerosis is a disease driven by the infiltration of immune cells, in particular monocytes, into the plaque.[11-13] It is also well established that the abundance of circulating monocytes predicts cardiovascular (CV) events and is directly linked to atherogenesis.[14,15] Interestingly, the SNS plays a direct role in regulating the hematopoietic system from which immune cells, including monocytes, arise.[16-19] In the context of CVD, the mobilization of hematopoietic stem and progenitor cells (HSPCs) from the BM to extramedullary tissues such as the spleen results in the generation of atherogenic monocytes that abundantly enter into the atherosclerotic plaque.[20] Mobilization of HSPCs can be mediated by sympathetic signaling within the BM, particularly in response to granulocyte-colony stimulating factor (G-CSF). The SNS synergizes with G-CSF to promote the breakdown of the HSPC BM microenvironment, which decreases the abundance of key HSPC retention factors and results in the liberation of HSPCs into the circulation.[16] This pathway has also been shown to be activated following a myocardial infarction (MI).[21] Sympathetic activation, along with raised G-CSF levels that are observed in apolipoprotein E knockout (*Apoe*$^{-/-}$) mice, caused HSPC mobilization from the BM and homing to the spleen where monocytes were subsequently produced that infiltrated atherosclerotic lesions. Interestingly, this promoted an unstable plaque phenotype, prone to rupturing and thus provides a plausible explanation for primary heart attack survivors being highly prone to a secondary, often fatal, CV event.[21,22] Importantly, the involvement of the SNS in driving aberrant hematopoiesis is not restricted to complications following a MI, as similarities in other models of stress and ischemic stroke are evident, suggesting this to be a more general mechanism. The augmented hematopoietic response in these pathologies caused by overactivation of the SNS were inhibited by administration of β-blockers or genetic deletion of β-adrenergic receptors.[21,23-25]

There appears to be an important role of the SNS in regulating hematopoiesis in acute stressors (i.e., MI, stroke, variable stress). However, it remains unknown if chronic sympathetic activation invokes this same atherogenic process. Thus, it is plausible that chronic sympathetic activation present in some cases of hypertension could play an important role in regulating atherogenesis by altering hematopoiesis. To address this question, we employed the Schlager hypertensive mice which were crossed onto an *Apoe*$^{-/-}$ background to produce hypertensive atherosclerosis-prone mice. The Schlager mouse was chosen as it represents a model of hypertension that is almost entirely driven by the SNS, with minimal contribution by the RAS.[26] We sought to characterize the contribution of SNS activation associated hypertension to the development of atherosclerosis, with the aim of understanding whether this form of hypertension was also associated with alterations to the hematopoietic system. Moreover, we aimed to investigate whether targeting the SNS could inhibit atherogenesis and, in turn, reveal an additional mechanism of hypertension associated atherosclerosis.

Methods

Detailed methods are available in *Online supplementary Methods.*

Animal Models

Apoe$^{-/-}$ mice were purchased from Jackson Laboratories and bred at the AMREP Animal centre. To generate hypertensive *Apoe*$^{-/-}$ mice, BPH/2J mice were crossed with Apoe-/- mice to produce BPH/2J x *Apoe*$^{-/-}$ (BPH/*Apoe*$^{-/-}$) mice. At 6 weeks of age, male *Apoe*$^{-/-}$ and BPH/ Apoe-/- mice were placed on a western type diet (WTD - SF00-219, Specialty Feeds, Australia; 21% fat, 0.15% cholesterol) for 16 weeks. In the first cohort of mice, age-matched mice *Apoe*$^{-/-}$ and BPH/*Apoe*$^{-/-}$ were placed on a WTD for 16 weeks for end-point analysis. In a second cohort of mice, obtained from a new set of breeders, three groups of aged-matched mice were employed: 1) Apoe-/-, 2) BPH/*Apoe*$^{-/-}$ and 3) BPH/ *Apoe*$^{-/-}$ + propranolol (0.5g/L; administered via drinking water for the duration of the WTD feeding). For the propranolol group, mice consumed on average 2.5ml of water amounting to an average daily dose of 35-40mg/kg/daily of propranolol.

To determine the effect of specific 2-adrenoreceptor blockade on HSPC mobilization and blood pressure we used BPH mice on an *Apoe*$^{+/+}$ background. The mice were injected daily with ICI-118551 (5mg/kg; Abcam, AUS) for 2 weeks.

All animal experiments were approved by the AMREP Animal Ethics Committee and conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes as stipulated by the National Health and Medical Research Council of Australia. All mice were housed in a normal light and dark cycle and had *ad libitum* access to food and water. Mice were randomly assigned to treatment and end-point analysis was blinded.

Statistics

Data are presented as mean ± SEM (unless stated otherwise) and were analysed using the two-tailed Student *t*-test or One-way ANOVA where appropriate. Analysis of baseline and final blood pressure between strains was performed using a two-way ANOVA with the factors strain (Pstrain) and time (Ptime) followed by a Sidak post-hoc test to account for multiple comparisons. A $P<0.05$ was considered significant. All tests were performed using the Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Hypertension associated with chronic sympathetic activation promotes an unstable atherosclerotic phenotype

To determine the contribution of chronic sympathetic activation in hypertension to atherosclerosis we crossed Schlager hypertensive mice with *Apoe*$^{-/-}$ mice (BPH/*Apoe*$^{-/-}$) and compared these to normotensive *Apoe*$^{-/-}$ mice. Mice were fed a high fat, high cholesterol western type diet (WTD) for 16 weeks. We preferenced this model over con-

tinual infusions of noradrenaline to allow for circadian fluctuations in blood pressure and heart rate, and to prevent the ongoing immune complications of surgery associated with the use of mini-pumps. Firstly, examining traditional cardiovascular risk factors revealed no change in body weight or cholesterol between the two groups and while blood pressure increased over the feeding period in both strains, the BPH/*Apoe*$^{-/-}$ mice maintained significantly higher blood pressure as measured by tail cuff and radio telemetry (Figure 1, A-C and *Online Supplementary Figure S1*). The mice were also equally active (*Online Supplementary Figure S1*). To explore the effect of chronic sympathetic activation associated with hypertension on atherosclerosis, we assessed the atherosclerotic burden in

