

## Original Article

# Mechanism of IFN- $\gamma$ in regulating OPN/Th17 pathway during vascular collagen remodeling of hypertension induced by ANG II

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**Abstract:** More and more researches show that hypertensive vascular remodeling is closely related to the imbalance of immune system in recent years. IFN- $\gamma$  is natural protein with the function of immune regulation and has resistance effect on vascular remodeling. However, the mechanism of IFN- $\gamma$  is to be defined. This paper is to explore the mechanism of IFN- $\gamma$  in regulating OPN/Th17 pathway. In this research, animal models of vascular collagen remodeling were established by inducing hypertensive mice with ANG II. There was no statistical significance when the systolic blood pressures and the percentages of wall thickness/lumen diameter in both groups of WT + AngII + IFN- $\gamma$  and WT + PBS were compared ( $P=0.219>0.05$ ,  $P=0.118>0.05$ ). The concentration of serum precollagen-type I and III and their ratio in WT + AngII + IFN- $\gamma$  group were decreased after the IFN- $\gamma$  being given ( $P<0.01$ ). Expression of OPN within tissue in WT + Ang II group was relatively high, but lowered after treated by IFN- $\gamma$ . Th17 cell ratio was decreased in WT + AngII + IFN- $\gamma$  group ( $P<0.01$ ). Expressions of ROR $\alpha$  and ROR $\gamma$ t mRNA within Th17 cell were decreased ( $P<0.01$ ). The content of IL-23 in WT + AngII + IFN- $\gamma$  group was increased, while IL-10 and TGF- $\beta$  decreased. It has proved that IFN- $\gamma$  can regulate the hypertensive vascular collagen remodeling induced by ANG II, lower the systolic pressure and reduce the pathological damage of vascular collagen remodeling and the collagen synthesis. The mechanism may that the differentiation of Th17 is inhibited by suppressing the OPN expression and regulating the secretion of inflammatory cytokines.

**Keywords:** ANG II, hypertension, vascular collagen remodeling, IFN- $\gamma$ , OPN/Th17

## Introduction

Vascular remodeling (VR) is a complicated and dynamic response process of blood vessel on stimulation, including changes of vascular structure and function caused by the migration, proliferation and apoptosis of vascular smooth muscle cell, as well as the degradation, synthesis and rearrangement of extracellular matrix, which are accompanied with hypertension [1]. The main pathological basis of development, progression and associated complications of hypertension is the vascular damage induced by vascular interstitial collagen remodeling [2]. Researches show that effectively reversing and alleviating the hypertensive VR, especially improving various types of collagen, can decrease the occurrence rate of cerebral apoplexy induced by hypertension [3]. Therefore,

trying to reverse the vascular collagen remodeling shall be the fundamental method for solving the hypertensive issues.

More and more researches show that hypertensive vascular remodeling is closely related to the imbalance of immune system in recent years. Both Treg cell and effector T cell are both involved in the physiopathologic process of hypertension and its target organ damage. The researches show that the inflammatory disorders caused by the immune imbalance of T helper cell 17 (Th17) and regulatory T cell (Treg) have participated in the inflammatory response and damage of target organ [4, 5]. Treg cell metastasis can improve the cardiac remodeling of mice interfered by Ang II, and also improve the oxidative stress and immune cell infiltration [6]. Osteopontin (OPN) is a kind of extracellular

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matrix protein secreted by multiple cells, which would be inflammatory factors and participate in the immuno-related pathological process of hypertension. OPN can regulate vascular remodeling with different approaches, such as adjusting the proliferation, differentiation, cell adhesion, migration and dystrophic calcification of vascular smooth muscle cells [7].

IFN- $\gamma$  is a natural protein with functions of tumor inhibition, anti-virus and immune regulation. Findings in this research show that angiogenesis inhibitor IFN- $\gamma$  can induce the generation of chemokines and indirectly inhibit angiogenesis [8]. It can also down-regulate the expression of matrix metalloproteinase to inhibit the degradation of endothelial basement membrane. Meanwhile, endothelial apoptosis will occur by promoting the expression of Fas with the resistant function on vascular remodeling [9]. However, the mechanism of IFN- $\gamma$  in regulating OPN/Th17 pathway to improve vascular collagen remodeling of hypertension induced by ANG II is to be defined. Therefore, this paper is to preliminarily clarify the regulating mechanism of IFN- $\gamma$  on vascular collagen remodeling by establishing hypertensive mice model induced by Ang II.

