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Original Article

Sodium nitroprusside alleviates hypertension mediated inflammation through down-regulating the expression of Cx40 in peripheral blood T lymphocytes from spontaneously hypertensive rats

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Abstract: Objective: The imbalance of circulating T lymphocytes, and Connexins (Cxs) in immune cells plays an essential role in the pathogenesis of hypertension-mediated inflammation. Nitric oxide (NO) is recognized as a key messenger in the regulation of adaptive immune responses. To expand our understanding of NO in treating hypertension-mediated inflammation, the present study was designed to investigate whether exogenous nitric oxide (NO) alleviates hypertension-mediated inflammation by regulating the Cx40 expression of peripheral blood lymphocytes in spontaneously hypertensive rats (SHR). Methods: SHR rats were treated with sodium nitroprusside (SNP) for 4 weeks. Wistar-Kyoto rats (WKYs) received daily intraperitoneally injections (i.p.) of a vehicle and were used as a control. We monitored arterial blood pressure (BP) and vascular remodeling and renal injury by the tail-cuff method and by hematoxylin and eosin staining, respectively. The percentage of $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD4^+CD25^+$ T cells in the peripheral blood, the surface expressions of Cx40 on T cells, and the serum cytokine levels were analyzed via flow cytometric analysis or ELISA. The protein levels of Cx40 in the peripheral blood lymphocytes were measured by Western blot. Results: SHR had a more pro-inflammatory peripheral immune profile than WKY. SNP treatment significantly decreased blood pressure elevation in SHR and significantly inhibited renal and vascular inflammation in SHR. In addition, exogenous NO could reverse hypertension-mediated inflammation in SHR, as evidenced by the decreased levels of IL-6 and TNF-α in the serum and culture supernatant, the decreased percentage of $CD4^+$ T cells, the CD4/CD8 ratio and the increased percentage of regulatory T cells. SNP treatment inhibited Cx40 expression in peripheral blood lymphocytes from SHR. Conclusion: exogenous NO alleviates hypertension-mediated inflammation, which is at least partly due to the regulation of adaptive immune responses by Cx40 expression inhibition.

Keywords: Sodium nitroprusside, hypertension-mediated inflammation, T lymphocytes, connexin40, spontaneously hypertensive rats

Introduction

Hypertension has been clearly recognized as a major risk factor for various cardiovascular diseases, and it contributes to more than 7 million deaths annually [1]. The participation of T lymphocytes exerts a crucial role in the development of hypertension-mediated inflammation, hypertensive end-organ damage, and blood pressure (BP) elevation [2-4]. The presence of T lymphocytes is considered a precondition for Ang II- or desoxycorticosterone acetate salt-induced hypertension [5, 6]. Subsequent studies suggest that inflammatory infiltration of T lymphocytes in SHR may be the cause of hypertension, not the result [6]. On the other hand, moderate BP elevation can cause the activation and proliferation of effector T lymphocytes [2]. Once activated, $CD4^+$ and $CD8^+$ T cells infiltrate the perivascular regions of blood vessels and renal tissues [2, 7], and then produce various pro-inflammatory cytokines [7], which lead to vascular remodeling and renal damage [8-11]. Pro-inflammatory cytokines produced by T lymphocytes, such as IL-1β, IL-2, IL-6, TNF-α, and IFN-γ, have been reported to be significantly up-regulated in different hypertensive models [12, 13]. Furthermore, an imbalance of regulatory T cells (Tregs) is also involved in the development of chronic hypertension-mediated inflammation [14]. Increasing evidence shows that that the suppression of the adaptive immune response, or a lack of effector T lymphocytes, and immunosuppressive drugs can attenuate the elevation of BP in some experimental models and in hypertensive patients; [12, 15, 16], however, there are many significant side-effects of these immunosuppressant drugs in hypertension therapy [17].

Despite the compelling evidence above suggesting that an imbalance of T lymphocytes and pro-inflammatory cytokines leads to the development of hypertension, the exact mechanisms of the imbalance of the adaptive immune system during the development and maintenance of hypertension remain to be elucidated. Previous and recent studies have demonstrated that connexins (Cxs)-based channels control the activation, proliferation and differentiation of T cells and cytokine secretion by forming gap junctional channels (GJCs) between T cells and other immune cells [18, 19]. In the adaptive immune system, Cx40 and Cx43 are the most important connexins regulating the inflammatory response [20]. Data from our laboratory and others have indicated that pro-inflammatory cytokines or primary hypertension contribute to the proliferation of T cells and the production of cytokines by enhancing Cx40/Cx43 expression and gap junctional intercellular communication among T cells [21-25]. Thus, Cxs provides a potential target for the therapy of hypertension-mediated inflammation.

During the past several decades, nitric oxide (NO) has been reported to have important regulatory roles in blood pressure (BP), acute and chronic inflammation, and host defense mechanisms [26, 27]. Although whether primary T lymphocytes express any of the NO synthase isoforms has long been debated, increasing evidence indicates that macrophage/inducible NO, synthase-derived NO, and exogenous NO donor inhibit T lymphocyte proliferation or even cause the death of T lymphocytes [28-30]. Inducible NO synthase also modulates the development, differentiation, and function of various types of T lymphocytes [29]. A recent study also showed that NO synthase is critical to maintaining BP and limiting a pro-inflammatory renal T cell profile in female SHR [31]. In addition, nitric oxide significantly increases the proliferation, division, and viability of $CD4^+CD25^-$ T cells and converts $CD4^+CD25^-$ effector cells to a population of $CD4^+CD25^+$ Treg cells [32]. On the other hand, it has been shown that NO may inhibit the expression of several cytokines (IL-1β or TNF-α, IL-6, IFN-γ) in lymphocytes [33].

Recently, NO has been reported to mediate the regulation of Cxs expression or different Cx mediated gap junctional intercellular communication in mesangial cells and endothelial cells [34, 35]. However, it is not well understood whether NO regulates immune homeostasis or protects against hypertensive inflammation by regulating Cx40 expression on T lymphocytes. Thus, this study was designed to determine if exogenous NO donor treatment will prevent hypertension-mediated inflammation by inhibiting Cx40 expression in peripheral blood T lymphocytes. These goals were met by analyzing the histopathological alteration in vascular/renal tissues, the percentage of peripheral blood T cell subsets, the serum levels of cytokines, and the protein levels of Cx40 in peripheral blood lymphocytes in SHR and WKY rats with and without sodium nitroprusside (SNP) treatment.

Materials and methods

Experimental animals and drug treatment

Age-matched 12-week-old male spontaneously hypertensive rats (SHR) (n = 60) and normotensive Wistar-Kyoto (WKY) rats (n = 60) (Vital River Laboratory Animal Technology Co., Ltd, Beijing, China; SCXK 2012-0001) were used in this study. All rats were housed in a temperature- and humidity-controlled quarters on a 12-h light-cycle and had free access to standard rat chow and water. Only SHR exhibiting a blood pressure (BP) of 150 mmHg or above were used. SHR were randomized to receive a vehicle or 10 $\mu g/kg^{-1} \cdot day^{-1}$ of SNP (the SNP solution was freshly prepared in normal saline) (Cat. No. 161527; Sigma Aldrich, St. Louis, Missouri, USA) via intraperitoneal injection until 16 weeks of age. The male WKY rats were intraperitoneally injected with the same volume of normal saline once daily. After treatment with SNP, BP was detected via the tail-cuff method as previously described [36]. All live animal experiments performed in this study complied with the Institutional Animal Care and Use Committees (IACUC) (No. A2046-047-02) of the Medical College of Shihezi University.

BP monitoring

The systolic blood pressure (SBP) of the rats was measured non-invasively using a tail-cuff apparatus (Chengdu Taimeng Software CO. Ltd., Chengdu, Sichuan, China) prior to the experiment, as described in our previous report [36]. The averaged BP of all the rats used was determined from at least three consecutive readings.

Histological analysis

The rats were euthanized by administering 30 mg/L pentobarbital sodium anesthesia (50 mg/kg, i.p.). The kidneys and basilar arteries (BA) were collected and fixed in a phosphate buffer (pH 7.4) containing 10% formalin. The formalin-fixed tissues were dehydrated and embedded in paraffin wax and cut into 4 μm thick sections. The renal and vascular injuries were evaluated by hematoxylin eosin staining as described previously [36] at ten different fields (100 × or 200 × magnification) per section.

Flow cytometric analysis

The peripheral blood mononuclear cells (PBMCs) from the whole blood (3 ml) of WKY and SHR rats were isolated using an isolation kit of mononuclear cells (Cat. No. P8630; Solarbio Science & Technology, Beijing, China). The percentages of $CD3^+CD4^+$, $CD3^+CD8^+$, and $CD4^+$ $CD25^+$ T cells in peripheral blood, and Cx40 expressions in T cells were analyzed via flow cytometric analysis according to our previous report [36]. All anti-rat CD3 (FITC-labelled), CD4 (APC-labelled), CD8 (PE-labelled), and CD25 (PE-labelled) monoclonal antibodies were purchased from Biolegend, Inc. (San Diego, CA, USA). The anti-Cx40 monoclonal antibodies and FITC-labeled secondary antibodies were purchased from Abcam (Cambridge, MA, USA) and Biolegend, Inc, respectively.