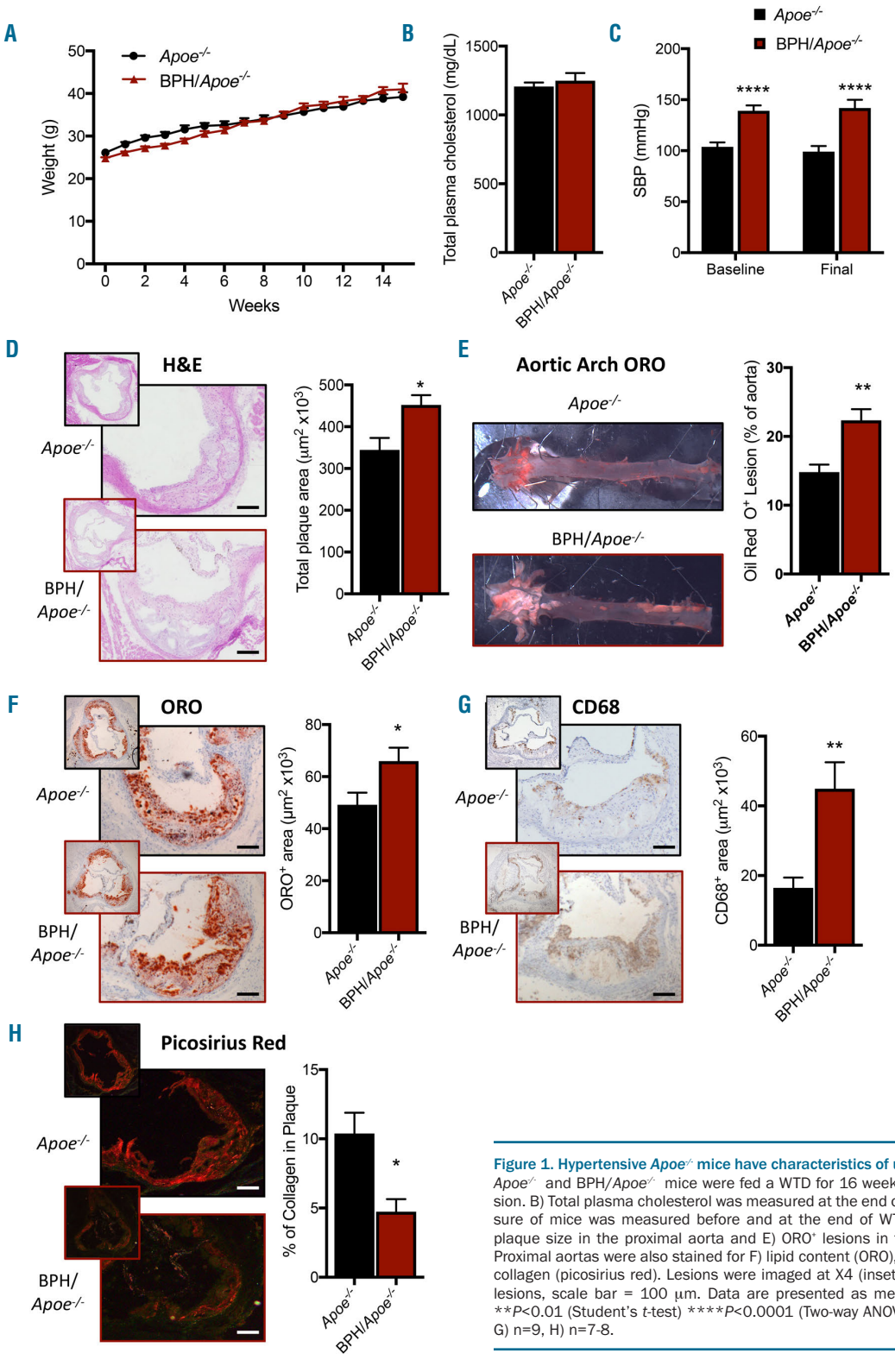

Figure 1. Hypertensive *Apoe*$^{-/-}$ mice have characteristics of unstable atherosclerotic lesions. *Apoe*$^{-/-}$ and BPH/*Apoe*$^{-/-}$ mice were fed a WTD for 16 weeks A) Weekly body mass progression. B) Total plasma cholesterol was measured at the end of feeding. C) Systolic blood pressure of mice was measured before and at the end of WTD feeding. D) H&E staining for plaque size in the proximal aorta and E) ORO^{+} lesions in the aortic arch were quantified. Proximal aortas were also stained for F) lipid content (ORO), G) macrophages (CD68) and H) collagen (picosirius red). Lesions were imaged at X4 (insets) and zoomed in to view single lesions, scale bar = 100 μm. Data are presented as mean ± SEM where *P<0.05 and **P<0.01 (Student's t-test) ****P<0.0001 (Two-way ANOVA). A,B,C) n= 5-10, D,E,F) n= 8, G) n=9, H) n=7-8.

the proximal aorta and aortic arch. We observed increases in plaque size between the groups (Figure 1D, E), suggesting that sympathetically driven hypertension may promote accelerated plaque growth. We further explored the lesion characteristics and noted a significant increase in the abundance of lipid within the lesions from the BPH/*Apoe*$^{-/-}$ mice (Figure 1F). A significant increase in plaque macrophages were accompanied by a decrease in plaque collagen in the BPH/*Apoe*$^{-/-}$ mice (Figure 1G, H), suggesting that chronic sympathetic activation was promoting remodeling of lesions in an adverse, unstable manner. This plaque phenotype in the BPH/*Apoe*$^{-/-}$ mice resonates with the findings of Dutta *et al.* in the context of acute SNS stimulation during a MI.[21]

Hypertensive *Apoe*$^{-/-}$ mice do not develop endothelial dysfunction

Endothelial dysfunction is a generally accepted consequence of hypertension.[27] To determine if the enhanced atherogenesis in BPH/*Apoe*$^{-/-}$ mice was the result of endothelial dysfunction we assessed the vascular responses in aortas from the BPH/*Apoe*$^{-/-}$ mice in comparison with