### Materials and methods

#### *Main reagents*

Materials, such as fetal calf serum, PBS buffered solution, 1640 culture medium, two antibodies (penicillin + streptomycin) and trypsin, were mainly bought from companies of HyClone and Gibco. PE-labeled human IL-17, FITC anti-human CD8 monoclonal antibody and PE-Cy5 anti-human CD3 monoclonal antibody were all bought from the BD Company in America. Reverse transcription-polymerase chain reaction (RT-PCR) kit and RT-PCR primer were bought from Takara Company in Dalian. RNA extraction reagent TRIzol was bought from Takara Company in Dalian, too. ELISA Kit of murine IL, TGF- $\beta$ , IL-17 and IL-23I was bought from Perprotech

#### *Experimental animal grouping*

Wild type C57BL/6J mice (Wild Type, WT) with weight of 18-20 g were selected and divided into three groups, eight for each: A. WT + PBS; B. WT + Ang II; C. WT + Ang II + IFN- $\gamma$ .

#### *Construction of hypertension models*

Hypertension models were constructed by adopting the method of perfusion of angiotensin for 7 days. Halothane inhalation or pentobarbital sodium injection was adopted to anesthetize mice. The micro pump was subcutaneously imbedded on the back of mice with the perfusion rate of 1500 ng/min/kg and concentration of 288-320  $\mu$ g/L. Blood pressure in mice (caudal artery) was monitored, recorded to see whether the ANG II perfusion model was success on the 4th-7th postoperative days or not. If success, they can be postoperatively adopted.

#### *Detection of systolic pressure, percentages of wall thickness/lumen diameter and collagen area*

Tail artery systolic pressure was measured in quiet and awake mice by blood pressure heart rate determinator. Thoracic aorta would be dissected rapidly with the paraformaldehyde being fixed after anesthesia in mice. Routine paraffin embedding would be produced and sliced up. HE and picric acid-sirius red staining would be used. The auto image analysis system would be used to analyze and measure the percentage of wall thickness/lumen diameter of thoracic aorta and the percentage of collagen area (percentage of vascular wall collagen area and vascular wall area).

#### *Detecting Th17 cell ratio with flow cytometry*

Corresponding reagents were successively added into cell samples (CD3<sup>+</sup> T cells) which were sorted out from normal mice spleen cells according to the following steps, well mixing, room temperature and incubation for 15 min in dark place: 20  $\mu$ L FITC-CD4 antibody and 20  $\mu$ L PE-Cy5-CD3 antibody, 100  $\mu$ L Fix&Perm stationary liquid, 100  $\mu$ L Fk&Perm membrane perforation and hemolysates, APC-IL-17 antibody. Finally, 300  $\mu$ L PBS suspended resuspension cells were added and Th17 cells (CD3<sup>+</sup>CD4<sup>-</sup>IL-17A<sup>+</sup>) were detected with flow cytometry shot by T cells population.

#### *Detecting the expression of mice spleen Th17 cell ROR $\alpha$ and ROR $\gamma$ t mRNA with RT-PCR*

Spleen tissue with appropriate sizes was added into liquid nitrogen and grinded quickly in mortar. Then TRIzol with corresponding volume was

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**Table 1.** Comparison of percentages of systolic pressure, wall thickness/lumen diameter and collagen area among groups ( $\bar{x} \pm S$ , n=8)

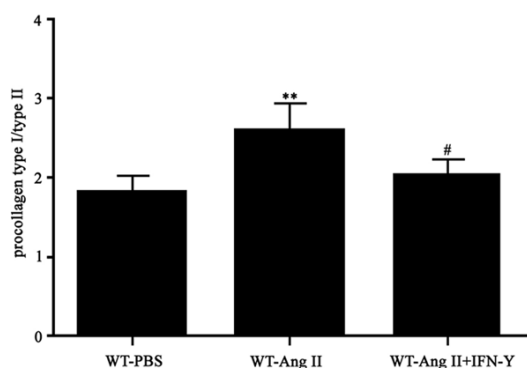
Groups	N	Systolic pressure	Wall thickness/lumen diameter percentage	Collagen area percentage
WT + PBS group	8	148.7 $\pm$ 10.0	6.06 $\pm$ 0.38	4.45 $\pm$ 0.18
WT + Ang II group	8	235.5 $\pm$ 17.3**	15.32 $\pm$ 0.55**	9.56 $\pm$ 0.43**
WT + AngII + IFN- $\gamma$ group	8	156.6 $\pm$ 14.2##	6.43 $\pm$ 0.50##	5.08 $\pm$ 0.30**##