Serum cytokine detection by ELISA assay

The SHR and WKY rats were euthanized by the administration of 30 mg/L sodium pentobarbital anesthesia (50 mg kg^{-1}, i.p.). Peripheral blood was collected into heparin-coated plain tubes. The serum was obtained by the centrifugation of the blood. We used ELISA to detect the levels of cytokines (IL-6 and TNF-α) in the serum as described by the manufacturer's instructions of ELISA kits (Cat. No. 70-EK306 for IL-6; Cat. No. 70-EK382 for TNF-α; Multi-Sciences Biotech Co., LTD., Hangzhou, China). The reaction was measured at 450 nm with a microplate reader (Dynatech, Germany). The cytokine levels were calculated according to the standard curve of each cytokine and expressed in pg/ml.

Cell culture and drug treatment

We isolated the PBMCs from the WKY and SHR rats using an isolation kit of mononuclear cells (Cat. No. P8630; Solarbio Science & Technology, Beijing, China). Next, the PBMCs were incubated for 3 h in 1 mL RPMI-1640 media (Cat. No. 11875085; Gibco brand; Invitrogen by Life Technologies, Carlsbad, California, USA) containing 10% fetal bovine serum (FBS; Cat. No. SH30084; HyClone, Logan, Utah, USA), 100 U penicillin and 100 μg/mL streptomycin (Cat. No. P0781; Sigma Aldrich, St. Louis, Missouri, USA) at 37°C in an incubator with 5% CO_2. After 3 h incubation, non-adherent T lymphocytes were collected following gentle pipetting in the medium, and then adjusted to 1×10^6 cells/ml in the medium. Cultured T lymphocytes from SHR were incubated for 48 hours with 200 μM SNP. After SNP treatment for 48 hours, all the cells and culture supernatant collected were used to measure the expression of Cx40 and the cytokine levels (TNF-α and IL-6) by flow cytometry and ELISA as described above, respectively. All the cells were cultured in RPMI-1640 with 10% FBS. All treatments were carried out in triplicate. The cultures were incubated at 37°C and 5% CO_2 in a humidified incubator.

Western blot

Peripheral blood lymphocytes from WKY and SHR with or without SNP treatment were lysed with a protein lysis buffer (Cat. No. 78510; Pierce Biotechnology Inc., Rockford, IL, USA) for 30 min. The lysed cells were sonicated and centrifuged at 10000 × *g* for 20 min at 4°C. The supernatant was harvested, and the total protein concentration was measured with a BCA protein assay kit (Cat. No. GK5021; Generay Biotechnology, Shanghai, China). Equal amounts of protein (20 μg/lane) for each gr-

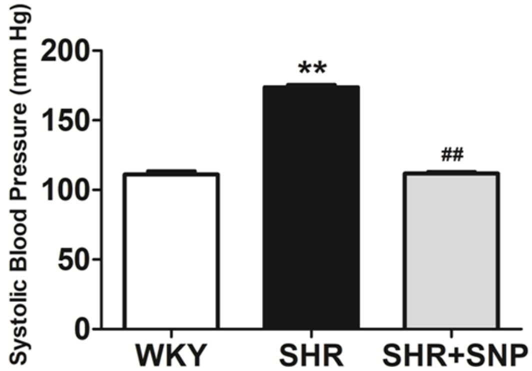


Figure 1. Systolic BP in SHR treated from 12 to 16 weeks of age with SNP. SHR had a higher blood pressure than WKY (**P < 0.01). SNP treatment significantly lowered BP in the SHR+SNP group (##P < 0.01 vs SHR). The data were analyzed by comparing the area under the curve values using one-way ANOVA and Student's t-test, n = 8 animals in each group.

oup were loaded onto SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) as described in our previous paper [36]. The detection of Cx40 expressions in the different groups were performed as described in our previous paper [36]. β-actin was used as internal reference.

Statistical analysis

All experimental data are presented as the mean ± SEM and assessed by Student's *t*-test for the comparison of two groups or by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test, when there was a significant difference between groups. The a analyses were carried out by GraphPad Prism version 5.0 software (GraphPad Software, San Diego, CA, USA), and for all comparisons, the differences were considered statistically significant with P < 0.05 or P < 0.01 (details described in the legend of the each figures).

Results

SNP treatment decreases blood pressure in SHR

To verify the effect of exogenous NO on lowering blood pressure, we measured BP in all rats at 16 weeks using tail cuff plethysmography. The SHR had a higher BP than the age-matched WKY (WKY vs SHR: (111.00 ± 2.50) mmHg vs (173.75 ± 1.60) mmHg; P < 0.01, **Figures 1** and S1). However, the SNP-treated SHR had lower BP than the vehicle-treated SHR (SHR vs SHR + SNP: (173.75 ± 1.60) mmHg vs (111.78 ± 1.18); P < 0.01, **Figures 1** and S1), and there was no difference in BP between the WKY rats and the SNP treated SHR (WKY vs SHR + SNP: (111.00 ± 2.50) vs (111.78 ± 1.18); P > 0.05, **Figures 1** and S1). The result confirms the role of NO in regulating BP.

Exogenous NO prevents vascular remodeling and renal injury

To investigate the effect of exogenous NO on hypertension-induced renal injury and vascular remodeling (arterial wall thickening), we assessed the histopathological changes of basilar arteries (BA) and kidneys via hematoxylin and eosin (H&E) staining. Compared to the WKY rats, the BA of the SHR showed an increased thickness of the vascular walls, severe endothelium injury, and a hypertension-induced infiltration of inflammatory cells (**Figure 2**). Furthermore, compared with the WKY rats, the vehicle-treated SHR showed severe pathological renal injuries after 4 weeks, enlarged renal tubules, and infiltration of immune cells (**Figure 2**). However, exogenous NO could significantly reverse these pathological alterations in cerebral arteries and renal tissues of the SHR (**Figure 2**).

Exogenous NO alleviates the imbalance of peripheral blood T lymphocyte subsets and hypertension-mediated inflammation in SHR

NO is known to play an important role in the regulation of the anti-inflammatory response [28-30]. To evaluate the effects of SNP on the hypertension-mediated inflammatory response of SHR, we analyzed the percentage of T cell subsets ($CD4^+$, $CD8^+$ and $CD4^+CD25^+$ T cells) by flow cytometry. Representative flow cytometry images and a bar graph indicating the percentage of T cell subsets are shown in **Figures 3** and S2. SHR had significantly more $CD3^+CD4^+$ T cells [WKY vs SHR: (62.11 ± 0.71)% vs (69.67 ± 0.55)%; P < 0.01, **Figures 3A** and S2A] and fewer $CD3^+CD8^+$ T cells than WKY rats [WKY vs SHR: (37.18 ± 1.05)% vs (30.22 ± 0.41)%; P < 0.01, **Figures 3B** and S2B], which led to a increased CD4/CD8 ratio in SHR [WKY vs SHR: (1.70 ± 0.04) vs (2.33 ± 0.04); P < 0.01, **Figures 3C** and S2C]. Furthermore, SHR had fewer circulating Tregs compared to the WKY rats [WKY vs SHR: (9.21 ± 0.39)% vs (5.77 ± 0.38)%; P < 0.01, **Figures 3D** and S2D]. In contrast, SHR exhibited a lower percentage of $CD4^+$ T cells [SHR vs SHR + SNP: (69.67 ± 0.55)% vs

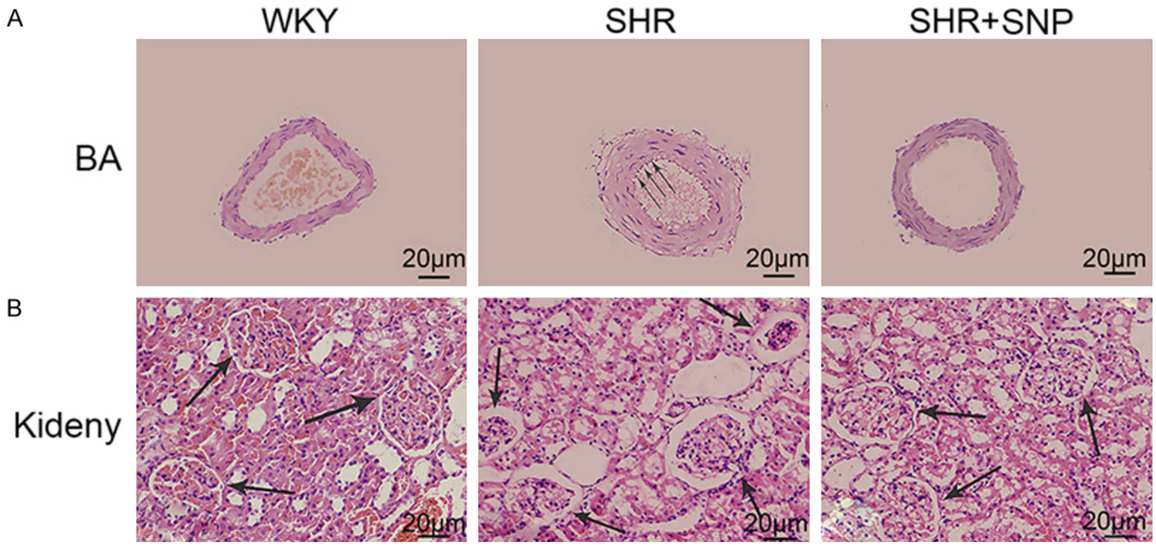


Figure 2. The protective effects of SNP on injuries of target organs of SHR. Cross-sections of basilar arteries (BA) (A) and the longitudinal-sections of kidney tissues (B) were stained with hematoxylin-eosin staining (magnification × 200 for BA and magnification × 100 for renal tissues. scalar bar = 20 μm) (n = 8).

