

# Connexin 43 in splenic lymphocytes is involved in the regulation of CD4<sup>+</sup>CD25<sup>+</sup> T lymphocyte proliferation and cytokine production in hypertensive inflammation

HAI-CHAO ZHANG<sup>1-3\*</sup>, ZHONG-SHUANG ZHANG<sup>1,2\*</sup>, LIANG ZHANG<sup>1,2\*</sup>, AI WANG<sup>1,2</sup>,  
HE ZHU<sup>1,2</sup>, LI LI<sup>1,2</sup>, JUN-QIANG SI<sup>1,2</sup>, XIN-ZHI LI<sup>2,4</sup> and KE-TAO MA<sup>1,2</sup>

<sup>1</sup>Department of Physiology, Medical College of Shihezi University;

<sup>2</sup>The Key Laboratory of Xinjiang Endemic and Ethnic Diseases, Medical College of Shihezi University,

Shihezi, Xinjiang 832000; <sup>3</sup>North China university of Science and Technology Affiliated Hospital, Tangshan,

Hebei 0630092; <sup>4</sup>Department of Pathophysiology, Medical College of Shihezi University, Shihezi, Xinjiang 832000, P.R. China

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**Abstract.** Chronic inflammation promotes the development of hypertension and is associated with increased T cell infiltration and cytokine production in impaired organs. Gap junction protein connexin 43 (Cx43), is ubiquitously expressed in immune cells and plays an important role in T cell proliferation and activation, and cytokine production. However, the correlation between Cx43 in T cells and the hypertensive inflammatory response remains unknown. Thus, in this study, we wished to examine this correlation. First, our results revealed that hypertension caused significant thickening of the vascular wall, inflammatory cell infiltration into part of the renal interstitium and glomerular atrophy, and it increased the tubular damage scores in the kidneys of spontaneously hypertensive rats (SHRs). Moreover, the SHRs exhibited stenosis in the central artery wall of the spleen with increased serum levels of interleukin (IL)-2 and IL-6 compared with normotensive Wistar-Kyoto (WKY) rats. The spleens of the SHRs exhibited a significantly decreased percentage of CD4<sup>+</sup>CD25<sup>+</sup> (Treg) T cells. However, the percentages of CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T cell and the levels of CD4<sup>+</sup>Cx43 and CD8<sup>+</sup>Cx43 did not differ significantly between the SHRs and WKY rats. In cultured lymphocytes from the SHRs and WKY rats, low percentages of Treg cells and reduced cyto-

kine (IL-2 and IL-6) mRNA expression levels were observed in the lymphocytes obtained from the SHRs and WKY rats treated with the connexin blocker, Gap27, or concanavalin A (ConA) plus Gap27. The effects of ConA and Gap27 differed between the SHRs and WKY rats. On the whole, our findings demonstrate that the splenic Treg cell-mediated suppression in SHRs may be involved in hypertensive inflammatory responses. Cx43 in the gap junctional channel may regulate lymphocyte activation and inflammatory cytokine production.

## Introduction

Low-grade inflammation is involved in the pathogenesis of cardiovascular diseases. The activation of the immune system contributes to the development of hypertension (1). Studies have demonstrated that T cells are required for the development of hypertension and the infiltration of target tissues (2,3). Previous studies have implicated changes in T cells in the immune status of experimental animals, such as angiotensin (Ang) II and DOCA-salt hypertension (2,4,5). Therefore, T cells are critical for the development of essential hypertension and targeting organ damage. However, exactly which type of T cells may contribute to inflammatory responses and renal end-organ damage during hypertension, as well as the mechanisms responsible for hypertensive inflammation remain speculative and unclear.

T cells can be divided into several subtypes, and each subtype plays differential roles in infection and immune homeostasis. T cells consist of T helper (Th) cells (CD4<sup>+</sup>) and cytotoxic T cells (CD8<sup>+</sup>). Th cells (CD4<sup>+</sup>) are classified into Th1, Th2, Th17 and regulatory T (Treg) cells. CD4<sup>+</sup> T cells remove pathogens by activating innate cells and B cells of the adaptive immune system. CD8<sup>+</sup> T cells kill pathogens by releasing cytotoxic molecules, such as perforin and granzyme B. The surface markers of Treg cells, which have an immunosuppressive T cell lineage, contain CD4<sup>+</sup>CD25<sup>+</sup> to prevent the elevation of blood pressure and the damage to target organs induced by Ang II (6,7). The abnormality of T cell subsets leads to hypertension and end-organ damage by releasing cytokines (1). Th1 cells are known to secrete adaptive cytokines, namely,

*Correspondence to:* Dr Ke-Tao Ma, Department of Physiology, Medical College of Shihezi University, 59 North 2nd Road, Shihezi, Xinjiang 832000, P.R. China  
E-mail: maketao@hotmail.com

Dr Xin-Zhi Li, Department of Pathophysiology, Medical College of Shihezi University, 59 North 2nd Road, Shihezi, Xinjiang 832000, P.R. China  
E-mail: lixinzhi@shzu.edu.cn

\*Contributed equally

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interleukin-2 (IL)-2, interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), whereas Th2 cells secrete IL-4, IL-6 and IL-13. It has previously been reported elevated Th1 activity in Ang-dependent hypertensive mice, as shown by the increased expression of lymphoid Th1-specific cytokines, namely, IFN- $\gamma$  and TNF- $\alpha$  (2). Central signals activate macrophages and T cells, which target the kidneys and vasculature, and release cytokines, including IL-6 and IL-17, thereby inducing renal and vascular dysfunction and elevating blood pressure (8,9).

It is known that gap junctions (GJs) between Tregs and T cells function in a novel signaling pathway to suppress T cell proliferation by mediating the transfer of Cyclic adenosine monophosphate (cAMP) (10). Gap junctional intercellular communication (GJIC) relies on the intercellular channels that span the lipid bilayers of contiguous cells, thereby allowing the direct bidirectional passage of 1-1.5 kDa molecules (11). A GJ is composed of two end-to-end connected hemichannels (also called connexons), each hemichannel is composed of six connexin (Cx) molecules (12). Cx43 is a major GJ protein of immune cells (13-15). Cx43-mediated GJs provide direct intercellular channels to link attached lymphocytes. Cx43 also plays an important role in the proliferation and activation of, as well as cytokine production in T lymphocytes (16,17). However, the involvement of GJs between lymphocytes in triggering inflammation during hypertension remains unknown.

In this study, we hypothesized that Cx43 in T cells can modulate the inflammatory response in hypertension by regulating lymphocyte proliferation and cytokine production. Therefore, the present study was carried out to examine whether Cx43 in splenic T cell subsets is associated with hypertension-induced inflammation.

## Materials and methods

**Source of reagents.** The fluorochrome-conjugated monoclonal antibodies (mAbs) to rat antigens for flow cytometric analysis, including anti-CD45 FITC (Cat. no. 202205), anti-CD3 FITC (Cat. no. 201403), anti-CD4 APC (Cat. no. 201509), anti-CD8a PE (Cat. no. 200608), anti-CD25 PE (Cat. no. 202105), mouse FITC-IgG1,  $\kappa$  Isotype Ctrl (Cat. no. 400107), mouse FITC-IgM,  $\kappa$  Isotype Ctrl (Cat. no. 401606), mouse APC-IgG1,  $\kappa$  Isotype Ctrl (Cat. no. 400122), mouse PE-IgG2a,  $\kappa$  Isotype Ctrl (Cat. no. 400212) and mouse PE-IgG1,  $\kappa$  Isotype Ctrl (Cat. no. 981804) were obtained from BioLegend (San Diego, CA, USA). Anti-Cx43 antibody (Cat. no. ab79010) was obtained from Abcam (Cambridge, MA, USA). The goat anti-mouse IgG/FITC secondary antibody (Cat. no. 405305) was acquired from BioLegend, and 10X erythrocyte lysis buffer was supplied by Mindray (Shenzhen, China). Rat IL-2 and rat IL-6 enzyme-linked immunosorbent assay (ELISA) kits were provided by MultiSciences Biotech Co., Ltd. (Hangzhou, China). RPMI-1640 medium was obtained from Gibco (Grand Island, NY, USA) and fetal bovine serum (FBS) was acquired from HyClone (Logan, UT, USA). Penicillin, streptomycin, concanavalin A (ConA) and TRI reagent were obtained from Sigma-Aldrich (St. Louis, MO, USA). Gap27 was supplied by Apexbio (Boston, MA, USA), and the cell counting kit-8 (CCK-8) kit was provided by Zomanbio (Beijing, China). The PrimeScript™ RT reagent kit was acquired from Takara (Shiga, Japan), and the QuantiNova SYBR-Green PCR

kit was obtained from Qiagen (Hilden, Germany). Primers were synthesized by Sangon Biotech (Shanghai, China).