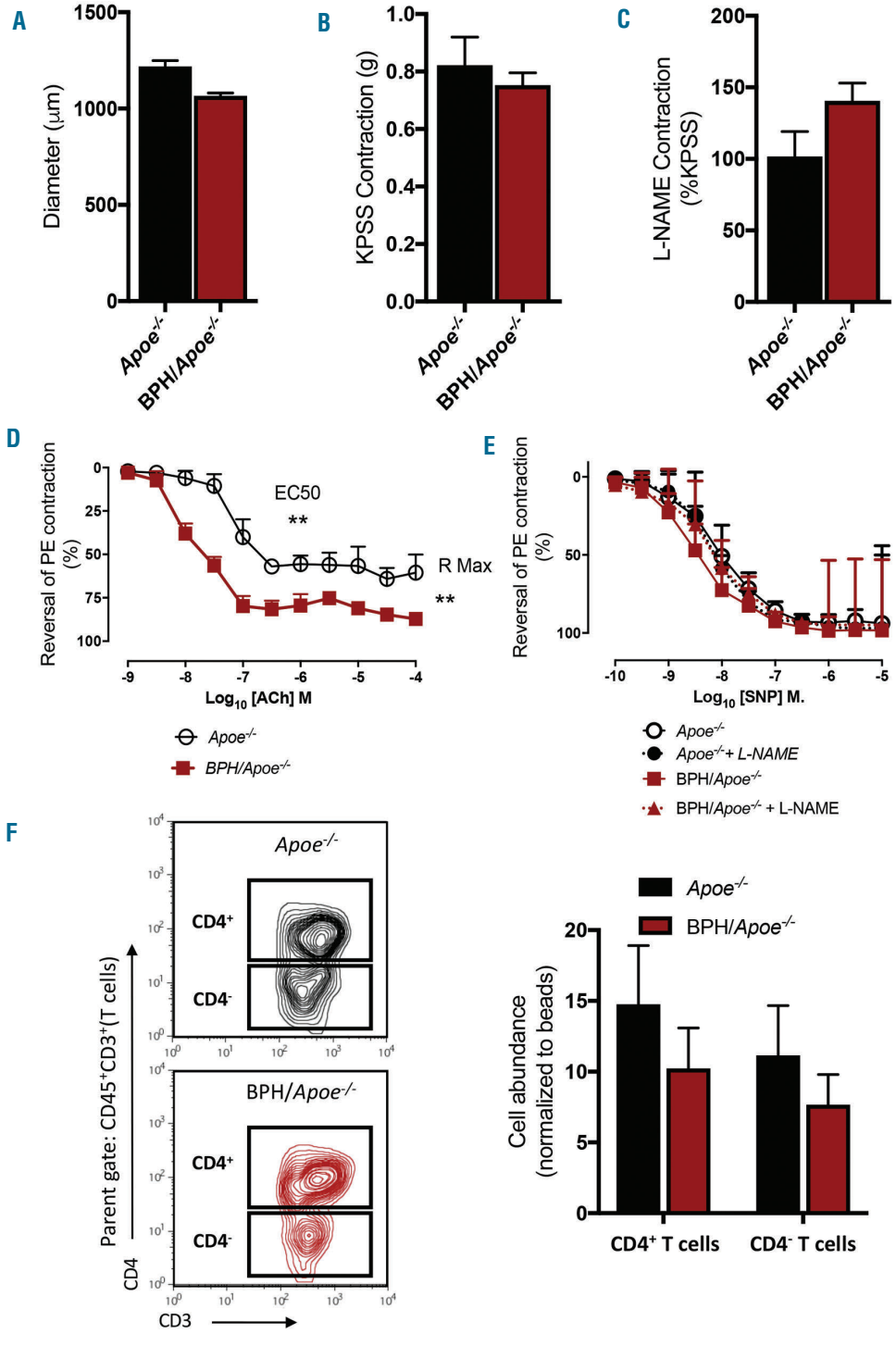

Figure 2. Hypertensive *Apoe*$^{-/-}$ do not have endothelial dysfunction. *Apoe*$^{-/-}$ and *BPH/Apoe*$^{-/-}$ mice were fed a WTD for 16 weeks after which aortas were harvested for *ex vivo* assessment of endothelial function. Aortas were assessed for A) diameter, B) KPSS and C) L-NAME contraction. Further myograph analyses were preformed to determine aortic relaxation in response to D) ACh and E) SNP with or without L-NAME administration. Aortic T-cell infiltration was assessed by flow cytometry for F) Abundance of CD4$^+$ T-helper cells. Data are presented as mean ± SEM where $^{**}P<0.01$ (Student's *t*-test). A,B) n=6-10,C) n= 6-8, D) n= 4-6, E,F) n=6.