(63.49 ± 0.60)%; P < 0.01, **Figures 3A** and S2A], and the $CD4^+/CD8^+$ ratio [SHR vs SHR + SNP: (2.33 ± 0.04) vs (1.82 ± 0.04); P < 0.01, **Figures 3C** and S2C] and a higher frequency of $CD4^+CD25^+$ T cells [SHR vs SHR + SNP: (5.77 ± 0.38)% vs (7.40 ± 0.43)%; P < 0.05, **Figures 3D** and S2D] in the peripheral blood after SNP treatment.

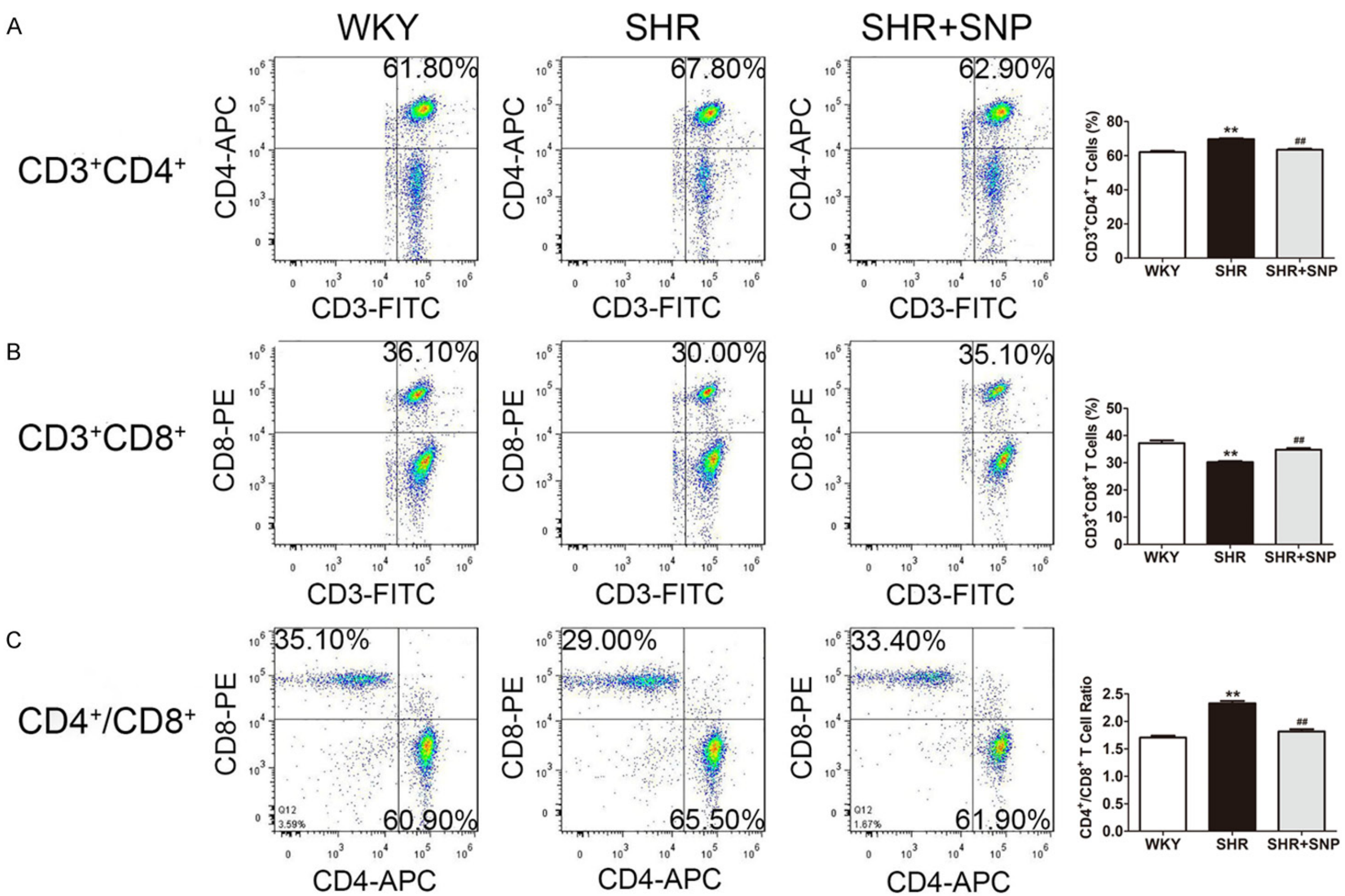
To further study the effect of NO on pro-inflammatory cytokine production in SHR, pro-inflammatory cytokines (IL-6 and TNF-α) were measured in the plasma of SHR with and without SNP treatment. **Figures 4** and S3 show that, compared with WKY, circulating IL-6 [WKY vs SHR: (8.47 ± 0.72) pg/ml vs (12.00 ± 0.72) pg/ml; P < 0.01, **Figures 4A** and S3A] and TNF-α [WKY vs SHR: (6.24 ± 0.19) pg/ml vs (9.33 ± 0.69) pg/ml; P < 0.01, **Figures 4B** and S3B] levels were elevated in SHR. In contrast, serum TNF-α and IL-6 of SHR were significantly decreased (P < 0.05 or P < 0.01, **Figures 4** and S3) after 4 weeks of SNP treatment. The results imply that NO inhibits the hypertension-mediated inflammatory response at the peripheral blood level.

Exogenous NO reduces the expression of Cx40 in $CD4^+$ and $CD8^+$ T lymphocytes from the peripheral blood of SHR

In recent studies of hypertension-mediated inflammation in SHR and hypertensive patients, we have shown that T lymphocyte subsets from hypertensive patients and SHR exhibited higher Cx40 expression compared with healthy controls [25]. The present results confirm our previous report that circulating $CD4^+$ and $CD8^+$ T cells of SHR have a higher expression level of Cx40 [(53.03 ± 2.09)% for $CD4^+$ T cell, P < 0.01; (38.88 ± 1.62)% for $CD8^+$ T cell, P < 0.01; **Figures 5A**, **5B**, S4A and S4B). To further determine whether NO-induced anti-inflammatory effects are associated with alterations of Cx40 expression in the T cells of peripheral blood from SHR, we determined the expression levels of Cx40 in T lymphocytes subsets (**Figures 5A**, **5B**, S4A and S4). The results showed that expressions of Cx40 in $CD4^+$ [(39.56 ± 0.88)%, P < 0.01; **Figures 5A** and S4A] and $CD8^+$ T cells [(24.12 ± 0.98)%, P < 0.01; **Figures 5B** and S4B] from the peripheral blood of SHR were significantly decreased by SNP treatment, and the expression levels of Cx40 showed no differences between the SNP-treated SHR and WKY rats (**Figures 5A**, **5B**, S4A and S4B).

Impact of SNP on the protein levels of Cx40 in peripheral blood lymphocytes of SHR

We used Western blot to further determine the effects of SNP on the protein levels of Cx40 in peripheral blood lymphocytes from SHR. SHR were found to increase Cx40 protein levels in peripheral blood lymphocytes (P < 0.01, **Figures 6A**, **6B** and S5). SNP markedly reduced


A
WKY
SHR
SHR+SNP
CD3+CD4+
CD4-APC
CD3-FITC
61.80%
67.80%
62.90%
CD3+CD4+ T Cells (%)
B
CD3+CD8+
CD8-PE
36.10%
30.00%
35.10%
CD3+CD8+ T Cells (%)
C
CD4+/CD8+
35.10%
60.90%
29.00%
65.50%
33.40%
61.90%
CD4+/CD8+ T Cell Ratio