**Experimental animals.** Age-matched 16- to 17-week-old male spontaneously hypertensive rats (SHRs) and normotensive Wistar-Kyoto (WKY) rats ( $n=15$  in each group) were used in this study. The experimental animals were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The protocol of this study was approved by the Institutional Animal Care and Use Committees (IACUC) of the Medical College of Shihezi University, Shihezi, China. The study was performed in accordance with the guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Public Health Service Policy on Humane Care and Use of Animals, DHEW Publication no. 96-01, PHS Policy revised in 2002).

**Blood pressure monitoring.** The systolic blood pressure (SBP) was measured non-invasively using a tail-cuff apparatus (Chengdu Thai Union Co., Chengdu, China) prior to the experiment. The rats were placed for 10 min in a chamber heated to 37°C. A cuff equipped with a pneumatic pulse sensor was wrapped around the tail of the rat and was placed in an individual plastic restrainer. Blood pressure was accurately recorded for each rat and averaged from at least 3 consecutive readings.

**Histological analysis.** The SHRs and WKY rats were euthanized under general anesthesia by an intraperitoneal injection of pentobarbital sodium (50 mg/kg). The kidneys and spleens were harvested, weighed and fixed in formalin. After 72 h, the samples were dehydrated, embedded in paraffin, and sliced into 5- $\mu$ m-thick sections. These sections were mounted on slides and stained with hematoxylin and eosin (H&E). The slides were photographed under a light microscope (Olympus, Tokyo, Japan). The damaged tubules were identified based on the presence and severity of the renal tubular epithelial cell degeneration, necrosis, loss of brush border cells, intraluminal casts and inflammatory cell infiltration, as assessed by two professional pathologists at 10 high-power fields (magnification,  $\times 400$ ) per section. The percentages of histological changes in the kidney tissues were scored with a semi-quantitative scale, which was designed to evaluate the degree of tubular damage as previously described (18): 0, normal kidney; 1, minimal damage (5% involvement); 2, mild damage (5-25% involvement); 3, moderate damage (25-50% involvement); 4, severe damage (50-75% involvement); and 5, most severe damage (>75% involvement).

**Detection of serum cytokine levels.** The SHR and WKY rats were euthanized by 30 mg/l sodium pentobarbital anesthesia (50 mg/kg, i.p.). Peripheral blood was collected with heparin-coated capillary glass tubes from abdominal aorta and allowed to clot at ambient temperature for 15 min. The blood samples obtained were centrifuged at 800  $\times$  g for 10 min at 4°C to obtain serum. ELISA was performed to quantify and compare the cytokine levels (IL-2 and IL-6) in serum according to the manufacturer's instructions with rat platinum ELISA kits. The reaction was measured at 450 nm with a microplate reader (Dynatech, Denkendorf, Germany). A standard curve was prepared from IL-2 and IL-6 standard dilutions before the IL-2

and IL-6 concentrations were determined in the rat samples. The concentration of each cytokine was expressed in pg/ml.

**Analysis of splenic lymphocyte subsets.** The spleens were harvested from the same rats as those above into a tissue culture dish and used to prepare a single cell suspension by gentle pressing with the plunger of a syringe. The isolated cells were passed through a 200  $\mu$ M cell strainer (BD Biosciences, San Jose, CA, USA) and collected. The cell suspensions were centrifuged at 200 x g for 10 min at room temperature. The red blood cells were isolated, treated with the erythrocyte lysis buffer, and washed twice with phosphate-buffered saline (PBS). After cell counting with a counting chamber and viability detection by trypan blue exclusion (>95%), they were resuspended in an appropriate volume of PBS to a final concentration of approximately  $1 \times 10^6$  cells/ml. The cells were incubated at 4°C for 15 min in the dark with fluorochrome-labelled mAbs for surface antigen markers, including T lymphocytes (CD3<sup>+</sup>), T-helper cells (CD3<sup>+</sup>CD4<sup>+</sup>), cytotoxic T cells (CD3<sup>+</sup>CD8<sup>+</sup>) and regulatory T cells (CD4<sup>+</sup>CD25<sup>+</sup>). The cells were resuspended in 500  $\mu$ l of PBS. Subsequently, approximately 20,000 cells were analyzed using a flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA), with an initial gate set by forward/side scatter (FSC/SSC) dot plots. Isotype-matched antibodies were used as negative control samples to confirm the specificity of antibody binding and eliminate nonspecific receptor binding or other cellular protein interactions. The proper fluorescence compensation was set to ensure that negative and positive cells were identical and used to gate the population. For the splenic cell suspension, CD45 staining was used to identify leukocytes, in the CD45<sup>+</sup> gate, T cells were identified by the anti-CD3, -CD4, -CD8 and -CD25 antibodies.

**Analysis of Cx43 in splenic lymphocyte subsets.** The spleens were harvested from the same SHR and WKY rats as those above into a tissue culture dish and used to prepare a single cell suspension via the method above. Cell suspension was resuspended in an appropriate volume of PBS to a final concentration of approximately  $1 \times 10^6$  cells/ml and fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Tween-20 for 20 min at room temperature. Following incubation in blocking buffer containing 10% goat serum, the cells were incubated with the anti-rat Cx43 antibodies at 4°C for 30 min prior to incubation with the FITC-labeled goat anti-mouse IgG secondary antibody in the dark for 30 min at 4°C with. Negative control samples were incubated with isotype-matched antibodies. The cells were washed twice with PBS and incubated with CD4 or CD8 antibodies for flow cytometry analysis as described above.

**Lymphocyte proliferation assay.** Splenic cells from the SHR and WKY rats were collected in respective centrifuge tube containing the lymphocyte separation medium. After density gradient centrifugation (400 x g for 30 min), the splenic mononuclear cells were isolated. The red blood cells were lysed by the addition of the erythrocyte lysis solution for 5 sec before washing the pellet twice with PBS. The pellet was resuspended in 1 ml of RPMI-1640 medium containing 10% FBS, 100 units penicillin and 100  $\mu$ g/ml streptomycin. Following 3 h of incubation at 37°C in 5% CO<sub>2</sub>, non-adherent cells were collected and adjusted to  $1 \times 10^6$  cells/ml in the culture medium. The mitogen ConA was used to stimulate the lymphocytes.

Furthermore, the Gap27 peptide was used to evaluate the effects on lymphocytes activation and proliferation (Gap27 dissolved in Hanks balanced salt solution was used as gap junctional communication inhibitor). The cells were dispensed into 96-well round-bottomed plates as 100  $\mu$ l triplicates. The wells were divided into the following 4 treatment groups: the control, ConA, Gap27 and Gap27 + ConA groups. The control group contained cells without stimulation. In the ConA group, the lymphocytes were stimulated with ConA (5  $\mu$ g/ml) for 24 h. In the Gap27 group, the lymphocytes were stimulated with Gap27 (500  $\mu$ M) for 48 h. In the Gap27 + ConA group, Gap27 (500  $\mu$ M) was used to incubate the lymphocytes for 48 h prior to stimulation with ConA (5  $\mu$ g/ml) for 24 h. The plates were incubated at 37°C under 5% CO<sub>2</sub> in a humidified incubator. After 72 h, the splenic lymphocytes from the SHR and WKY rats were harvested to determine the cell proliferation with CCK-8 kits according to the manufacturer's instructions. The optical density was measured using a microplate reader (Dynatech), with a test wavelength of 450 nm.