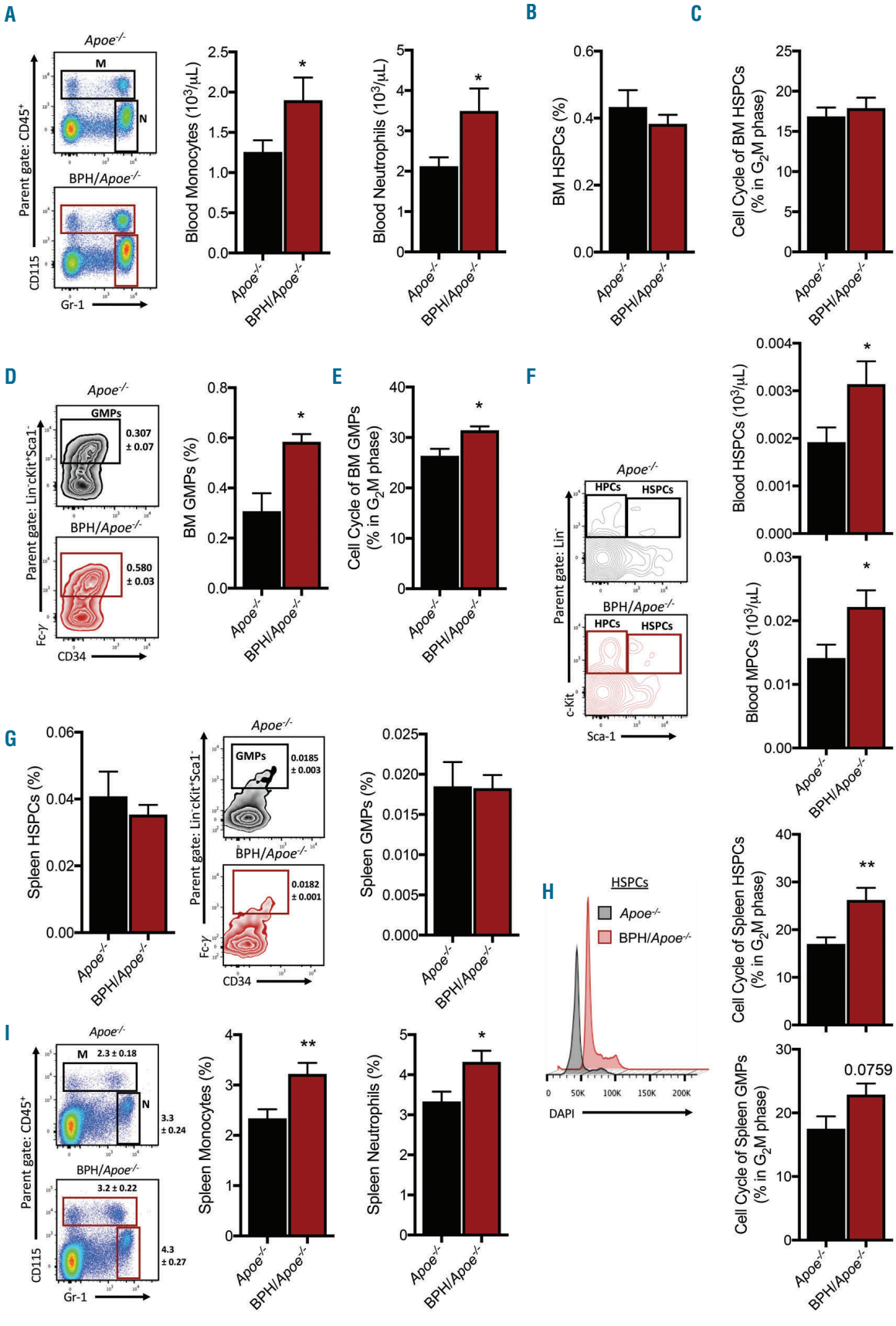

Figure 3. *BPH/Apoe*$^{-/-}$ mice have extramedullary hematopoiesis. *Apoe*$^{-/-}$ and BPH/ *Apoe*$^{-/-}$ mice were fed a WTD for 16 weeks after which flow cytometry was used to assess A) circulating monocytes(M)/neutrophils(N). B) Levels of BM HSPCs and C) proportion of HSPCs proliferating. D) Levels of BM GMPs and E) proportion of GMPs proliferating. F) Blood HSPC and MPC populations. G) Spleen HSPCs and GMPs were assessed along with H) proliferating HSPCs and GMPs. I) splenic monocyte(M)/neutrophil(N) populations. Data are presented as mean ± SEM where **P*<0.05 and ***P*<0.01 (Student's *t*-test). A-I) n= 5-11.

control *Apoe*$^{-/-}$ mice. Firstly, no change in vessel diameter or constrictor responses to a high potassium solution was evident. Nor were there differences in basal nitric oxide (NO) levels when the constriction to L-NAME (L-NG-Nitroarginine methyl ester) was examined (Figure 2, A-C). These data suggest that alterations in vascular reactivity are not biased by differences in constrictor responses. Surprisingly, endothelium-dependent NO-mediated relaxation in response to acetylcholine (ACh) was worse in the *Apoe*$^{-/-}$ mice when compared to BPH/*Apoe*$^{-/-}$ mice (Figure 2D). These differences between the strains were endothelium independent since there were no differences in the constriction and relaxation response to the NO donor sodium nitroprusside (SNP) in the presence or absence of L-NAME (Figure 2E). To further confirm no decline in vascular function in these mice, we examined the abundance of T cells, which have been linked to the pathogenesis of hypertension.[28] We observed no differences in aortic T cells between the *Apoe*$^{-/-}$ and BPH/*Apoe*$^{-/-}$ mice (Figure 2F). Moreover, there was no difference in the activation state of these CD4^{+} T cells, as assessed by CD62L expression (MFI; *data not shown*). These data suggest that the enhanced atherogenesis in the BPH/*Apoe*$^{-/-}$ mice occurs independently of changes to the endothelium.