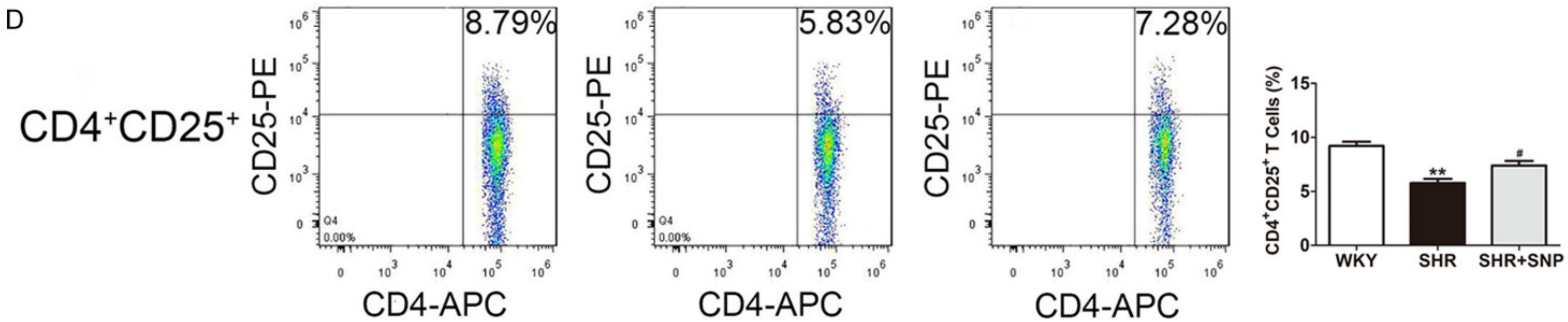


Figure 3. The profile of T lymphocyte subtypes in SHR treated from 12 to 16 weeks of age with SNP. A-D. Representative flow cytometry analysis showing percentages of circulating T lymphocyte subtypes in the peripheral blood of 8 SHR and 8 age-matched WKY rats. Bar graph in the right of each scatter plot of flow cytometry shown are the percentages of $CD4^+$, $CD8^+$ and $CD25^+$ T cells expressing $CD4^+$ as well as the ratio of $CD4^+/CD8^+$ in the peripheral blood of SHR and WKY rats. The vertical axis represents the frequency of various T lymphocyte subtypes. Quantitative analysis of the mean percentage of cells ± SEM. $^{**}P < 0.01$, compared with the WKY rats; $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$, compared with SHR ($n = 8$ animals in each group).

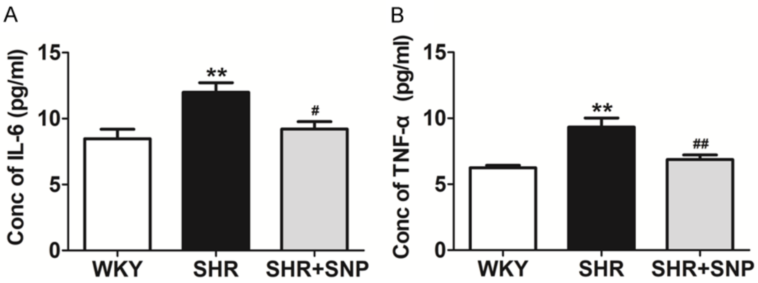


Figure 4. Pro-inflammatory cytokine profile in the serum of SHR treated from 12 to 16 weeks of age with SNP. Shown are the serum levels of IL-6 (A) and TNF-α (B). Data shown are the mean ± SEM; $^{**}P < 0.01$, compared with the WKY rats; $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$, compared with SHR (*n* = 9 animals in each group).

the expression of Cx40 in the peripheral blood lymphocytes of SHR ($P < 0.01$, **Figures 6A**, **6B** and S5). Therefore, these results are consistent with observations in different T cells subsets from SHR that express lower Cxs in the presence of SNP.

Exogenous NO reduces the expression of Cx40 in peripheral blood lymphocytes from SHR and the secretion of cytokines in vitro

To further evaluate the effects of exogenous (lower-cased E) NO on Cx40 expression and inflammatory cytokines release, an in vitro study using peripheral blood lymphocytes from WKY and SHR was carried out. The results showed that cultured lymphocytes from SHR expressed higher levels of Cx40 ($P < 0.01$, **Figures 7A**, **7B** and S6), and produced higher levels IL-6 and TNF-α in the supernatant than those from the WKY rats ($P < 0.01$, **Figures 8A**, **8B** and S7). After 48 hours of SNP incubation, the expression levels of Cx40 and the supernatant levels of IL-6 and TNF-α in the SHR were lower than in the SHR without SNP incubation ($P < 0.01$, **Figures 7**, **8**, S6 and S7). These results are consistent with observations of the in vivo model.

Discussion

The results of this study identify the possible therapeutic effect of nitric oxide on hypertension-mediated inflammation. This was achieved by studying the effects of the supplementation of exogenous NO on BP, the target organs and the adaptive immune system in SHR. The major novel finding of the current study is that exogenous NO significantly inhibits vascular remodeling and renal injury and improves immune balance in SHR. This study supports previous reports [27-33] about the roles of NO in anti-hypertension and anti-inflammation and expands our understanding of NO in treating hypertension.

Hypertensive stimuli like Ang II, high salt, and excessive catecholamines lead to the formation of effector T cells, resulting in the development of prehypertension [37]. Ang II and DOCA-salt also significantly increase the vascular and renal infiltration of $CD4^+$ and $CD8^+$ T cells in male animals [7]. There is evidence to suggest that major factors inducing BP elevation, like Ang II promotes the elevation of blood pressure and increases the expression of pro-inflammatory cytokines and induces the proliferation of splenic lymphocytes and cytokines production through its receptors on immune cells [38]. The initial elevations of BP during prehypertension may in turn lead to T-cell activation [37]. Activated T cells and T cell driven cytokines cause vascular remodelling, ultimately contributing to the development of hypertension [37]. Morphological changes in cerebral arteries during chronic BP elevation are involved in the development of ischemic cerebrovascular diseases [39], so BA were used as the primary blood vessels in our study. Our results showed significantly higher BP and vascular wall thickening in BA of SHR compared to WKY rats, which is consistent with previous studies [40, 41]. Moreover, the infiltration of T cells in the blood vessels and kidneys are a consistent feature of hypertension [37]. The results of the present study showed an increased infiltration of immune cells and damage in BA and the renal tissues of SHR. These changes of vascular morphology induced by inflammation lead to an increase in vascular tone and impair arterial relaxation, and thus lead to BP elevation [42].

Several studies from hypertensive animal models showed that both $CD4^+$ and $CD8^+$ T cells are involved in the pathogenesis of hypertension, and $CD4^+$ cells are the main adaptive immune players in experimental models of hypertension and hypertensive patients [7, 8, 43]. In our