**Analysis of cultured splenic lymphocyte subsets in vitro.** To calculate the frequency of different lymphocyte subpopulations in response to ConA stimulation and treatment with the GJ inhibitor, Gap27, each group of cultured cell suspensions from the WKY rats and SHR was incubated and stained for 15 min at 4°C in the dark for flow cytometric analysis with the following antibodies: FITC-anti-CD3, APC-anti-CD4, PE-anti-CD8 and PE-anti-CD25. The samples were analyzed with the experimental procedure for flow cytometry as mentioned above. The cell proliferation rate was calculated as follows: cell proliferation rate = (ConA group-control group)/ConA group x100%. The cell inhibition rate was calculated as follows: cell inhibition rate = [ConA group-(Gap27 + ConA group)]/ConA group x100%.

**Analysis of IL-2 and IL-6 mRNA expression by reverse transcription-quantitative PCR (RT-qPCR).** Total RNA from the cultured lymphocytes from the SHR and WKY rats was extracted using TRI reagent. The RNA concentration and purity were determined by measuring spectrophotometric absorbance at 260 nm (A<sub>260</sub>) and the A<sub>260</sub>/280 ratio with a NanoVue™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A 1  $\mu$ l aliquot of RNA was used to generate cDNA in 10  $\mu$ l reaction volumes with the PrimeScript™ RT reagent kit. Reverse transcriptase reactions were operated in a thermocycler (Takara, Mountain View, CA, USA) for 15 min at 37°C, followed by incubation at 85°C for 5 sec. For each diluted cDNA sample (performed in triplicate for each gene), 2  $\mu$ l of cDNA was amplified in 10  $\mu$ l reactions with 5  $\mu$ l of 2X SYBR-Green PCR master mix as gene-specific primers: IL-2 forward, 5'-GCA CCT GTA AGT CCA GCA AC-3' and reverse primer, 3'-ACG CTT GTC CTC CTT GTC A-5'; IL-6 forward, 5'-TTG GGA CTG ATG TTG TTG AC-3' and reverse primer, 3'-TGT GGG TGG TAT CCT CTG T-5'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward, 5'-CTC TCT GCT CCT CCC TGT TC-3', and reverse primer, 3'-GCC AAA TCC GTT CAC ACC G-5'. The real-time PCR system (Bio-Rad, Hercules, CA, USA) was operated with the following reaction program: 20 sec at 37°C and 2 min at 95°C, followed by 40 cycles of 5 sec at 95°C and 10 sec at 60°C. All assays were done in triplicate and the  $\Delta$ Cq value was



computed. A relative quantification assay was performed to evaluate the ratio of each cytokine normalized to the GAPDH expression in each sample.

**Statistical analysis.** All results were presented as the means  $\pm$  SEM for each assessment. All statistical analyses were performed with GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). One-way ANOVA, followed by Tukey's multiple comparison test, was performed to compare more than 3 groups. An unpaired two-tailed t-test was used to establish 2 group comparisons. Differences with  $p < 0.05$  were considered statistically significant.

## Results

**Systematic analysis of body weight and systolic blood pressure between SHR and WKY rats.** The basal SBP of the WKY rats and SHRs was measured prior to the start of the experiments. At 16–17 weeks of age, the SBP was significantly higher in the SHRs ( $188.9 \pm 3.7$  mmHg) than in the age-matched WKY rats ( $109.3 \pm 2.6$  mmHg;  $p < 0.01$ ). A tendency toward an increased spleen weight (SW) was observed in the SHRs, although the difference was not significant as compared with the WKY rats. No significant difference was observed in the body weight (BW) or the ratio of the spleen weight to the body weight (SW/BW) ratio between the SHRs and WKY rats ( $p > 0.05$ ) (Table I).

**Target organ damage caused by hypertensive inflammation.** To observe the pathological changes in the target organs resulting from hypertension, we determined the pathological changes in the kidneys and spleens of the SHRs by H&E staining. H&E staining revealed a significant thickening of the vascular wall, inflammatory cell infiltration into part of the renal interstitium, and glomerular atrophy in the kidneys of the SHRs as compared with those of the WKY rats. After blinded semi-quantitative scoring, the tubular damage score in the SHRs ( $3.1 \pm 0.2$ ) was greater than that in the WKY rats ( $0.7 \pm 0.2$ ;  $p < 0.01$ ) (Fig. 1). The SHRs exhibited stenosis in the wall of the central artery in the spleen (Fig. 1).

We evaluated the hypertensive inflammatory response by measuring the concentration of typical Th1 (IL-2) and Th2 (IL-6) cytokines in the serum of the WKY rats and SHRs, as these cytokines are generally regarded as signs of the pro-inflammatory response. The serum concentrations of IL-2 ( $100.5 \pm 15.5$  pg/ml) and IL-6 ( $28.5 \pm 1.5$  pg/ml) in the SHRs were significantly higher than the IL-2 ( $50.8 \pm 10.0$  pg/ml) and IL-6 ( $22.1 \pm 1.0$  pg/ml;  $p < 0.05$ ) levels in the WKY rats (Fig. 2). These results suggested that the development of hypertension was accompanied by an inflammatory response and the impairment of kidney and spleen function was caused by inflammation. Simultaneously, the elevated blood pressure may promote lymphocyte activation and the secretion of pro-inflammatory cytokines.

**Hypertension-induced reduction of the percentage of Tregs in spleen.** We investigated whether pro-inflammatory lymphocytes can be detected in the spleens of normotensive and hypertensive rats. Flow cytometric analysis (Fig. 3) was used to determine the percentages of CD3<sup>+</sup> T cells (WKY,  $41.8 \pm 1.0\%$ ;

Table I. General information of SHRs and WKY rats.

Rats	BW (g)	SBP (mmHg)	SW (mg)	SW/BW (mg/g)
WKY (n=15)	282.0 $\pm$ 3.2	109.3 $\pm$ 2.6	629.0 $\pm$ 15.6	2.2 $\pm$ 0.2
SHR (n=15)	290.8 $\pm$ 2.9	188.9 $\pm$ 3.7 <sup>a</sup>	652.5 $\pm$ 11.2	2.2 $\pm$ 0.1

Results are the means  $\pm$  SEM, <sup>a</sup> $p < 0.01$  compared with the WKY rats. SHRs, spontaneously hypertensive rats; WKY, Wistar-Kyoto; SW, spleen weight; SBP, systolic blood pressure; BW, body weight.