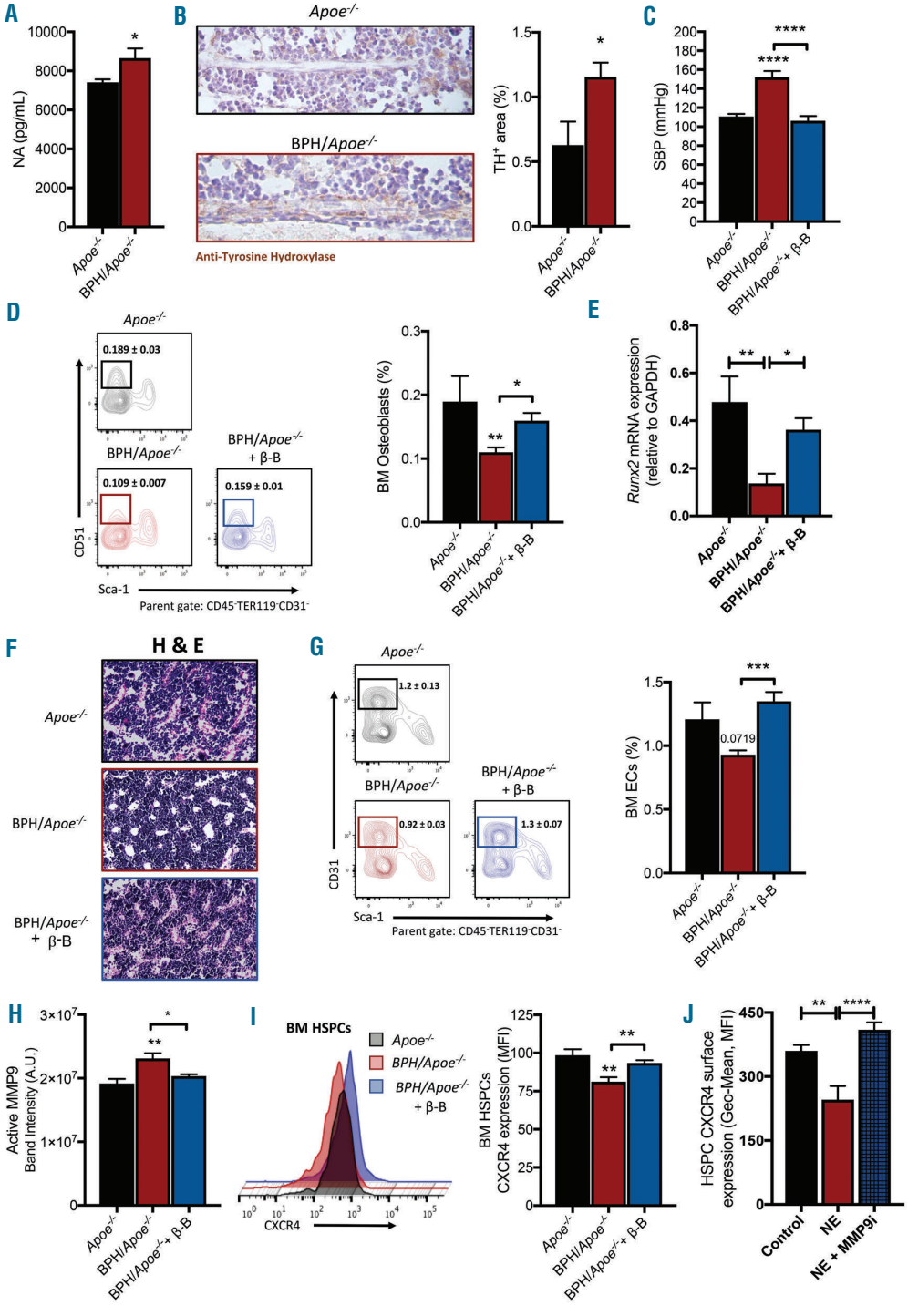

Figure 4. Sympathetic nervous system signalling induces a breakdown of the HSPC niche in the BM. *Apoe*$^{-/-}$ and *BPH/Apoe*$^{-/-}$ mice were fed a WTD for 16 weeks with *BPH/Apoe*$^{-/-}$ mice either treated with or without Propranolol (0.5g/L) in drinking water. A) Plasma NA was quantified by HPLC. B) BM sections were immunostained for sympathetic activity as indicated by tyrosine hydroxylase, imaged at X20; scale bar = 25 μm. C) Systolic blood pressure of mice following 16 weeks treatment. D) Osteoblastic lineage cells were quantified by flow cytometry. E) mRNA levels of *Runx2* in the BM. F) H&E stained representation of BM vascular morphology, imaged at X10 scale bar = 100 μm. G) BMECs were measured by flow cytometry. H) MMP9 content in the BM extracellular fluid was determined via Zymography. I) CXCR4 expression levels on HSPCs was assessed by flow cytometry from and J) neutrophil supernatant cultured HSPCs. Data are presented as mean ± SEM where **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 (Student's *t*-test or One-Way ANOVA). A) n= 8, B) n=5, C) n=7, D) n= 7-9, E) n= 5-9, G) n= 7-9, H) 5-9, I) n= 12-15, J) n= 7.

BPH/Apoe$^{-/-}$ mice have enhanced myelopoiesis

An overactive SNS has recently been shown to promote the mobilization of BM HSPCs to the spleen, resulting in the generation of splenic monocytes that can infiltrate into atherosclerotic lesions. Therefore, we next assessed the hematopoietic system in these mice.[21] We discovered prominent monocytosis and neutrophilia in the BPH/*Apoe*$^{-/-}$ mice in the blood (Figure 3A). Next, to determine if the increased monocyte and neutrophil numbers were due to activated myelopoiesis, we examined the abundance and proliferation of HSPCs and myeloid progenitor cells in the BM. While the levels and proliferation of HSPCs within the BM were similar (Figure 3B, C), we did observe more granulocyte-macrophage progenitors (GMPs) in the BPH/*Apoe*$^{-/-}$ mice, which were proliferating at a higher rate (Figure 3D,E). Consistent with SNS activation in promoting the mobilisation of HSPCs from the BM, we detected elevated levels of circulating HSPCs and myeloid progenitors (MPCs) in the BPH/*Apoe*$^{-/-}$ mice (Figure 3F). Given the higher circulating HSPCs, we were expecting to see more HSPCs in the spleen. However, no such change in the abundance of HSPCs was detected (Figure 3G). Of note, a higher proportion were in the G2M phase of the cell cycle (Figure 3H), suggesting that the chronic activation of the SNS was influencing the HSPCs to proliferate more in the spleens of BPH/*Apoe*$^{-/-}$ mice. Indeed, monocytes and neutrophils were elevated in the spleens of the BPH/*Apoe*$^{-/-}$ mice, confirming extramedullary myelopoiesis was occurring in this chronic sympathetic driven model (Figure 3I).

Sympathetic activation contributes to the breakdown of the HSPC bone marrow microenvironment

The Schlager mice are an established model of sympathetic activation-mediated hypertension.[26] However, we wanted to confirm that there was evidence of increased sympathetic activation in the BM, which could account for the enhanced mobilization of HSPCs observed in Figure 3D. Firstly, to confirm an overall increase in sympathetic tone, we quantified plasma noradrenaline levels, which we found to be higher in the BPH/*Apoe*$^{-/-}$ mice (Figure 4A). More central to our proposed mechanism for enhanced HSPC mobilization in the BPH/*Apoe*$^{-/-}$ mice, we found enhanced expression of tyrosine hydroxylase (TH), the rate-limiting enzyme found in nerve terminals responsible for noradrenaline (NA) production, around the blood vessels in the BM of the BPH/*Apoe*$^{-/-}$ mice (Figure 4B). Together, these data reveal a more global increase in sympathetic tone in SNS-driven hypertension.

Next, we sought to determine if overactive sympathetic signaling in the BM led to changes within the BM microenvironment and whether these changes could be reversed with the use of a β-blocker. Given the importance of sympathetic overdrive in mediating hypertension, as expected, propranolol normalized blood pressure in the BPH/*Apoe*$^{-/-}$ mice (Figure 4C).

Having demonstrated that propranolol could reverse the systemic responsiveness of β-receptors to sympathetic activation in BPH/*Apoe*$^{-/-}$ mice, we examined key HSPC niche cells in the BM to determine if sympathetic overdrive influenced myelopoiesis via effects on the BM niche. Interestingly, we found a significant reduction in the abundance of CD51^{+} osteoblasts in the BM on the BPH/*Apoe*$^{-/-}$ mice, which were restored when these mice were treated with propranolol (Figure 4D). Consistent with this finding, analysis of BM mRNA for *Runx2*, the transcription factor that drives osteoblast production, showed a reduction in *Runx2* expression in the BPH/*Apoe*$^{-/-}$ mice relative to *Apoe*$^{-/-}$ mice. Similar to our flow cytometry data, treatment with propranolol prevented the suppression of *Runx2* expression (Figure 4E). When we assessed the gross morphological changes in the BM, it appeared that the vascular structures were altered with the BPH/*Apoe*$^{-/-}$ mice showing smaller sinusoidal structures relative to the *Apoe*$^{-/-}$ mice, with propranolol reverting the sinusoids back to that seen in the *Apoe*$^{-/-}$ mice (Figure 4F). Furthermore, in examining the endothelial cell population, we noted a trend towards a decrease in the abundance of these cells, which, again, could be restored with the administration of propranolol (Figure 4G). As these niche cells are an important source of the HSPC retention factor CXCL12, we measured its mRNA expression and found that propranolol greatly increased *Cxcl12* expression, thereby potentially aiding in promoting HSPC retention and reduced quiescence in the BM (*Online Supplementary Figure S2, A*). The changes in these two key niche cells may provide a mechanism for increased HSPC release from the BM in BPH/*Apoe*$^{-/-}$ mice. Other cells within the BM express β-adrenoreceptors, which we profiled using gene array data from Novershtern *et al.* and analysed using online software (BloodSpot) to generate a hierarchical differentiation tree.[29,30] Firstly, HSPCs and myeloid progenitors did not display any enrichment for the adrenoceptors. However, neutrophils were identified as one of the cells enriched in transcripts for the β2-adrenoreceptor, but not β1- or β3-adrenoreceptors (*Online Supplementary Figure S2, B-D*). We pharmacologically confirmed the requirement for β2-adrenoreceptor stimulation in HSPC mobilization using the BPH mice on an *Apoe*$^{+/+}$ background by administering the β2 specific antagonist ICI-118551 (*Online Supplementary Figure S2, E*). Furthermore, neutrophils have previously been shown to be responsive to NA *in vitro*.[31,32] Mechanistically, activated neutrophils can release MMP9 which can cleave CXCR4 on HSPCs, providing another avenue to HSPC liberation from the BM.[22,33] We measured levels of MMP9 in the BM extracellular fluid (BMEF) *via* zymography and found that both active and latent MMP9 levels increased in the BPH/*Apoe*$^{-/-}$ mice, a phenotype reversed with propranolol treatment (Figure 4H and *Online Supplementary Figure S2, F*). In support of this we found reduced surface CXCR4 expression on the HSPCs from the BPH/*Apoe*$^{-/-}$ mice, which was restored in mice treated with propranolol (Figure 4I). These data were further supported by BM mRNA analysis indicating that propranolol treatment increases *Cxcr4* expression (*Online Supplementary Figure S2, G*). To explore this mechanism further, we cultured HSPCs in the supernatants of neutrophils treated with NA and examined CXCR4 cell surface abundance. We found significantly less CXCR4 on HSPCs cultured in supernatants from NA activated neutrophils (isolated from wild-type mice), compared to vehicle treated neutrophils, which was prevented when MMP9 was inhibited (Figure 4J). When we included the β2-adrenoreceptor specific inhibitor ICI-118551 into the BM neutrophil stimulation media with NA, the harvested supernatant caused less efficient cleavage of CXCR4 (*Online Supplementary Figure S2, H*) thereby confirming the role for neutrophil β2-adrenoreceptors. These data support the hypothesis that sympathetic activation is present in the BM of the BPH/*Apoe*$^{-/-}$ mice and responsible for the