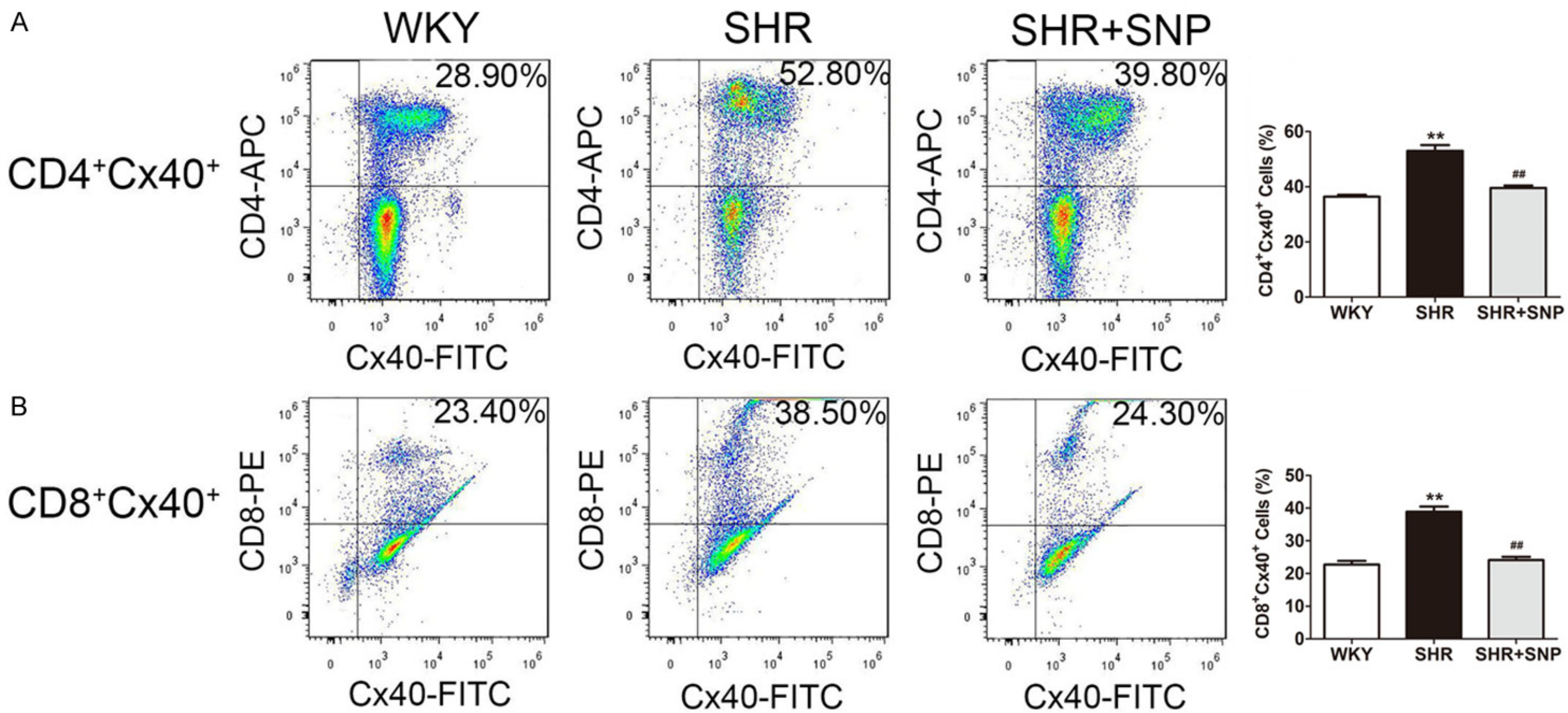


Figure 5. The effect of SNP treatment on the expressions of Cx40 in different T lymphocyte subtypes of SHR. A and B. Representative flow cytometry plots are presented for Cx40 expression levels on gated single-positive $CD4^+$ T lymphocytes or $CD8^+$ T lymphocyte populations in the peripheral blood from 8 SHR and 8 WKY rats. The cells were stained with unlabeled anti-Cx40 plus FITC-labelled secondary antibodies. Based on the $CD4^+$ or $CD8^+$ gates, the cells were further gated based on Cx40 expression levels, and the frequency of $CD4^+$ or $CD8^+$ T cells expressing Cx40 was determined. The bar graph in the right of each scatter plot of flow cytometry shows the percentage of the $CD4^+$ or $CD8^+$ T cell population expressing Cx40. Both Cx40 expression levels are significantly increased in $CD4^+$ or $CD8^+$ T cells of SHR compared with those of WKY rats. SNP treatment inhibited the expressions of Cx40 in $CD4^+$ and $CD8^+$ T cell from the peripheral blood of SHR. Values are the mean ± SEM. $^{**}P < 0.01$, compared with WKY rats; $^{##}P < 0.01$, compared with SHR rats (n = 8 animals in each group).

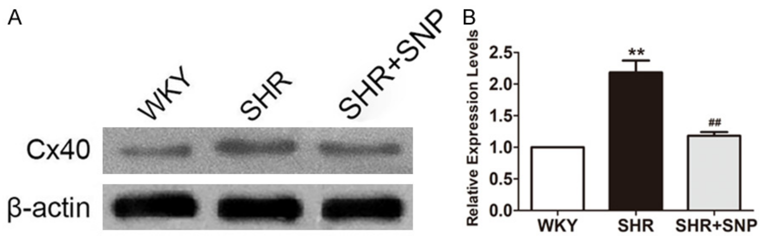


Figure 6. Western blot analysis of the effect of SNP treatment on the expression of Cx40 protein in peripheral blood lymphocytes of SHR. A. Representative bands of total Cx40 expression by Western blot in the peripheral blood lymphocytes of SHR treated with SNP. B. The bar graph represents ratios of Cxs to β-actin. The data represent the mean ± SEM of three experiments (n = 3 animals in each group). $^{**}P < 0.01$ vs WKY rats; $^{\#\#}P < 0.01$ vs SHR.

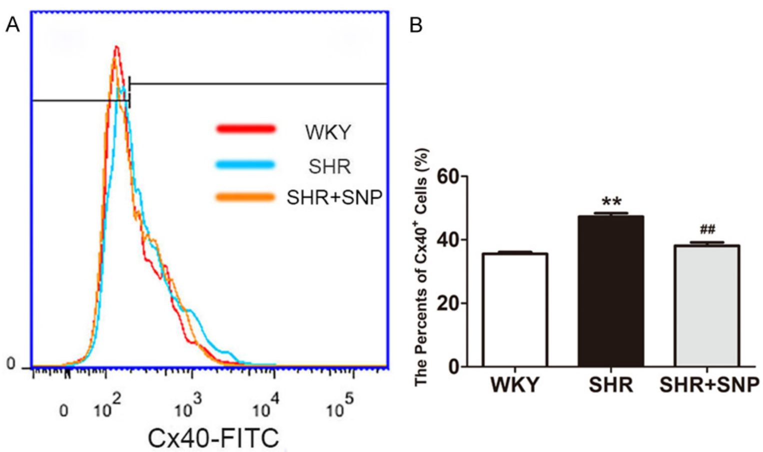


Figure 7. The effect of SNP incubation on the expression of Cx40 in vitro cultured peripheral blood lymphocytes of SHR. PBMCs from SHR and WKY rats were incubated with or without SNP (200 μM) for 48 h in a culture medium and then were harvested and examined by flow cytometry for the expression of Cx40. A. The X-axis of the histogram represents the parameter's signal value in the channel numbers (count) and the Y-axis represents the number of events per channel number; B. The bar graph represents the mean expressional level of Cx40 positive cells of three independent experiments ± SEM. $^{**}P < 0.01$, vs the WKY group; $^{\#\#}P < 0.01$, vs the SHR group (n = 6 animals in each group).

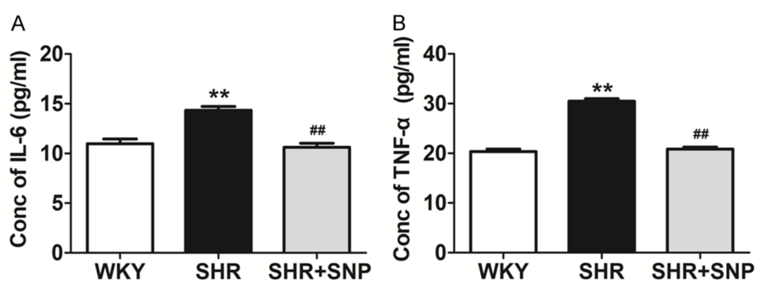


Figure 8. The effect of SNP incubation on the supernatant levels of IL-6 and TNF-α in the culture supernatant from the PBMCs of the SHR. A. The supernatant levels of IL-6 in supernatant of lymphocyte culture fluid; B. The supernatant levels of TNF-α in supernatant of lymphocyte culture fluid. Data represent the mean ± SEM. $^{**}P < 0.01$, vs the WKY group; $^{\#\#}P < 0.01$, vs the SHR group (n = 6 animals in each group).

study, SHR also have more $CD4^+$ T cells and a higher $CD4^+/CD8^+$ ratio than WKY rats. However, the decrease in the number of activated $CD8^+$ T cells may result from an enhanced infiltration of $CD8^+$ T cells into other tissues. Thus, we can speculate here that the alteration in the percentage of $CD8^+$ T cells represents a general immunological imbalance in hypertensive rats, although the causes remain unknown. Meanwhile, the current study also demonstrates that SHR also have fewer $CD4^+CD25^+$ T cells, suggesting that a Tregs imbalance in number improves hypertensive inflammation and is an important factor in the development of hypertension. In addition, several pro-inflammatory cytokines secreted by T cells were shown to be elevated in the serum of many hypertensive models and hypertensive patients, contributing to the inflammation of blood vessels [13, 44]. In the present study, compared with the WKY rats, the SHR had higher serum levels of TNF-α and IL-6. Among the two proinflammatory cytokines, high levels of IL-6 are positively correlated with enhanced BP and may be an independent risk factor for hypertension [45]. Increased IL-6 levels suppress $CD4^+$ naïve T cell differentiation into Tregs [46]. Above all, our findings together with others demonstrate that T lymphocytes and cytokines contribute to the elevation of BP.

NO has been recognized as a key effector in the modulation of BP and T cell-mediated immunity. The NG-nitro-L-arginine methyl ester (L-NAME) induced hypertensive animal model of chronic inhibition of NO synthesis exhibit early inflammation (monocyte infiltration in kidney) and late cardiovascular remodeling in rats or female SHR [31, 47]. There is ample evidence that NO bioavailability is decreased in SHR [48], and that the chronic inhibition of NO accelerates hypertension and induces perivascular inflammation in SHR [49]. Increasing TNF-α also impairs the ability of the endothelium to produce NO [3]. Thus, impaired dynamic NO release in the spontaneously hypertensive rat (SHR) may be a key factor causing the BP elevation and hypertension-mediated inflammatory infiltration in this study. To explore the anti-inflammatory effect in SHR after exogenous NO donor administration for 4 weeks, we observed that SNP treatment in SHR could reduce vascular remodeling (arterial wall thickening) and leukocyte infiltration of the vascular wall and kidneys in SHR, which suggests an anti-inflammatory role of NO. Indeed, increasing evidence indicates that NO may play a role in acute and chronic inflammation [27]. Inducible NO synthase regulates the development, differentiation, and/or function of immune cells of various types [29]. It has been shown that concanavalin-A induces NO synthase II expression in macrophages and subsequently produced NO impairs DNA synthesis as well as mitochondrial function in T cells, thereby suppressing cell proliferation [27]. Exogenous NO has also been shown to inhibit T lymphocyte proliferation [30]. Moreover, NO markedly increases the proliferation, division and viability of $CD4^+CD25^-$ T cells and converts $CD4^+CD25^-$ effector cells to a population of $CD4^+CD25^+$ Treg cells [32]. Our results also demonstrate that NO donors significantly attenuate the imbalance between effector and regulatory T cell subsets in SHR by reversing the proportion of $CD4^+$ and $CD8^+$ T cells, the CD4/CD8 ratio, and the percentage of Tregs in the peripheral blood in SHR. Interestingly, the pro-inflammatory effect or anti-inflammatory effect of NO depends on different immune processes. At low concentrations, NO has been shown to protect cells from apoptosis; high doses of NO induce thymocyte and splenic T cell apoptosis or necrosis [27]. Based on this observation, the concentration of the NO donor is high enough to completely inhibit the T lymphocyte mediated inflammatory response by inhibiting T lymphocyte proliferation or even causing the apoptosis of T lymphocytes in the current study. Furthermore, it has been shown that nitric oxide may inhibit the expression of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IFN-γ) in lymphocytes, monocytes, and other cells [33]. We also observed a decrease in TNF-α, and IL-6 of serum and culture supernatant. Overall, we believe that exogenous NO treatment ameliorates vascular dysfunction, counteracts BP elevation and associated kidney and vascular damage by limiting the proliferation of circulating effector T cells and the expression or production of pro-inflammatory mediators (TNF-α and IL-6).