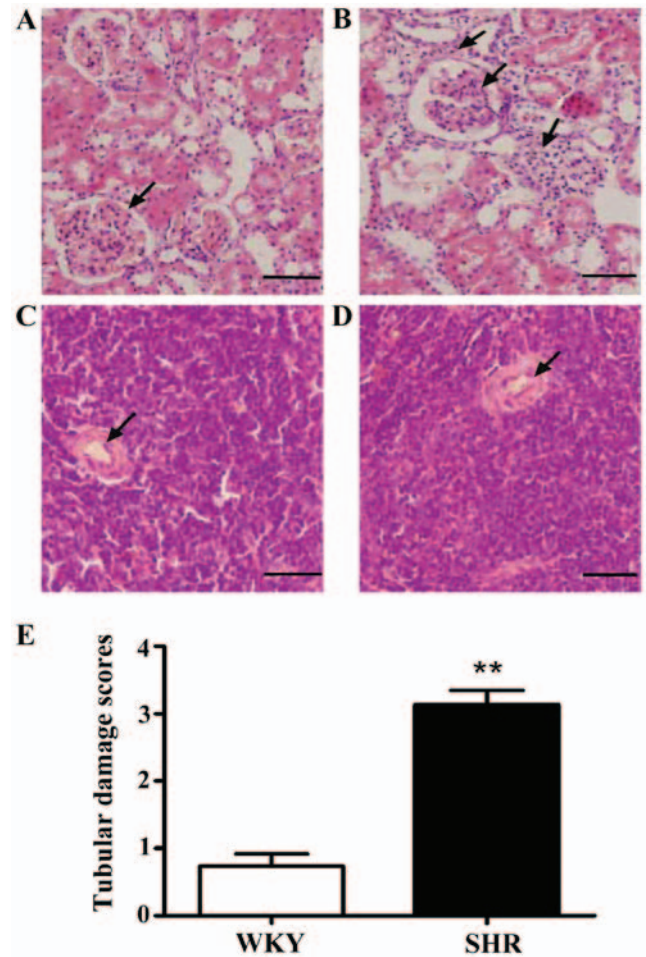


Figure 1. Histopathological changes in the kidney and spleen sections as shown by hematoxylin and eosin (H&E) staining. (A) Representative histological images of the kidney; Wistar-Kyoto (WKY) rat glomerular basement membrane (arrow). (B) Representative histological images of the kidney; thickening of the vascular wall, atrophy of the glomerulus, inflammatory cell infiltration into part of renal interstitium (arrows) in spontaneously hypertensive rats (SHRs). (C) Representative histological images of the spleen; WKY rat central artery (arrow). (D) Representative histological images of the spleen; stenosis in the wall of the central artery (arrow) in SHR. Scale bar, 20  $\mu$ m. (E) Semi-quantitative tubular damage scores in the kidneys of WKY rats and SHRs. Results are the means  $\pm$  SEM. <sup>\*\*</sup> $p < 0.01$  compared to WKY rats (n=15).

SHR,  $41.5 \pm 1.6\%$ ;  $p > 0.05$ ), CD3<sup>+</sup>CD4<sup>+</sup> T cells (WKY,  $52.5 \pm 1.4\%$ ; SHR,  $54.9 \pm 0.7\%$ ;  $p > 0.05$ ), CD3<sup>+</sup>CD8<sup>+</sup> T cells (WKY,  $37.2 \pm 0.7\%$ ; SHR,  $36.2 \pm 0.9\%$ ;  $p > 0.05$ ) and CD4<sup>+</sup>CD25<sup>+</sup> T cells (WKY,  $4.9 \pm 0.1\%$ ; SHR,  $2.6 \pm 0.1\%$ ;  $p < 0.01$ ), as well as the ratio of CD4<sup>+</sup>/CD8<sup>+</sup> (WKY,  $1.47 \pm 0.02$ ; SHR,  $1.52 \pm 0.03$ ;  $p > 0.05$ ) cells. The frequencies of CD4<sup>+</sup>CD25<sup>+</sup> T cells were

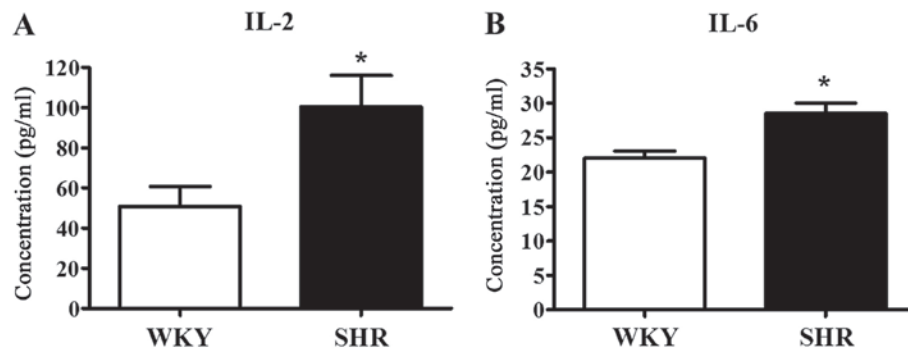


Figure 2. Determination of the concentration of pro-inflammatory cytokines in the serum of spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) rats by ELISA. (A) Interleukin (IL)-2. (B) IL-6. Results represent the means  $\pm$  SEM. \* $p < 0.05$  compared with the WKY rats ( $n=15$ ).

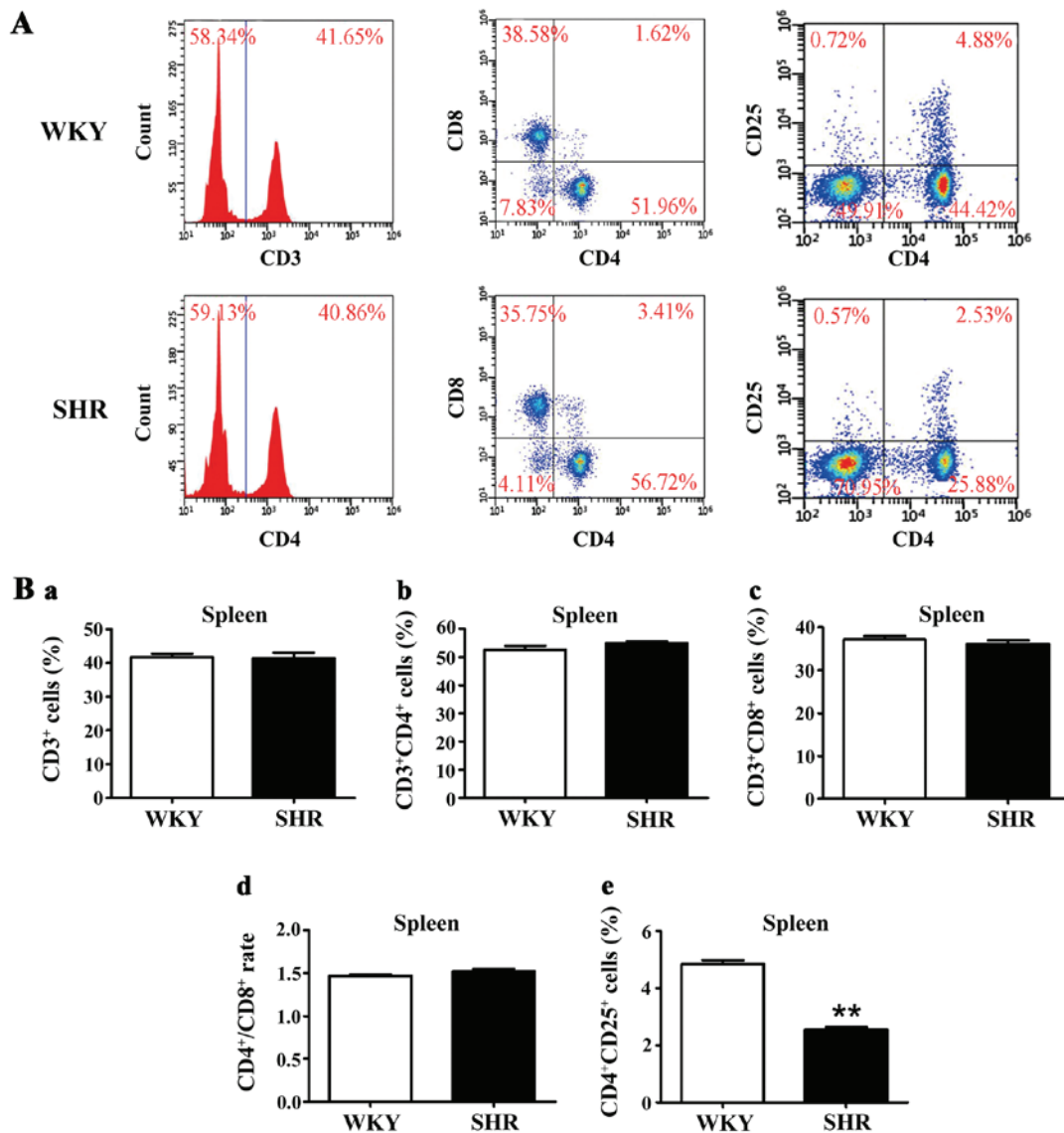


Figure 3. Flow cytometric analysis of lymphocyte subsets in the spleens of spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) rats. (A) Representative scatter plots indicate the percentage of CD3<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells in spleen samples. (B) Bar graph shows the the percentage of T cell subsets in the spleens of SHRs and WKY rats. A statistically significant decreased percentage of Treg cells (phenotype CD4<sup>+</sup>CD25<sup>+</sup>) in the spleen of SHRs was observed as compared with the WKY rats. Results are the means  $\pm$  SEM. \*\* $p < 0.01$  compared with the WKY rats ( $n=15$ ).

significantly lower in the spleens of SHRs as compared with those of the WKY rats. The percentage of other T cell subtypes, including the CD3<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, and CD3<sup>+</sup>CD8<sup>+</sup> T cells in the

spleen and the CD4<sup>+</sup>/CD8<sup>+</sup> ratio did not differ significantly between the SHRs and WKY rats. These results demonstrated that a population of Tregs may be required for hypertension.

