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 significant-
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ly 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 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 Cxs 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 μg/kg·day 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
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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). 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% CO₂. After 3 h incubation, non-adherent T lymphocytes were collected following gentle pipetting in the medium, and then adjusted to 1 × 10⁶ 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 cultures were carried out in triplicate. The cultures were incubated at 37°C and 5% CO₂ 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: General Biotechnology, Shanghai, China). Equal amounts of protein (20 µg/lane) for each gr-
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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 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 715

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(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

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).
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A

CD3^+CD4^+

CD3-FITC

CD4-APC

WKY 61.80%  SHR 67.80%  SHR+SNP 62.90%

B

CD3^+CD8^+

CD3-FITC

CD8-PE

WKY 36.10%  SHR 30.00%  SHR+SNP 35.10%

C

CD4^+/CD8^+

CD3-FITC

CD4-APC

CD8-PE

WKY 35.10%  SHR 29.00%  SHR+SNP 33.40%

CD4/CD8 T Cell Ratio

WKY 60.90%  SHR 65.50%  SHR+SNP 61.90%
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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<sup>+</sup>, CD8<sup>+</sup> and CD25<sup>+</sup> T cells expressing CD4<sup>+</sup> as well as the ratio of CD4<sup>+</sup>/CD8<sup>+</sup> 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).
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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 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.

**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).

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]. 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
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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).
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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 Cx40 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 expression 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).

In the present 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 pro-inflammatory 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 study...
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⁺ Tregs [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 nitrrosylation [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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References

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

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### Table B

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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.
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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.
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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.
Figure S5. Western blot analysis of the expression of Cx40 protein in peripheral blood lymphocytes of WKY, SHR and SHR + SNP group.
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**Figure S6.** The expression of Cx40 in vitro cultured peripheral blood lymphocytes of WKY, SHR and SHR + SNP group.
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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.