mobilization of HSPCs by acting on key niche cells along with stimulating neutrophils to secrete proteases that cleave the retention receptor CXCR4 on HSPCs.

Suppressing chronic sympathetic signaling dampens myelopoiesis in hypertensive *Apoe*$^{-/-}$ mice

Having observed a restoration in the HSPC BM microenvironment when BPH/*Apoe*$^{-/-}$ mice were treated with propranolol, we explored if this was also reflected by normalization of myelopoiesis in these mice. Following treatment with propranolol, we observed a reduction in circulating monocytes and neutrophils (Figure 5A). We determined if this reduction was echoed by changes in the BM stem and progenitor populations. Following administration of propranolol, the abundance of BM HSPCs was not affected; however, these cells were proliferating at lower rates and giving rise to fewer GMPs (Figure 5, B-D). Consistent with an improvement in the HSPC microenvironment, there were fewer mobilized HSPCs and MPCs in the blood of the BPH/*Apoe*$^{-/-}$ mice treated with propra-

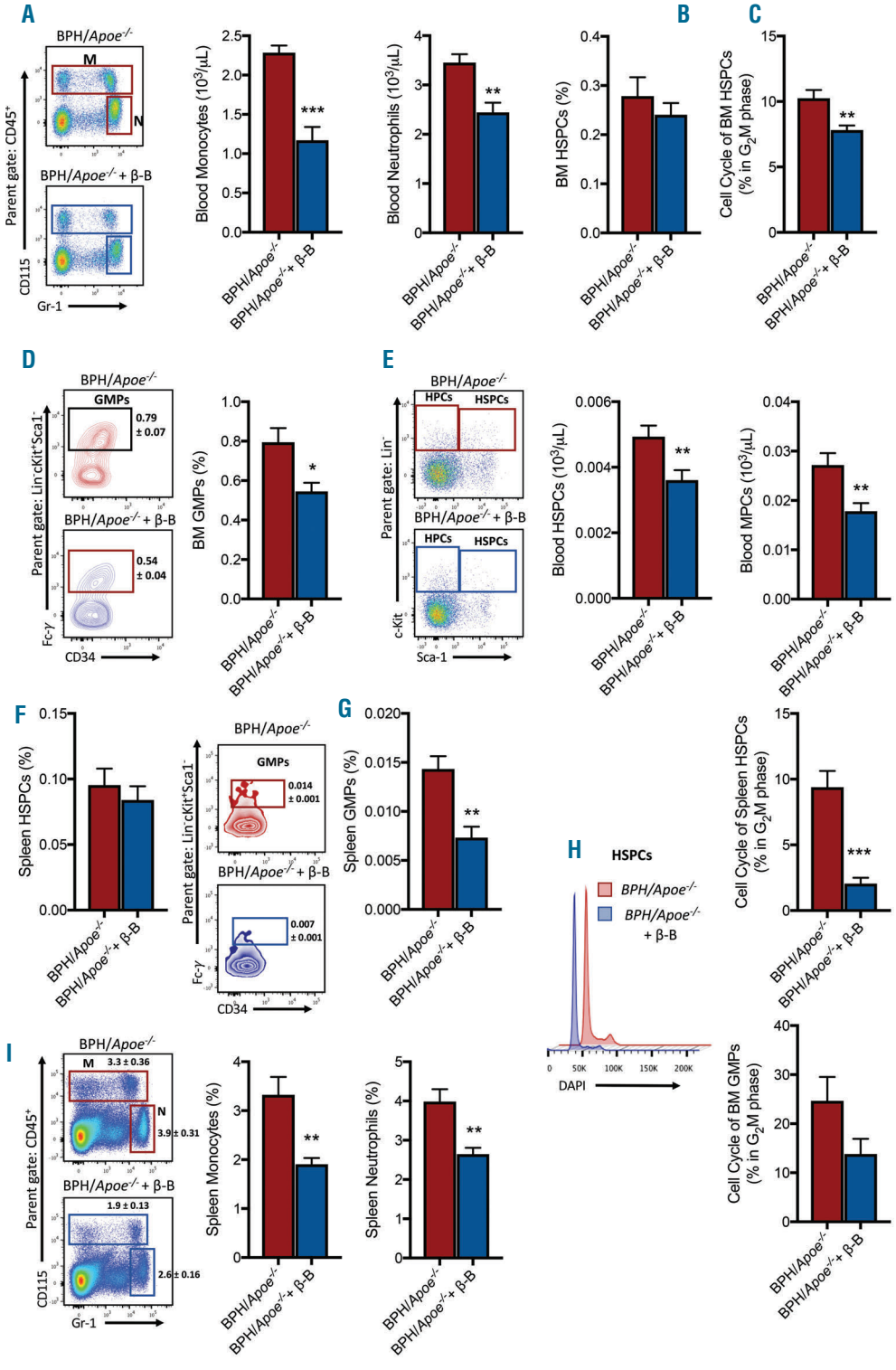

Figure 5. Propranolol prevents extramedullary hematopoiesis in *BPH/Apoe*$^{-/-}$ mice. *BPH/Apoe*$^{-/-}$ mice were fed a WTD for 16 weeks and treated with vehicle or Propranolol (0.5 g/L) in drinking water. Flow cytometry was used to assess A) circulating monocytes(M)/neutrophils(N), B) BM HSPCs and C) proportion of which are proliferating along with D) of BM GMPs. E) Blood HSPC and MPC populations. F) Spleen HSPC and G) GMP populations along with H) proliferating splenic HSPCs and GMPs were assessed by flow cytometry. I) Splenic monocyte(M)/neutrophil(N) populations. Data are presented as mean ± SEM where **P*<0.05, ***P*<0.01 and ****P*<0.001 (Student's *t*-test). A-I) n= 6-10.

nolol (Figure 5E). This was paralleled by a decrease in extramedullary hematopoiesis in the spleen as evidenced by fewer proliferating HSPCs, GMPs and less monocytes and neutrophils (Figure 5, F-I). Taken together, these data suggest that lowering responsiveness to chronic sympathetic signaling in the BPH/*Apoe*$^{-/-}$ mice results in an overall dampening of myelopoiesis.