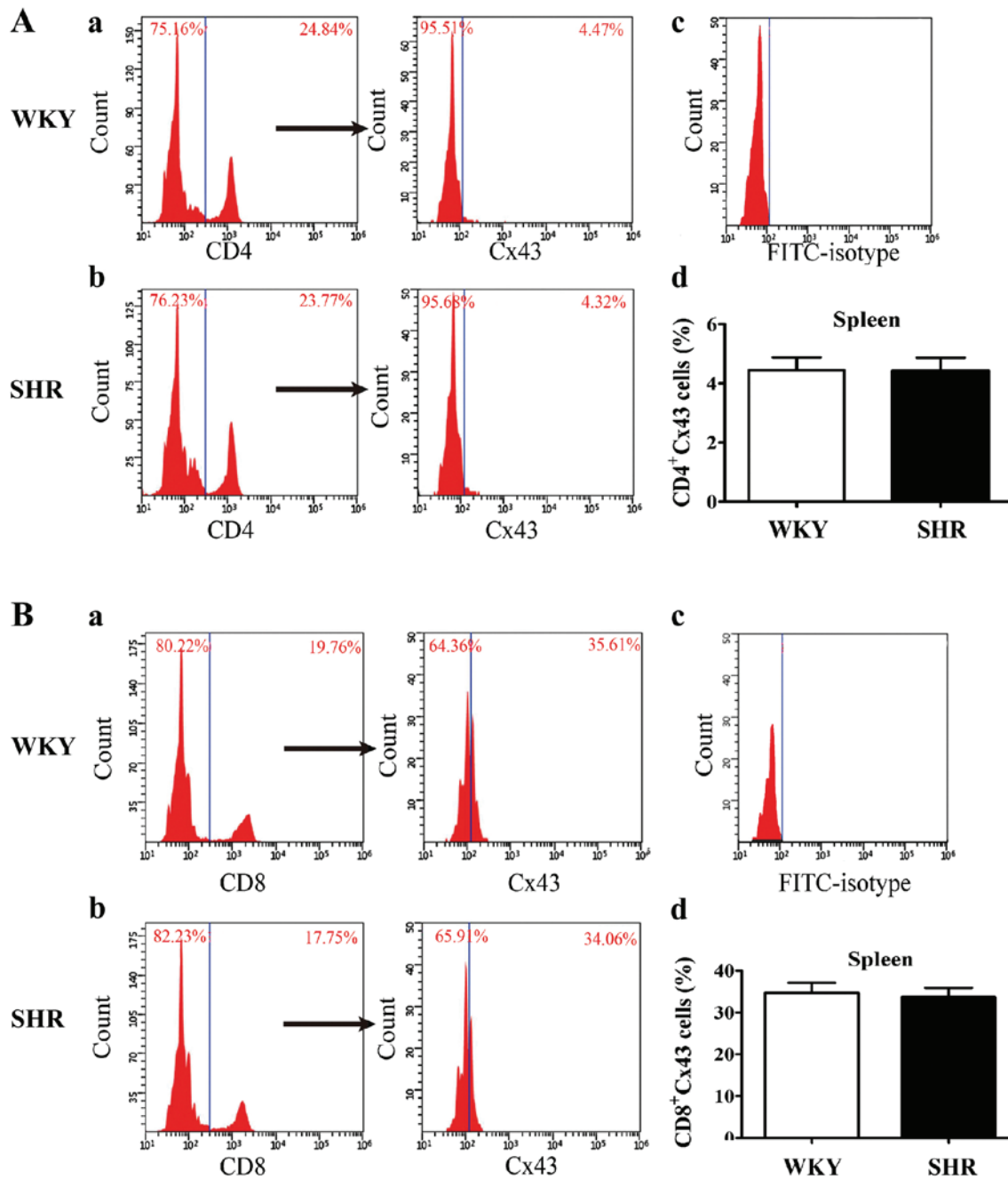


Figure 4. Flow cytometric analysis of connexin 43 (Cx43) surface expression in splenic lymphocyte subsets between spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) rats. (A) (a) Representative density plots indicating the percentage of CD4<sup>+</sup>Cx43 in spleens of WKY rats. (b) Representative density plots indicating the percentage of CD4<sup>+</sup>Cx43 in spleen of SHRs. (c) Representative density plots indicating FITC-isotype of Cx43. (d) Bar graph showing the percentage of CD4<sup>+</sup>Cx43 in spleens of SHRs compared with WKY rats. (B) (a) Representative density plots indicating the percentage of CD8<sup>+</sup>Cx43 in spleens of WKY rats. (b) Representative density plots indicating the percentage of CD8<sup>+</sup>Cx43 in spleens of SHRs. (c) Representative density plots indicating FITC-isotype of Cx43. (d) Bar graphs showing the percentage of CD8<sup>+</sup>Cx43 in spleen of SHRs compared with WKY rats. Results are the means  $\pm$  SEM (n=15).

After analyzing the phenotypic characterization of lymphocyte subsets, we compared Cx43 surface expression in splenic lymphocyte subsets between the SHRs and WKY rats. A representative scatter plot in Fig. 4 shows the percentage of CD4<sup>+</sup>Cx43 and CD8<sup>+</sup>Cx43 cells in the spleens from the SHRs and WKY rats. Flow cytometric analysis revealed the absence of significant statistical differences in the percentage of CD4<sup>+</sup>Cx43 or CD8<sup>+</sup>Cx43 in the spleens between the SHRs and WKY rats. However, Cx43 surface expression could not be

detected in the SHRs and WKY rats due to the low percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells (Fig. 4).

*Role of Gap27 in lymphocyte proliferation and activation between the SHRs and WKY rats.* To confirm the involvement of GJs in hypertensive inflammatory responses, cultured splenic lymphocytes from the SHRs and WKY rats were treated with ConA (5  $\mu$ g/ml) alone or ConA for 24 h following treatment with the Cx43 mimetic peptide, Gap27 (500  $\mu$ M) for 48 h. The



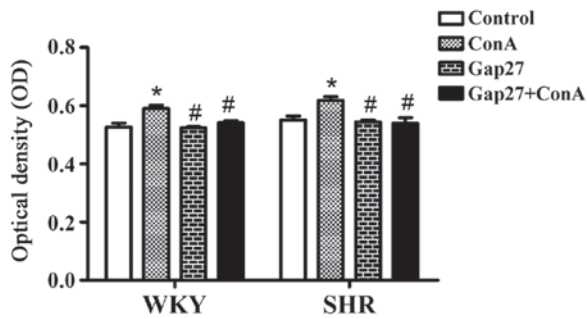


Figure 5. Cell counting kit-8 (CCK-8) assay of the effects of Gap27 on lymphocyte proliferation. Cultures of splenic lymphocyte from spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) rats treated with the vehicle (control), 5  $\mu$ g/ml concanavalin A (ConA), 500  $\mu$ M Gap27 (Gap27), 500  $\mu$ M Gap27+5  $\mu$ g/ml ConA (Gap27 + ConA). Results are the means  $\pm$  SEM from 3 replicate experiments. \* $p$ <0.05 compared with the control group; # $p$ <0.05 compared with the ConA group ( $n$ =3).