Note: Compared with WT + PBS group, \*\*P<0.01; compared with WT + Ang II group, ##P<0.01.

**Table 2.** Comparison of concentrations of serum precollagen-type I and III

Groups	N	precollagen-type I ( $\mu$ g/L)	precollagen-type III ( $\mu$ g/L)
WT + PBS group	8	82.45 $\pm$ 8.95	43.45 $\pm$ 5.39
WT + Ang II group	8	159.89 $\pm$ 17.49**	62.19 $\pm$ 7.53*
WT + AngII + IFN- $\gamma$ group	8	95.56 $\pm$ 12.13#	48.34 $\pm$ 4.39#

Note: Compared with WT + PBS group, \*P<0.05, \*\*P<0.01; compared with WT + Ang II group, #P<0.05.



**Figure 1.** Statistical figure analysis of concentration ratio of serum precollagen-type I and III among groups.



**Figure 2.** Level expression difference of OPN protein among groups. A. WT + PBS; B. WT + Ang II; C. WT + Ang II + IFN- $\gamma$ .

added into for extracting RNA and turn into cDNA under reverse transcription action. 1  $\mu$ l cDNA was selected as a template to carry out the RT-PCR. Primer sequence of ROR $\alpha$  was: upstream: 5'-AAACAAGCAGCGGGAGG-

TGA-3'; downstream: 5'-TGGCAA CTCCACC ACAT-AC-3'. Primer sequence of ROR $\gamma$  was: upstream: 5'-CCTGGGCTCCT CGCCTG ACC-3'; downstream: 5'-TC-TCTCT GCC CTCA GCCTG-CC-3'. Reaction conditions were: 95 $^{\circ}$ C pre-denaturation 5 min; 95 $^{\circ}$ C 30 s, 55 $^{\circ}$ C 30 s, 72 $^{\circ}$ C elongation 1 min (30 cycle); 72 $^{\circ}$ C final elongation 10 min, preserving at 4 $^{\circ}$ C.

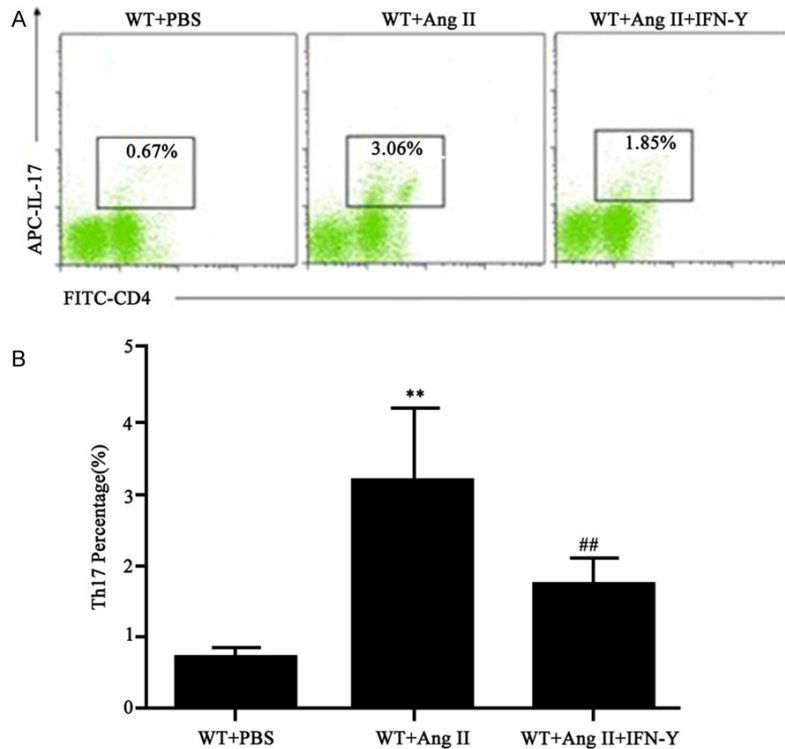
*Detecting the expression level of OPN protein with western blot in spleen tissue*