Blocking sympathetic signalling decreases atherosclerosis in BPH/*Apoe*$^{-/-}$ mice

To examine if the reduction in sympathetic tone and dampening of myelopoiesis was associated with reduced atherosclerotic plaque progression, we assessed the size and complexity of lesions in the proximal aorta. Firstly, we noted a reduction in lesion size in the proximal aorta and aortic arch of BPH/*Apoe*$^{-/-}$ mice treated with propranolol (Figure 6A, B). Exploring the lesion characteristics, we noted that propranolol treated mice had reduced plaque lipid accumulation along with a reduction in plaque macrophages (Figure 6C, D). We also observed a trend for increased collagen (Figure 6E). These changes were seen in the absence of any changes in plasma cholesterol levels (Figure 6F). Given that the hypertension in the BPH/Apoe-/- mice did not promote endothelial dysfunction, it sug-

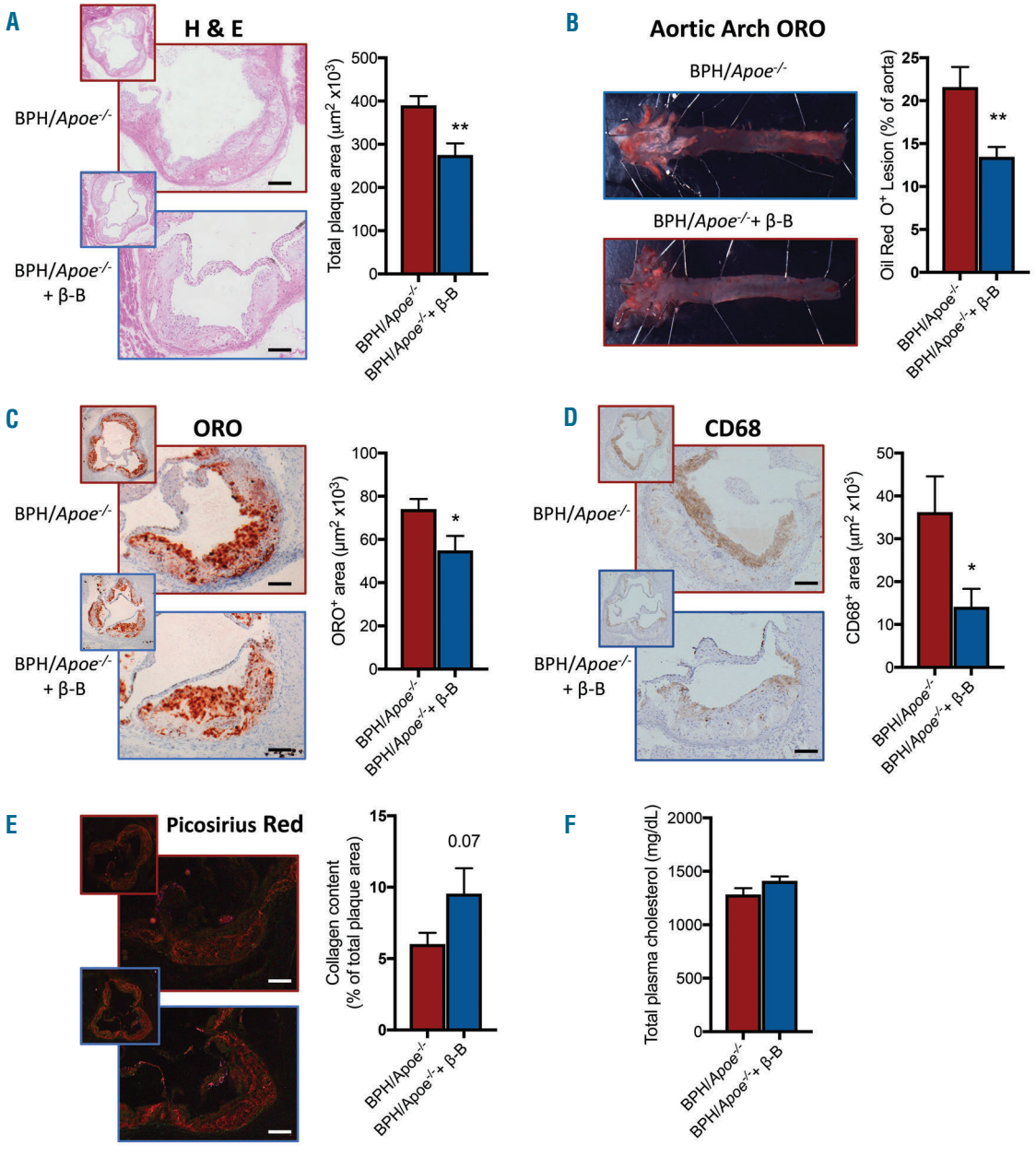

Figure 6. Propranolol inhibits plaque progression in *BPH/Apoe*$^{-/-}$ mice. *BPH/Apoe*$^{-/-}$ mice were fed a WTD for 16 weeks and treated with vehicle or Propranolol (0.5g/L) in drinking water. At the end point, atherosclerosis in the proximal aorta was assessed for A) H&E staining for plaque size in the proximal aorta and B) lipid content (ORO+) lesions in the aortic arch were quantified. Proximal aortas were also stained for C) lipid content (ORO), D) macrophages (CD68) and E) collagen (picosirius red). Lesions were imaged at X4 (insets) and zoomed in to view single lesion, scale bar = 100 μm. F) Total plasma cholesterol levels. Data are presented as mean ± SEM where **P*<0.05 and ***P*<0.01 (Student's *t*-test). A,B) n= 9, C-E) n=7, F) n=9.

gests that the improvements in plaque size and complexity are due to dampened myelopoiesis and, subsequently, reduced monocyte infiltration.