activation of lymphocytes *in vitro* was determined by CCK-8 assay. Statistical analysis of the results is shown in Fig. 5. In the lymphocytes from the WKY rats, compared with the control group ( $0.53 \pm 0.01$ ), significant lymphocyte proliferation was observed in the ConA group ( $0.59 \pm 0.01$ ;  $p$ <0.05). Compared to the ConA group, lymphocyte proliferation was significantly decreased in the Gap27 group ( $0.52 \pm 0.01$ ) and Gap27 + ConA group ( $0.54 \pm 0.01$ ;  $p$ <0.05). No statistically significant differences were noted between the Gap27 + ConA and control groups. In the lymphocytes from the SHRs, significant lymphocyte proliferation was observed in the ConA group ( $0.62 \pm 0.01$ ) as compared with the control group ( $0.55 \pm 0.01$ ;  $p$ <0.05). The lymphocytes in the Gap27 group ( $0.54 \pm 0.01$ ) and Gap27 + ConA group ( $0.54 \pm 0.02$ ) exhibited a significantly decreased proliferation as compared with the ConA group ( $p$ <0.05). By contrast, lymphocyte proliferation following Gap27 intervention was similar to that of the control group even after ConA stimulation ( $p$ >0.05) (Fig. 5). These data demonstrated that the proliferation of T lymphocytes may be directly regulated by GJs during essential hypertension.

The percentage of T cell subsets was determined for the SHRs and WKY rats treated with Gap27. In the lymphocytes from the WKY rats, the percentages of total T cells ( $CD3^+$ ) and  $CD4^+CD25^+$  T cells differed significantly among the 4 groups. The percentages of  $CD3^+$  and  $CD4^+CD25^+$  T cells were higher in the ConA-treated lymphocytes than in the control group lymphocytes ( $p$ <0.05) (Fig. 6). Under the effects of Gap27, the percentages of  $CD3^+$  and  $CD4^+CD25^+$  T cells in the Gap27 and Gap27 + ConA groups were significantly decreased compared to those of the ConA group ( $p$ <0.05). However, the percentages of  $CD3^+$  and  $CD4^+CD25^+$  T cells did not differ significantly between the Gap27 + ConA and control groups. More specifically, the  $CD4^+/CD8^+$  ratio was statistically similar in all comparisons (Fig. 6). In the lymphocytes from the SHRs, the trends for the percentages of  $CD3^+$  and  $CD4^+CD25^+$  T cells, as well as the  $CD4^+/CD8^+$  ratio, among the 4 groups were consistent with those of the WKY rats (Fig. 6). In addition, the proliferation rates of the  $CD3^+$  and  $CD4^+CD25^+$  T cells, as well as the inhibition rate of the  $CD4^+CD25^+$  T cells in the lymphocytes from the SHRs were higher than those of the WKY rats ( $p$ <0.01) (Fig. 7).

Table II. The expression of IL-2, IL-6 mRNA in cultured lymphocytes.

Rats	mRNA	Control	ConA	Gap27	Gap27+ConA
WKY	IL-2	1	$2.13 \pm 0.18^a$	$0.94 \pm 0.10^d$	$1.52 \pm 0.20^c$
	IL-6	1	$2.00 \pm 0.19^a$	$1.25 \pm 0.15^c$	$1.24 \pm 0.19^c$
SHR	IL-2	1	$2.76 \pm 0.69^b$	$0.99 \pm 0.17^d$	$1.05 \pm 0.20^c$
	IL-6	1	$3.13 \pm 0.69^a$	$1.08 \pm 0.27^d$	$1.07 \pm 0.27^c$

Results are the means  $\pm$  SEM,  $n$ =3. <sup>a</sup> $p$ <0.01 and <sup>b</sup> $p$ <0.05 compared with the control group; <sup>c</sup> $p$ <0.05 and <sup>d</sup> $p$ <0.01 compared with the ConA group. IL, interleukin; ConA, concanavalin A; WKY, Wistar-Kyoto; SHR, spontaneously hypertensive rat.

The blocking of direct intercellular communications among adjacent lymphocytes suppresses the synthesis and release of pro-inflammatory cytokines, such as IFN- $\gamma$  and IL-2 (17,19). Thus, to further investigate involvement of Cx43-mediated GJIC in the synthesis of pro-inflammatory cytokines in changed T cells subsets during hypertension, the IL-2 and IL-6 cytokine levels were detected following intervention with Gap27 by RT-qPCR. The results obtained from the 4 groups are presented in Table II. In the SHRs and WKY rats, the IL-2 and IL-6 mRNA expression levels were significantly increased following ConA stimulation ( $p$ <0.05). However, the IL-2 and IL-6 mRNA expression levels decreased following treatment with Gap27 in the Gap27 and ConA + Gap27 groups ( $p$ <0.05). Notably, the IL-2 and IL-6 mRNA expression levels did not differ significantly between the Gap27 + ConA and control groups (Table II). In addition, the proliferation rate of the lymphocytes from the SHRs expressing IL-2 and IL-6 was higher than that of those from the WKY rats ( $p$ <0.05) (Fig. 8). Compared with the lymphocytes from the WKY rats, the Gap27 + ConA group lymphocytes from the SHRs exhibited a significant decrease in IL-2 and IL-6 mRNA expression compared with the ConA group (Table II), and thus the inhibition rate of lymphocytes expressing IL-2 and IL-6 was significantly higher in the lymphocytes from the SHRs than in those from the WKY rats ( $p$ <0.01) (Fig. 8). This trend may be explained by the obvious responsiveness of these cytokines to ConA, such that cytokine secretion was immediately inhibited by Gap27. The effects of Gap27 also differed in the lymphocytes obtained from the hypertensive and normotensive rats.

## Discussion

The findings of the present study demonstrate that hypertension causes renal and splenic injury by downregulating the percentage of  $CD4^+CD25^+$  T cells in secondary lymphoid organ (i.e., the spleen), and upregulating serum inflammatory cytokine (IL-2 and IL-6) expression. The kidney plays an important role in the regulation of water and electrolyte balance, with various endocrine functions that are closely related to hypertension. Wang *et al* reported that elevated systemic blood pressure accelerated the progression of kidney injury in rats (20). Gestational hypertension, particularly in preeclampsia, also causes significant kidney damage (21). In accordance with previous studies (3,22,23), significant