Although a large number of studies and our previous and present work have shown that a disorder of lymphocyte subtypes plays an important role in hypertension, the precise mechanisms underlying this role remain unclear. Therefore, comprehending how T lymphocyte subsets become imbalanced and participate in the hypertensive inflammation is crucial. However, increasing evidence indicates that Cxs-based channels play an essential role in the promotion of the activation, proliferation, and differentiation of T lymphocytes, and cytokine production [50]. Cx40 and Cx43 are the main Cxs in almost all immune cells, with the predominant expression of Cx43 in circulating lymphocytes [51], and Cx43 acts in a pro-inflammatory way [52, 53]. Interestingly, additional data from our lab showed that an increase in Cx40 expression was positively correlated with T lymphocyte proliferation and pro-inflammatory cytokine synthesis in the peripheral blood of hypertensive patients and splenic/peripheral lymphocytes of SHR [25, 36, 54]. Similarly, in the current study, we also observed an enhanced expression of Cx40 in T cells of the peripheral blood in the SHR compared with the WKY rats. Although T, B and NK cells from secondary lymphoid organs have been shown to express Cx40 at low levels [22], the contribution of Cx40 to the activation and proliferation of lymphocyte is still unknown. It has been proposed that Cx40 formed hemi-channels facilitate the ATP-mediated propagation of calcium ions, but this is speculative [55, 56]. ATP release by Cx-based hemi-channels results in a proliferation of immune cells, the production of cytokines, and the perpetuation of the inflammasome cycle [20]. Although the role of Cx40 in T-lymphocytes remains to be further investigated, our data provides an association between hypertension-mediated inflammation and the up-regulation of Cx40 expression in peripheral blood lymphocytes.

To further investigate whether NO inhibits hypertension-mediated inflammation by altering Cx40 expression in peripheral blood lymphocytes, we assessed the impact of SNP on the Cx40 expression of different T cell subsets. We have demonstrated that SNP significantly decreased the expression of Cx40 in $CD4^+$/$CD8^+$ T cells as well as in the total peripheral blood lymphocytes of SHR, and this may result in a reduction of Cx40 based channels and the remodeling of gap junctions. Our data demonstrate that NO may exert its anti-inflammatory effect in hypertension-mediated inflammation by lowering Cx40 expression.

Our study also had some limitations. First, whether the therapeutic effects of different NO donors on inflammation induced by the cardiovascular disease can be repeated in other hypertensive or cardiovascular disease models is a key question which has yet to be further explored. Secondly, in our experiment, we did not detect the effect of NO on hypertension-mediated vascular remodeling in the main peripheral resistance arteries. Thirdly, the limitation is that we only observed the anti-inflammatory effects of NO on hypertension-mediated inflammation, but we did not investigate the detailed anti-inflammatory mechanism of NO by regulating the function of Cx40 in the context of hypertensive inflammation, although NO may react with Cx40 by cysteine residue nitrosylation [57]. Lastly, how NO decreases the expressions of Cxs also needs to be better defined.

Conclusion

Taken together, we have also shown that exogenous NO inhibits hypertension-mediated inflammation and chronic inflammation induced target organ damage by reversing the immunological imbalance. The mechanisms may be at least partially related to NO, especially its modulation of Cx40 expression.

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Disclosure of conflict of interest

None.

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1way ANOVA Column statistics	A	B	C
	WKY	SHR	SHR+SNP
	Y	Y	Y
1 Number of values	8	8	8
2			
3 Minimum	102.9	167.6	107.5
4 25% Percentile	104.6	169.9	109.0
5 Median	109.3	173.7	111.3
6 75% Percentile	118.6	178.1	115.4
7 Maximum	121.1	180.2	116.7
8			
9 Mean	111.0	173.8	111.8
10 Std. Deviation	7.060	4.525	3.338
11 Std. Error	2.496	1.600	1.180
12			
13 Lower 95% CI	105.1	170.0	109.0
14 Upper 95% CI	116.9	177.5	114.6

	1way ANOVA Tabular results					
1	Table Analyzed	血压-SNP				
2						
3	One-way analysis of variance					
4	P value	< 0.0001				
5	P value summary	***				
6	Are means signif. different? (P < 0.05)	Yes				
7	Number of groups	3				
8	F	382.0				
9	R squared	0.9732				
10						
11	Bartlett's test for equal variances					
12	Bartlett's statistic (corrected)	3.723				
13	P value	0.1555				
14	P value summary	ns				
15	Do the variances differ signif. (P < 0.05)	No				
16						
17	ANOVA Table	SS	df	MS		
18	Treatment (between columns)	20740	2	10370		
19	Residual (within columns)	570.3	21	27.16		
20	Total	21310	23			
21						
22	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
23	WKY vs SHR	-62.75	34.06	Yes	***	-69.32 to -56.18
24	WKY vs SHR+SNP	-0.7750	0.4207	No	ns	-7.346 to 5.796
25	SHR vs SHR+SNP	61.98	33.64	Yes	***	55.40 to 68.55

Figure S1. Systolic Blood Pressure in the WKY, SHR and SHR + SNP group.

A

	1way ANOVA Column statistics	A WKY	B SHR	C SHR+SNP	D Title
		Y	Y	Y	Y
1	Number of values	13	12	13	
2					
3	Minimum	58.57	66.53	60.85	
4	25% Percentile	60.12	67.86	61.72	
5	Median	61.88	69.76	63.25	
6	75% Percentile	63.59	71.10	64.26	
7	Maximum	67.51	73.13	68.02	
8					
9	Mean	62.11	69.67	63.49	
10	Std. Deviation	2.576	1.918	2.164	
11	Std. Error	0.7144	0.5536	0.6001	
12					
13	Lower 95% CI	60.56	68.45	62.18	
14	Upper 95% CI	63.67	70.89	64.80	

	1way ANOVA Tabular results					
1	Table Analyzed	CD4-SEM-SNP				
2						
3	One-way analysis of variance					
4	P value	< 0.0001				
5	P value summary	***				
6	Are means signif. different? (P < 0.05)	Yes				
7	Number of groups	3				
8	F	39.67				
9	R squared	0.6939				
10						
11	Bartlett's test for equal variances					
12	Bartlett's statistic (corrected)	0.9850				
13	P value	0.6111				
14	P value summary	ns				
15	Do the variances differ signif. (P < 0.05)	No				
16						
17	ANOVA Table	SS	df	MS		
18	Treatment (between columns)	399.5	2	199.8		
19	Residual (within columns)	176.2	35	5.036		
20	Total	575.8	37			
21						
22	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
23	WKY vs SHR	-7.556	11.90	Yes	***	-9.757 to -5.356
24	WKY vs SHR+SNP	-1.378	2.214	No	ns	-3.534 to 0.7782
25	SHR vs SHR+SNP	6.178	9.727	Yes	***	3.978 to 8.379

B

	1way ANOVA Column statistics	A WKY	B SHR	C SHR+SNP
		Y	Y	Y
1	Number of values	8	8	8
2				
3	Minimum	34.43	28.77	31.78
4	25% Percentile	35.40	28.99	33.57
5	Median	36.29	30.51	35.07
6	75% Percentile	38.38	31.10	36.19
7	Maximum	43.79	31.93	36.84
8				
9	Mean	37.18	30.22	34.82
10	Std. Deviation	2.974	1.150	1.667
11	Std. Error	1.052	0.4066	0.5894
12				
13	Lower 95% CI	34.69	29.26	33.42
14	Upper 95% CI	39.67	31.18	36.21

	1way ANOVA Tabular results					
1	Table Analyzed	CD8-SEM-SNP				
2						
3	One-way analysis of variance					
4	P value	< 0.0001				
5	P value summary	***				
6	Are means signif. different? (P < 0.05)	Yes				
7	Number of groups	3				
8	F	23.23				
9	R squared	0.6887				
10						
11	Bartlett's test for equal variances					
12	Bartlett's statistic (corrected)	5.959				
13	P value	0.0508				
14	P value summary	ns				
15	Do the variances differ signif. (P < 0.05)	No				
16						
17	ANOVA Table	SS	df	MS		
18	Treatment (between columns)	200.5	2	100.3		
19	Residual (within columns)	90.64	21	4.316		
20	Total	291.2	23			
21						
22	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
23	WKY vs SHR	6.961	9.477	Yes	***	4.342 to 9.581
24	WKY vs SHR+SNP	2.361	3.215	No	ns	-0.2585 to 4.981
25	SHR vs SHR+SNP	-4.600	6.262	Yes	***	-7.220 to -1.980