Discussion

Chronic hypertension is arguably one of the most common risk factors associated with atherosclerotic CVD.[1] However, delving into the responsible mechanism(s), it remains unclear if an increase in blood pressure alone, or in conjunction with a change in concurrent signaling events such activation of the RAS or sympathetic activation, directly contributes to atherosclerotic CVD. Using a genetic model of hypertension driven by sympathetic activation, we show that this form of hypertension (compared to atherosclerotic prone mice without hypertension) alters the characteristics of the atherosclerotic lesion to a more unstable phenotype, hallmarked by increased macrophage accumulation. We also found that chronic sympathetic activation caused changes in hematopoiesis. In particular, increased sympathetic activity was found in the BM, altering the HSPC microenvironment and causing the liberation of certain stem cells to the spleen where monocytes were generated. This was accompanied by an increase in blood monocytes, likely explaining the increased macrophage burden observed in the atherosclerotic lesions. These atherogenic pathways could all be inhibited pharmacologically by blocking sympathetic signalling through β-adrenoreceptors using propranolol. These findings suggest that chronic sympathetic activation, present in many forms of hypertension, likely contributes to the increased CVD risk by modulating hematopoiesis, independent of endothelial dysfunction.

With respect to understanding the contribution of hypertension to vascular disease, the majority of research has focused on the effects on the endothelium. Perhaps the most common belief is that hypertension causes endothelial dysfunction and activation, which in turn recruits immune cells and forms the main mechanism propagating the atheroma. Interestingly, we found no evidence of endothelial dysfunction in our hypertensive mice, at least in this model of a dominant sympathetic driven form of hypertension, the endothelial dysfunction was not contributing significantly to atherogenisis.[34] Supporting our theory that underlying sympathetic nervous signaling, that may be independent of pressure itself, can drive atherogenesis, moderate increases in AngII are sufficient to promote accelerated atherogenesis, without elevations in blood pressure.[3,4] Additionally, AngII has also been shown to invoke a T-helper cell (TH1) immune response to promote atherogenesis independent of its hemodynamic effects. Thus, signaling events that can cause hypertension are likely important in driving CVD through their immune modulatory responses.[7-10,35,36] Further, with the discovery of accelerated vascular disease driven by acute events triggering sympathetic activation leading to enhanced monocyte production, it is plausible that this pathway is triggered in chronic SNS-driven hypertension and would contribute to accelerated atherosclerosis.[21,23,24] We hypothesized that the overactive SNS seen in subgroups of patients with hypertension would contribute to atherogenesis by stimulating hematopoiesis. Importantly, elevated WBCs are associated with the incidence of hypertension and predicts CV outcomes in this patient group.[37-39] However, the cause of increased WBCs in hypertensive patients has not been resolved.

Consistent with recent studies which have observed monocytosis following acute scenarios of sympathetic activation, we too observed monocytosis in the hypertensive BPH/*Apoe*$^{-/-}$ mice.[21,24] The initial predominant change driven by the overactive sympathetic signaling in our study, relevant to increased myelopoiesis, appears to occur within the BM. We noted a decreased abundance of two key niche cells, endothelial cells and osteoblasts, which harbour anchoring points in the marrow for HSPCs, preventing their release into circulation.[40-43] The contribution of the SNS in regulating this process was first described by a seminal study from the Frenette laboratory, detailing the requirement of a functional SNS in the BM, which is required for G-CSF mediated HSPC mobilization.[16] Almost a decade later, the Nahrendorf group discovered the importance of this pathway in respect to CVD, revealing that sympathetic activation following an acute myocardial infarction promotes HSPC liberation to the spleen where the production of an additional atherogenic pool of monocytes occurs.[21] The absence of an expanded HSPC population in the spleen is likely due to the chronic nature of our study and suggests that these cells likely rapidly matured into myeloid committed progenitors. The recent studies then suggest that the monocytes generated, migrated into the atherosclerotic lesion, and enhanced macrophage burden, potentiating the risk of a secondary CV event. Our data reveal that this process is occurring chronically and identifies an important mechanism that likely contributes to atherogenesis and the increased risk of a CV event in hypertension. We also identified another pathway by which sympathetic signaling can induce the liberation of BM HSPCS by causing a decrease in the HSPC-expressed retention receptor CXCR4. Given that HSPCs do not appear to express β adrenoreceptors, it suggested a cell extrinsic mechanism resulting in less HSPC cell surface CXCR4. Interestingly, neutrophils express β2 adrenoceptors and can be activated after sensing NE (*Online Supplementary Figure S2, B-C*). Modelling this *in vitro* revealed that NE-activated neutrophils produce MMP9, which cleaves CXCR4 on HSPCs. Thus, BM sympathetic activation likely liberates HSPCs via multiple mechanisms, some of which are independent of the previously described SNS/G-CSF axis.

As mentioned above, there are several studies that have identified a role for β adrenoreceptors in influencing HSPC release via modulating the BM niche. There is strong evidence for the role of β-3 adrenergic receptor in regulating nestin+ stromal cell production of key factors such as CXCL12, angiopoietin and stem cell factor, thereby influencing HSPC retention and proliferation. β-3 antagonism following ischemic events has shown reduced HSPC mobilisation and proliferation leading to dampened extramedullary hematopoiesis.[19,21,23,24] However, there is also strong evidence pointing to a role for the β-2 adrenergic receptor in regulating BM niche components and HSPC mobilisation. Although this has been suggested to occur through other niche components such as osteoblasts and other stromal cells and not specifically nestin+ cells.[16,18] These findings regarding the role of β adrenoceptors in modulating HSPC mobilisation suggest that in our study there is a likely contribution of both β-2 and β-3 receptors to changes in the BM. However, considering the role of β-2 in the setting of hypertension and elevated

blood pressure we focused on the specific role of β-2. Interestingly, a recent study by Mendez-Ferrer's group has also highlighted the chronic role of nestin+ cells present in the BM and other tissues in regulating myeloid cell movement in the setting of atherosclerosis.[44] Given that nestin+ cells in the BM express β-3 receptors and the data we have presented above regarding chronic sympathetic driven hypertension and its contribution to atherosclerosis, it is likely that this pathway may also play a role and warrants further investigation.

The obvious limitation of this study is that our findings were generated in mice. However, this also allowed us to isolate a prominent form of hypertension to reveal a novel atherogenic mechanism, which appears to be independent of endothelial dysfunction, and thus our findings also permit the current dogma to be challenged. While we revealed the effectiveness of propranolol in this model, there is a need to further investigate the effects of directly reducing enhanced hematopoiesis without targeting systemic blood pressure. It is likely that with the development of anti-inflammatory drugs targeted at the hematopoietic system, it would be possible to dampen the effects on the hematopoietic system without affecting blood pressure which would hypothetically provide the same conclusions as in the present study. Finally, we only studied one form of hypertension, driven by sympathetic signaling. It would be of specific importance to extend a modified version of this hypothesis to hypertension driven by the RAS.

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