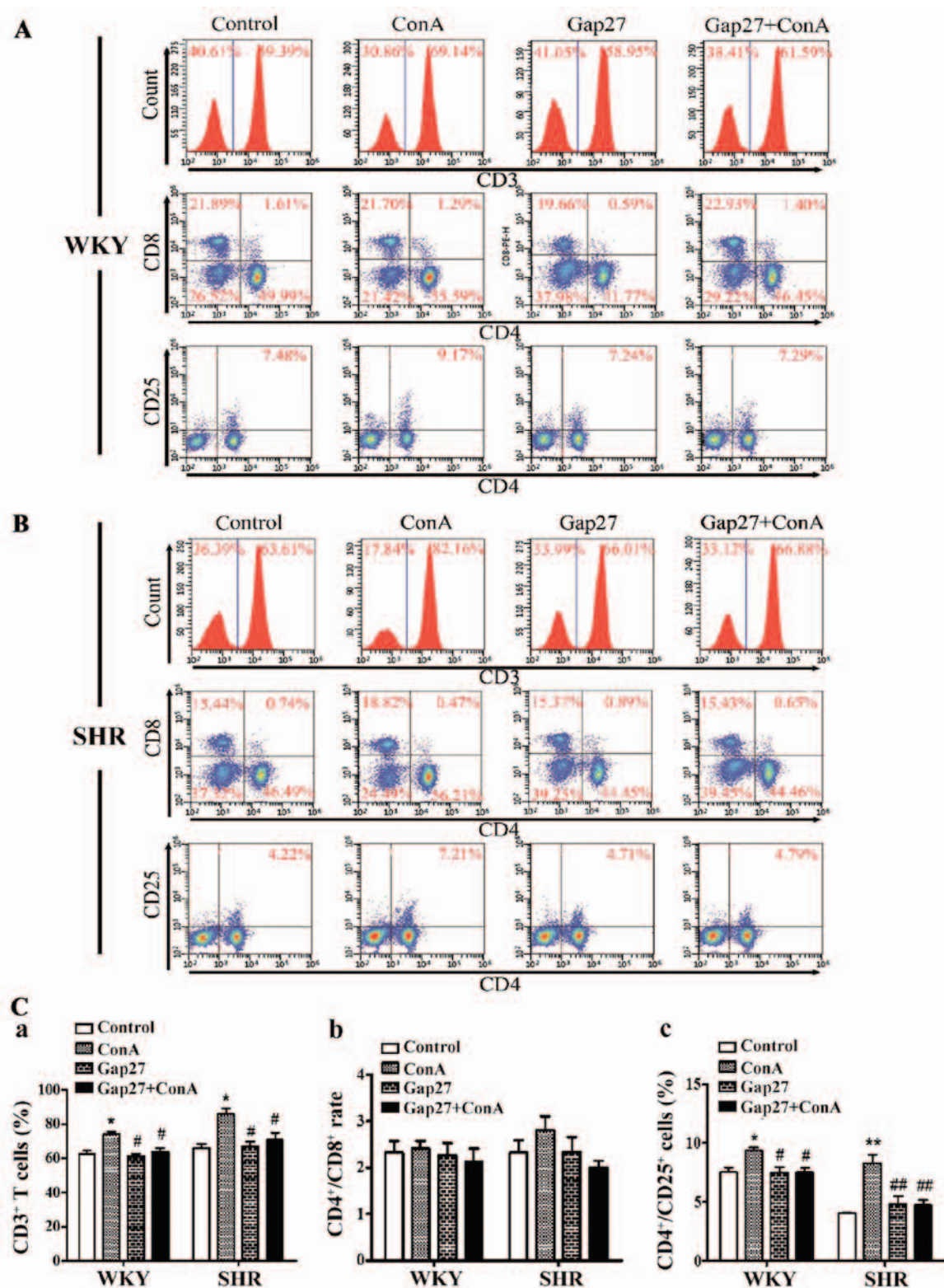


Figure 6. Flow cytometric analysis of the effects of Gap27 on lymphocyte subsets. (A) Representative scatter plots indicating the percentage of CD3<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells of different groups in Wistar-Kyoto (WKY) rats. (B) Representative scatter plots indicating the frequency of CD3<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells of different groups in spontaneously hypertensive rats (SHRs). (C) (a) Bar graphs showing the percentage of CD3<sup>+</sup>, (b) CD4<sup>+</sup>/CD8<sup>+</sup>, (c) CD4<sup>+</sup>/CD25<sup>+</sup> cells in different groups of SHRs and WKY rats. Results are the means  $\pm$  SEM from 3 replicate experiments. \* $p < 0.05$  and \*\* $p < 0.01$  compared with the control group; # $p < 0.05$  and ## $p < 0.01$  compared with the concanavalin A (ConA) group ( $n = 3$ ).

thickening of the vascular wall, inflammatory cell infiltration into part of the blood vessels and glomerular atrophy were observed in the kidneys of hypertensive rats in this

study (Fig. 1). A number of different types of infiltrating immune cells, such as macrophages, T lymphocytes and B lymphocytes have been identified in the kidneys of hyper-



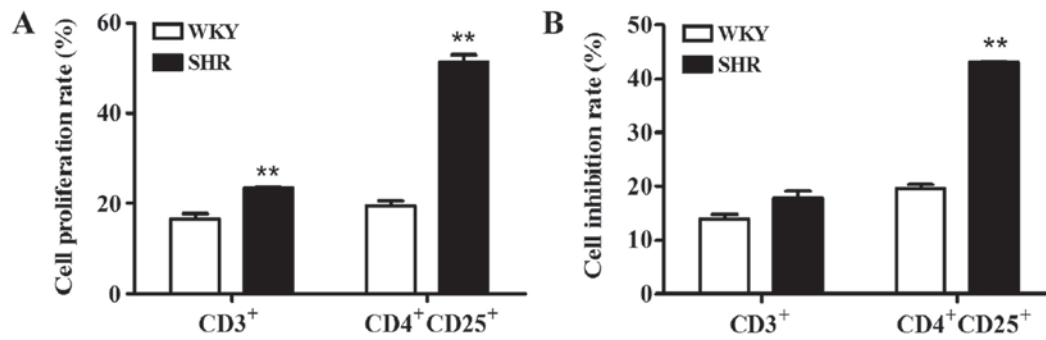


Figure 7. Comparisons of the proliferation and inhibition rate of lymphocyte subsets between spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) rats. (A) Lymphocyte proliferation rate. (B) Lymphocyte inhibition rate. \*\* $p < 0.01$  compared with the WKY rats.

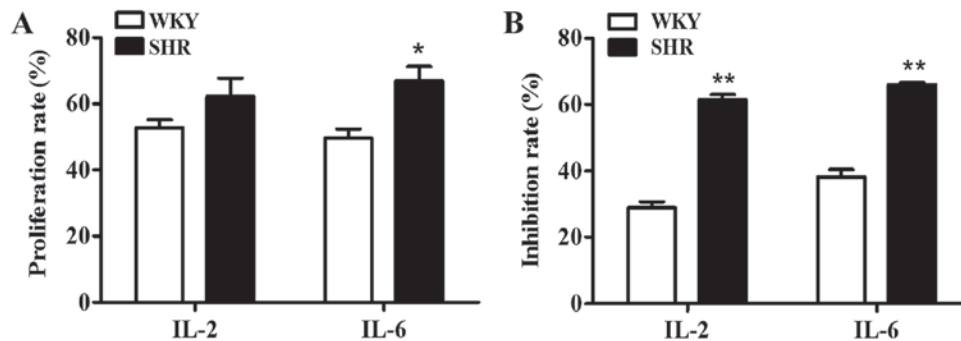


Figure 8. Comparisons of the proliferation and inhibition rate of lymphocytes expressing interleukin-2 (IL-2) and IL-6 between spontaneously hypertensive rats (SHRs) and WKY rats. (A) Proliferation rate. (B) Inhibition rate. \* $p < 0.05$  and \*\* $p < 0.01$  compared with the WKY rats.

tensive rats (24,25). However, the mechanisms leading to the infiltration of these inflammatory cells into the kidneys during hypertension remain to be determined. We speculate that the infiltration of immune cells in the kidneys of hypertensive rats is a secondary effect, which may be mediated by a primary increase in arterial pressure.

As a secondary lymphoid organ and a source of vasoactive factors, the spleen controls the amount of peripheral neuroendocrine and immune mediators in the blood, and maintains a close interaction with the central system via sympathetic innervation in response to stress (26,27). Spleen removal can induce hypertension and lead to tissue injury. Spleen re-implantation reverses the elevation of blood pressure and reduce tissue injury induced by Ang II (28). In the present study, the spleens from SHRs exhibited central artery wall thickening and stenosis (Fig. 1). The mammalian spleen is conventionally considered to be the main filter for blood-borne pathogens and antigens, and this organ is also important for maintaining the lymphocyte populations and immune homeostasis (29). T cells are involved in the pathophysiology of chronic hypertension and target organ damage (30-32). However, the association between hypertensive inflammation and splenic lymphocytes and the mechanisms implicating immune response in hypertension remain elusive. In this study, we compared the different lymphocyte subsets, including T cells (CD3<sup>+</sup>), T-helper cells (CD3<sup>+</sup>CD4<sup>+</sup>), cytotoxic T cells (CD3<sup>+</sup>CD8<sup>+</sup>) and Treg (CD4<sup>+</sup>CD25<sup>+</sup>) in the spleens between SHRs and WKY rats. The percentages of CD3<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells, as well as the CD4<sup>+</sup>/CD8<sup>+</sup> ratio exhibited similar trends in the spleens of the SHRs and WKY rats (Fig. 3). Similar findings have been reported by

Pascual *et al* in the spleens of SHRs younger than 12 weeks (33). Treg populations are decreased; likewise, the adoptive transfer of Treg cells and their anti-inflammatory effects are prevented in Ang II-induced hypertension (7,34). Consistent with the literature, we observed the significantly decreased number of CD4<sup>+</sup>CD25<sup>+</sup> T cells in the spleens of SHRs compared with those of WKY rats (Fig. 3). This result suggests that splenic Treg cells are involved in the pathogenesis of hypertensive inflammation and are associated with vascular diseases. Our findings imply that the prevention of regulatory T cell reduction may be a potential therapeutic technique with which to attenuate inflammation in hypertension.