C

1way ANOVA Column statistics	A WKY	B SHR	C SHR+SNP
	Y	Y	Y
1 Number of values	12	8	8
2			
3 Minimum	1.401	2.155	1.652
4 25% Percentile	1.674	2.194	1.703
5 Median	1.698	2.365	1.825
6 75% Percentile	1.768	2.434	1.909
7 Maximum	1.886	2.462	1.997
8			
9 Mean	1.704	2.329	1.816
10 Std. Deviation	0.1241	0.1206	0.1180
11 Std. Error	0.03584	0.04265	0.04171
12			
13 Lower 95% CI	1.626	2.228	1.717
14 Upper 95% CI	1.783	2.430	1.915

1way ANOVA Tabular results					
1 Table Analyzed	CD4/CD8-SNP				
2					
3 One-way analysis of variance					
4 P value	< 0.0001				
5 P value summary	***				
6 Are means signif. different? (P < 0.05)	Yes				
7 Number of groups	3				
8 F	67.12				
9 R squared	0.8430				
10					
11 Bartlett's test for equal variances					
12 Bartlett's statistic (corrected)	0.02184				
13 P value	0.9891				
14 P value summary	ns				
15 Do the variances differ signif. (P < 0.05)	No				
16					
17 ANOVA Table	SS	df	MS		
18 Treatment (between columns)	1.980	2	0.9901		
19 Residual (within columns)	0.3688	25	0.01475		
20 Total	2.349	27			
21					
22 Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
23 WKY vs SHR	-0.6244	15.93	Yes	***	-0.7625 to -0.4862
24 WKY vs SHR+SNP	-0.1116	2.848	No	ns	-0.2498 to 0.02653
25 SHR vs SHR+SNP	0.5127	11.94	Yes	***	0.3614 to 0.6641

D

1way ANOVA Column statistics	A WKY	B SHR	C SHR+SNP
	Y	Y	Y
1 Number of values	8	8	8
2			
3 Minimum	7.470	4.230	5.580
4 25% Percentile	8.480	4.875	6.525
5 Median	9.090	5.710	7.130
6 75% Percentile	10.14	6.520	8.648
7 Maximum	10.96	7.660	9.110
8			
9 Mean	9.209	5.769	7.395
10 Std. Deviation	1.106	1.089	1.214
11 Std. Error	0.3911	0.3849	0.4293
12			
13 Lower 95% CI	8.284	4.859	6.380
14 Upper 95% CI	10.13	6.679	8.410

1way ANOVA Tabular results					
1 Table Analyzed	CD4CD25-SNP				
2					
3 One-way analysis of variance					
4 P value	< 0.0001				
5 P value summary	***				
6 Are means signif. different? (P < 0.05)	Yes				
7 Number of groups	3				
8 F	18.30				
9 R squared	0.6354				
10					
11 Bartlett's test for equal variances					
12 Bartlett's statistic (corrected)	0.09342				
13 P value	0.9544				
14 P value summary	ns				
15 Do the variances differ signif. (P < 0.05)	No				
16					
17 ANOVA Table	SS	df	MS		
18 Treatment (between columns)	47.38	2	23.69		
19 Residual (within columns)	27.18	21	1.294		
20 Total	74.56	23			
21					
22 Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
23 WKY vs SHR	3.440	8.552	Yes	***	2.005 to 4.875
24 WKY vs SHR+SNP	1.814	4.509	Yes	*	0.3791 to 3.248
25 SHR vs SHR+SNP	-1.626	4.043	Yes	*	-3.061 to -0.1916

Figure S2. A. The percentage of $CD3^+CD4^+$ T cell subset in the WKY, SHR and SHR + SNP group. B. The percentage of $CD3^+CD8^+$ T cell subset in the WKY, SHR and SHR + SNP group. C. The CD4/CD8 ratio in the WKY, SHR and SHR + SNP group. D. The percentage of $CD4^+CD25^+$ T cell subset in the WKY, SHR and SHR + SNP group.

A

	1way ANOVA Column statistics	A WKY Y	B SHR Y	C SHR+SNP Y
1	Number of values	9	9	9
2				
3	Minimum	6.053	8.347	6.956
4	25% Percentile	6.822	10.29	7.852
5	Median	7.483	11.90	8.698
6	75% Percentile	9.845	14.03	10.38
7	Maximum	12.86	14.38	12.46
8				
9	Mean	8.474	12.00	9.202
10	Std. Deviation	2.169	2.161	1.701
11	Std. Error	0.7232	0.7203	0.5671
12				
13	Lower 95% CI	6.806	10.34	7.895
14	Upper 95% CI	10.14	13.66	10.51

	1way ANOVA Tabular results					
1	Table Analyzed	IL-6-SNP				
2						
3	One-way analysis of variance					
4	P value	0.0028				
5	P value summary	**				
6	Are means signif. different? (P < 0.05)	Yes				
7	Number of groups	3				
8	F	7.608				
9	R squared	0.3880				
10						
11	Bartlett's test for equal variances					
12	Bartlett's statistic (corrected)	0.5531				
13	P value	0.7584				
14	P value summary	ns				
15	Do the variances differ signif. (P < 0.05)	No				
16						
17	ANOVA Table	SS	df	MS		
18	Treatment (between columns)	62.24	2	31.12		
19	Residual (within columns)	98.16	24	4.090		
20	Total	160.4	26			
21						
22	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
23	WKY vs SHR	-3.523	5.225	Yes	**	-5.904 to -1.142
24	WKY vs SHR+SNP	-0.7286	1.081	No	ns	-3.110 to 1.652
25	SHR vs SHR+SNP	2.794	4.145	Yes	*	0.4129 to 5.175

B

	1way ANOVA Column statistics	A WKY Y	B SHR Y	C SHR+SNP Y
1	Number of values	9	9	9
2				
3	Minimum	5.441	6.580	5.500
4	25% Percentile	5.865	7.053	5.877
5	Median	6.155	9.548	7.157
6	75% Percentile	6.519	11.14	7.576
7	Maximum	7.408	11.99	8.603
8				
9	Mean	6.243	9.332	6.871
10	Std. Deviation	0.5668	2.063	1.030
11	Std. Error	0.1889	0.6878	0.3432
12				
13	Lower 95% CI	5.807	7.746	6.080
14	Upper 95% CI	6.678	10.92	7.663

	1way ANOVA Tabular results					
1	Table Analyzed	TNF-α-SNP				
2						
3	One-way analysis of variance					
4	P value	0.0002				
5	P value summary	***				
6	Are means signif. different? (P < 0.05)	Yes				
7	Number of groups	3				
8	F	12.77				
9	R squared	0.5155				
10						
11	Bartlett's test for equal variances					
12	Bartlett's statistic (corrected)	11.53				
13	P value	0.0031				
14	P value summary	**				
15	Do the variances differ signif. (P < 0.05)	Yes				
16						
17	ANOVA Table	SS	df	MS		
18	Treatment (between columns)	47.99	2	24.00		
19	Residual (within columns)	45.11	24	1.880		
20	Total	93.10	26			
21						
22	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
23	WKY vs SHR	-3.090	6.761	Yes	***	-4.704 to -1.476
24	WKY vs SHR+SNP	-0.6289	1.376	No	ns	-2.243 to 0.9852
25	SHR vs SHR+SNP	2.461	5.385	Yes	**	0.8468 to 4.075

Figure S3. A. Content of IL-6 in the plasma of WKY, SHR and SHR + SNP group. B. Content of TNF-α in the plasma of WKY, SHR and SHR + SNP group.

A

	1way ANOVA Column statistics	A WKY Y	B SHR Y	C SHR+SNP Y
1	Number of values	8	8	8
2				
3	Minimum	33.63	42.12	35.01
4	25% Percentile	34.35	48.86	37.88
5	Median	36.54	53.60	40.03
6	75% Percentile	38.28	57.34	41.33
7	Maximum	38.98	60.78	42.94
8				
9	Mean	36.35	53.03	39.56
10	Std. Deviation	2.043	5.919	2.499
11	Std. Error	0.7223	2.093	0.8835
12				
13	Lower 95% CI	34.64	48.09	37.47
14	Upper 95% CI	38.06	57.98	41.65

B

	1way ANOVA Column statistics	A WKY Y	B SHR Y	C SHR+SNP Y
1	Number of values	8	8	8
2				
3	Minimum	18.58	32.04	21.78
4	25% Percentile	20.37	34.02	22.06
5	Median	21.87	39.44	23.16
6	75% Percentile	25.66	42.47	25.60
7	Maximum	28.10	45.00	30.08
8				
9	Mean	22.71	38.88	24.12
10	Std. Deviation	3.179	4.578	2.777
11	Std. Error	1.124	1.618	0.9819
12				
13	Lower 95% CI	20.05	35.05	21.80
14	Upper 95% CI	25.37	42.70	26.44