Cx43 is ubiquitously expressed in different types of immune cells (35). The transfer of information by GJs plays an important role in various immune response processes. However, the effects of Cx43 in splenic lymphocytes on inflammation in hypertension are largely unknown. In our experiments, the level of CD4<sup>+</sup>Cx43 and CD8<sup>+</sup>Cx43 in the spleen did not differ significantly between the SHRs and WKY rats (Fig. 4). Of note, the expression of Cx43 in CD8<sup>+</sup> T cells was higher than that in CD4<sup>+</sup> T cells, thereby indicating of the differential expression of Cx43 in different lymphocyte subsets. Of course, we also detected the expression of Cx43 in CD4<sup>+</sup>CD25<sup>+</sup> T cells; however, the surface expression of Cx43 was not detected due to the low percentage of CD4<sup>+</sup>CD25<sup>+</sup> cells. The function of Cx43 in CD4<sup>+</sup>CD25<sup>+</sup> cells and their relationship requires further investigation.

A vital role of Cx43 is to mediate the GJs, regulating key inflammation processes (36). The transmission of signals mediated by Cx43 depends on hemichannels-independent and

channel-independent mechanisms through GJs (37). It has also been shown that the upregulation of Cx43 expression can promote the activation of CD4<sup>+</sup> T lymphocytes (10,16,38). Given that arterial hypertension activates T lymphocytes (1), ConA is used as a T cell activator that induces various cytokine secretions (39,40). In addition, to confirm the involvement of GJs in lymphocyte proliferation after activation, lymphocytes were treated with a Cx extracellular loop mimetic peptide (Gap27), which can restrict intercellular communication across GJs and reduce or block Cx hemichannel function in CD4<sup>+</sup> T cells and other cell systems (i.e., non-diabetic cells) (16,41). The results revealed that ConA significantly increased the proliferation of lymphocytes from WKY rats and SHR rats compared with the controls. Gap27 significantly inhibited the ConA-stimulated proliferative response of lymphocytes, whereas no significant differences were noted between the Gap27 + ConA and Gap27 groups (Fig. 5). Second, the proliferative response of CD3<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> lymphocytes from the WKY rats and SHR rats were significantly inhibited by Gap27 as compared with the ConA group. No significant differences were observed for the CD4<sup>+</sup>/CD8<sup>+</sup> ratio in all comparisons (Fig. 6). The proliferation rates of CD3<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells, as well as the inhibition rate of CD4<sup>+</sup>CD25<sup>+</sup> T cells, were higher in the lymphocytes from the SHR rats than those from the WKY rats (Fig. 7). We mainly focused on the proportion of the different cell subsets in these *in vitro* stimulation experiments, and the absolute cell numbers were not considered. Therefore, variations in the percentages of cell subsets cannot exclude the possible loss or increased cell survival during the cell culture. The results indicated that the Cx43-induced mediation of GJ channels or hemichannels may be involved in the proliferation of lymphocyte subsets, particularly in CD4<sup>+</sup>CD25<sup>+</sup> T cells. The precise mechanisms by which Cx43 regulate the proliferative response of lymphocyte subsets remain unknown.

IL-2, is a Th1 cytokine which participates in the inflammatory responses, antitumor activity and anti-graft rejection. Elevated IL-6 levels also contribute to hypertension in pregnant rats with chronic reductions in uterine perfusion (42). As previously demonstrated, Ang II-mediated hypertension was attenuated in IL-6-knockout mice (43-45). Knocking down IL-6 by RNA interference has been shown to block cold-induced hypertension (46). Luther *et al* demonstrated that Ang II induced IL-6 expression via a mineralocorticoid receptor-dependent mechanism (47). These findings strongly support the essential role of IL-2 and IL-6 in the development of hypertension. Kumral *et al* reported that renovascular hypertension induced the elevation of IL-2 and IL-6 in serum (48). In the present study, the concentration of IL-2 and IL-6 significantly increased in the serum from SHR rats compared with that from WKY rats (Fig. 2), thereby verifying that hypertension is chronic low-grade inflammation. The interruption of GJIC between lymphocytes or blocking of Cx43 hemichannel causes the reduced synthesis and release of cytokines, such as IFN- $\gamma$ , IL-2 and IL-10 (49). Mendoza-Naranjo *et al* demonstrated that Cx43 regulated IFN- $\gamma$  secretion, but the blockade of the Cx43-GJ function obviously decreased IFN- $\gamma$  secretion by the primed T cells (15). Cytokines also positively regulate Cx expression in immune cells (14,50), suggesting the bidirectional regulation between GJs and cytokines. In our study, a marked decrease in IL-2 and IL-6 mRNA expres-

sion after Gap27 treatment was observed (Table II), which suggests the inhibitory effect of Gap27 on pro-inflammatory cytokine production. These data are consistent with those of recent study by Oviedo-Orta *et al* (16). The study by Oviedo-Orta *et al* indicated that Cx43 is a vital functional component of the immunological synapse between DCs and T lymphocytes (16). However, there were differences in hypertensive and normotensive rats with the action of ConA and Gap27 (Fig. 8). These results clearly demonstrate that Cx mimetic peptide Gap27 inhibits the ConA-induced lymphocyte activation and proliferative response *in vitro*. The proliferation and activation of T lymphocytes and the synthesis and secretion of pro-inflammatory cytokines are regulated by intracellular Ca<sup>2+</sup> levels (51). The increase in intracellular Ca<sup>2+</sup> is a key step in the cascade of signals that results in T cell proliferation (52). More recently, Mendoza-Naranjo *et al* reported that the impaired Ca<sup>2+</sup> signals result from GJs blocking in DC-T cell reduced lymphocyte activation and IFN- $\gamma$  secretion. This finding suggests that Ca<sup>2+</sup> signals, which are regulated by GJs, may be some of the important mechanisms that control T cell activation and proinflammatory cytokines levels (17). Therefore, it is conceivable that Gap27 is likely to block CxHc and thus blocks ATP release and transfer of second messengers such as Ca<sup>2+</sup> entry (49,53). Further studies are required to demonstrate this hypothesis. Above all, these findings indicate that GJIC may regulate the production of inflammatory cytokines after lymphocyte activation.

In conclusion, our results suggest that splenic Tregs may be involved in the suppression of inflammation in hypertension. Cx43 may serve as an important functional component in the modulation of the pro-inflammatory. The action of ConA and Gap27 differed in hypertensive and normotensive rats. The mechanisms by which Gap27 is involved in the control of lymphocyte activation have not yet been established and elucidated. The functional interplays between GJs, cytokines and signaling pathways in Treg-mediated immunosuppressive properties are of important interest. Further studies are warranted to further investigate the interplay between the immune response and the expression of Cx43; some non-specific inhibitor such as niflumic acid (NFA) and 18 $\beta$ -glycyrrhetic acid affecting Cx43 expression can be used to analyze whether the alteration of Cx43 expression will affect hypertensive inflammatory response *in vivo* or *in vitro*. Further studies using Cx43 tissue-specific gene knockout mutants may also provide useful tools with which to discern the contribution of Cx to T cell activation. In addition, further investigations into the role of Cxs and the electrophysiological characteristics of GJs in Treg-mediated immune response suppression and effector T cell activation may lead to the discovery of novel immune therapeutic targets for the treatment of hypertension.

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