	1way ANOVA Tabular results					
1	Table Analyzed	CD4Cx40-SNP				
2						
3	One-way analysis of variance					
4	P value	< 0.0001				
5	P value summary	***				
6	Are means signif. different? (P < 0.05)	Yes				
7	Number of groups	3				
8	F	41.36				
9	R squared	0.7975				
10						
11	Bartlett's test for equal variances					
12	Bartlett's statistic (corrected)	8.803				
13	P value	0.0123				
14	P value summary	*				
15	Do the variances differ signif. (P < 0.05)	Yes				
16						
17	ANOVA Table	SS	df	MS		
18	Treatment (between columns)	1254	2	626.8		
19	Residual (within columns)	318.2	21	15.15		
20	Total	1572	23			
21						
22	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
23	WKY vs SHR	-16.68	12.12	Yes	***	-21.59 to -11.77
24	WKY vs SHR+SNP	-3.213	2.334	No	ns	-8.121 to 1.696
25	SHR vs SHR+SNP	13.47	9.788	Yes	***	8.562 to 18.38

	1way ANOVA Tabular results					
1	Table Analyzed	CD8Cx40-SNP				
2						
3	One-way analysis of variance					
4	P value	< 0.0001				
5	P value summary	***				
6	Are means signif. different? (P < 0.05)	Yes				
7	Number of groups	3				
8	F	49.62				
9	R squared	0.8254				
10						
11	Bartlett's test for equal variances					
12	Bartlett's statistic (corrected)	1.836				
13	P value	0.3993				
14	P value summary	ns				
15	Do the variances differ signif. (P < 0.05)	No				
16						
17	ANOVA Table	SS	df	MS		
18	Treatment (between columns)	1283	2	641.3		
19	Residual (within columns)	271.4	21	12.92		
20	Total	1554	23			
21						
22	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
23	WKY vs SHR	-16.16	12.72	Yes	***	-20.70 to -11.63
24	WKY vs SHR+SNP	-1.407	1.107	No	ns	-5.941 to 3.126
25	SHR vs SHR+SNP	14.76	11.61	Yes	***	10.22 to 19.29

Figure S4. A. Cx40 expression level on gated single-positive $CD4^+$ T lymphocytes populations in the peripheral blood from WKY, SHR and SHR + SNP group. B. Cx40 expression level on gated single-positive $CD8^+$ T lymphocytes populations in the peripheral blood from WKY, SHR and SHR + SNP group.

1way ANOVA Column statistics	A WKY	B SHR	C SHR+SNP
	Y	Y	Y
1 Number of values	3	3	3
2			
3 Minimum	1.000	1.817	1.098
4 25% Percentile	1.000	1.817	1.098
5 Median	1.000	2.318	1.150
6 75% Percentile	1.000	2.421	1.297
7 Maximum	1.000	2.421	1.297
8			
9 Mean	1.000	2.185	1.182
10 Std. Deviation	0.0	0.3234	0.1033
11 Std. Error	0.0	0.1867	0.05967
12			
13 Lower 95% CI	1.000	1.382	0.9249
14 Upper 95% CI	1.000	2.989	1.438

1way ANOVA Tabular results					
1 Table Analyzed	Cx40-WB-SNP				
2					
3 One-way analysis of variance					
4 P value	0.0006				
5 P value summary	***				
6 Are means signif. different? (P < 0.05)	Yes				
7 Number of groups	3				
8 F	31.83				
9 R squared	0.9139				
10					
11 ANOVA Table	SS	df	MS		
12 Treatment (between columns)	2.445	2	1.223		
13 Residual (within columns)	0.2305	6	0.03842		
14 Total	2.676	8			
15					
16 Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17 WKY vs SHR	-1.185	10.47	Yes	***	-1.676 to -0.6943
18 WKY vs SHR+SNP	-0.1816	1.605	No	ns	-0.6726 to 0.3094
19 SHR vs SHR+SNP	1.004	8.870	Yes	**	0.5127 to 1.495

Figure S5. Western blot analysis of the expression of Cx40 protein in peripheral blood lymphocytes of WKY, SHR and SHR + SNP group.

1way ANOVA Column statistics		A WKY	B SHR	C SHR+SNP
		Y	Y	Y
1	Number of values	6	6	6
2				
3	Minimum	33.83	43.80	35.20
4	25% Percentile	34.56	44.87	35.43
5	Median	35.45	46.94	38.05
6	75% Percentile	36.63	49.70	40.93
7	Maximum	37.66	51.54	41.25
8				
9	Mean	35.58	47.25	38.14
10	Std. Deviation	1.310	2.758	2.558
11	Std. Error	0.5348	1.126	1.044
12				
13	Lower 95% CI	34.21	44.36	35.46
14	Upper 95% CI	36.95	50.15	40.83

1way ANOVA Tabular results						
1	Table Analyzed	Cx40-体外-SNI				
2						
3	One-way analysis of variance					
4	P value	< 0.0001				
5	P value summary	***				
6	Are means signif. different? (P < 0.05)	Yes				
7	Number of groups	3				
8	F	42.69				
9	R squared	0.8506				
10						
11	Bartlett's test for equal variances					
12	Bartlett's statistic (corrected)	2.523				
13	P value	0.2833				
14	P value summary	ns				
15	Do the variances differ signif. (P < 0.05)	No				
16						
17	ANOVA Table	SS	df	MS		
18	Treatment (between columns)	451.7	2	225.8		
19	Residual (within columns)	79.35	15	5.290		
20	Total	531.0	17			
21						
22	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
23	WKY vs SHR	-11.67	12.43	Yes	***	-15.12 to -8.224
24	WKY vs SHR+SNP	-2.563	2.730	No	ns	-6.013 to 0.8864
25	SHR vs SHR+SNP	9.110	9.702	Yes	***	5.660 to 12.56

Figure S6. The expression of Cx40 in vitro cultured peripheral blood lymphocytes of WKY, SHR and SHR + SNP group.

A

1way ANOVA Column statistics	A WKY	B SHR	C SHR+SNP
	Y	Y	Y
1 Number of values	6	6	6
2			
3 Minimum	9.542	13.00	9.022
4 25% Percentile	9.996	13.52	9.981
5 Median	10.77	14.37	10.48
6 75% Percentile	12.02	15.10	11.71
7 Maximum	12.86	15.69	11.74
8			
9 Mean	10.97	14.34	10.62
10 Std. Deviation	1.194	0.9330	1.010
11 Std. Error	0.4876	0.3809	0.4123
12			
13 Lower 95% CI	9.718	13.36	9.561
14 Upper 95% CI	12.23	15.32	11.68

	1way ANOVA Tabular results					
1	Table Analyzed	IL-6-体外-SNP				
2						
3	One-way analysis of variance					
4	P value	< 0.0001				
5	P value summary	***				
6	Are means signif. different? (P < 0.05)	Yes				
7	Number of groups	3				
8	F	22.87				
9	R squared	0.7530				
10						
11	Bartlett's test for equal variances					
12	Bartlett's statistic (corrected)	0.2988				
13	P value	0.8612				
14	P value summary	ns				
15	Do the variances differ signif. (P < 0.05)	No				
16						
17	ANOVA Table	SS	df	MS		
18	Treatment (between columns)	50.57	2	25.29		
19	Residual (within columns)	16.59	15	1.106		
20	Total	67.16	17			
21						
22	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
23	WKY vs SHR	-3.367	7.843	Yes	***	-4.944 to -1.790
24	WKY vs SHR+SNP	0.3512	0.8181	No	ns	-1.226 to 1.928
25	SHR vs SHR+SNP	3.718	8.662	Yes	***	2.141 to 5.295

B

1way ANOVA Column statistics	A WKY	B SHR	C SHR+SNP
	Y	Y	Y
1 Number of values	6	6	6
2			
3 Minimum	18.05	28.73	19.42
4 25% Percentile	19.78	29.34	20.05
5 Median	20.55	30.73	20.83
6 75% Percentile	21.23	31.40	21.73
7 Maximum	21.44	31.99	22.17
8			
9 Mean	20.35	30.49	20.85
10 Std. Deviation	1.203	1.175	0.9902
11 Std. Error	0.4910	0.4797	0.4042
12			
13 Lower 95% CI	19.09	29.26	19.81
14 Upper 95% CI	21.61	31.72	21.89

	1way ANOVA Tabular results					
1	Table Analyzed	TNF-α-体外-SNP				
2						
3	One-way analysis of variance					
4	P value	< 0.0001				
5	P value summary	***				
6	Are means signif. different? (P < 0.05)	Yes				
7	Number of groups	3				
8	F	154.4				
9	R squared	0.9537				
10						
11	Bartlett's test for equal variances					
12	Bartlett's statistic (corrected)	0.1983				
13	P value	0.9056				
14	P value summary	ns				
15	Do the variances differ signif. (P < 0.05)	No				
16						
17	ANOVA Table	SS	df	MS		
18	Treatment (between columns)	392.0	2	196.0		
19	Residual (within columns)	19.04	15	1.269		
20	Total	411.1	17			
21						
22	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
23	WKY vs SHR	-10.14	22.04	Yes	***	-11.83 to -8.449
24	WKY vs SHR+SNP	-0.4963	1.079	No	ns	-2.186 to 1.193
25	SHR vs SHR+SNP	9.642	20.97	Yes	***	7.953 to 11.33

Figure S7. A. The supernatant levels of IL-6 in culture supernatant from PBMCs of WKY, SHR and SHR + SNP group. B. The supernatant levels of TNF-α in culture supernatant from PBMCs of WKY, SHR and SHR + SNP group.