Spleen tissue with appropriate sizes was added into liquid nitrogen and grinded quickly in mortar. Then frozen lysis buffer with corresponding volume was added into and placed on the ice lysing for 60 min. Lysis buffer was placed in EP tube with conditions of 4 $^{\circ}$ C, 1200 r, centrifugal 30 min, and was cryopreserved in -80 $^{\circ}$ C after sub-package. Expression changes of OPN protein were detected with method of Western Blotting. Firstly, steps, such as manufacturing glue, SDS-PAGE gel electrophoresis, transmembran and blocking, primary antibody incubation 4 $^{\circ}$ C overnights and second antibody incubation for 1 h, were carried out. Relative content was reflected by scanning strap with chemiluminescence method on the Bio-RAP and by the ratio of  $\beta$ -actin grey level.

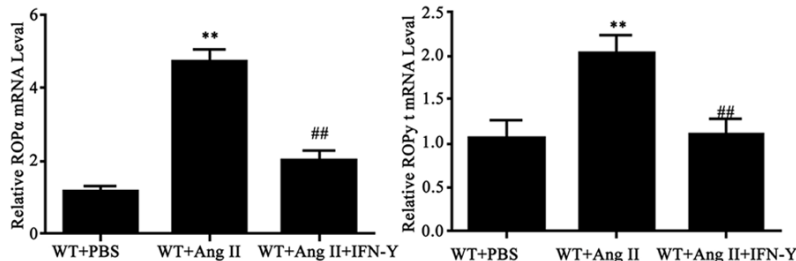
*Detecting the concentrations of serum precollagen-type I and III, as well as the spleen cultural supernatant IL-10, TGF- $\beta$  and IL-23*

Heart blood of intraperitoneal anesthesia mice under indoor temperature was selected with conditions of 3000 r and centrifugal 10 min. Supernatant was absorbed to separate serum and placed in refrigerator to store in -20 $^{\circ}$ C after sub-package. ELISA technique was adopted for

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**Figure 3.** Comparison of Th17 cell ratios in spleen among groups. A. Th17 cell subset ratio in mice spleen after treated with IFN- $\gamma$ ; B. statistical chart of Th17 cell subset ratio (compared with WT + PBS group, \* $P < 0.05$ , \*\* $P < 0.01$ ; compared with WT + Ang II group, ## $P < 0.05$ , ### $P < 0.01$ ).



**Figure 4.** Statistical charts of expressions of ROR $\alpha$  and ROR $\gamma$ t mRNA among groups (Compared with WT + PBS group, \* $P < 0.05$ , \*\* $P < 0.01$ ; compared with WT + Ang II group, ## $P < 0.05$ , ### $P < 0.01$ ).

detecting concentrations of serum precollagen-type I and III in all the groups.

Cultural supernatant of spleen was selected to detect concentrations of serum precollagen-type I and III in all the groups with ELISA technique. ELISA detecting method was operated in accordance with company's specifications. By draw calibration curve, concentrations would be found out on the calibration curve by the OD value of the specimen.

### Statistical analyses

All of the data were input into computer with statistical software of SPSS18.0 and then statistical analysis and disposal were conducted. Variance analysis could be used if there were more than two groups of measurement data. T test was adopted when conducting two-group comparison. Rank-sum test was used for enumeration data.

### Results

#### Comparison of percentages of systolic pressure, wall thickness/lumen diameter and collagen area among groups

Compared with WT + PBS group, percentages of systolic pressure, wall thickness/lumen diameter and collagen area of WT + Ang II group were higher. Differences were statistically significant ( $P = 0.000 < 0.01$ ). Whereas, differences of percentages of systolic pressure, wall thickness/lumen diameter in WT + AngII + IFN- $\gamma$  group were not statistically significant when compared with WT + PBS group ( $P = 0.219 > 0.05$ ,  $P = 0.118 > 0.05$ ), which declared that IFN- $\gamma$  could improve the elevated systolic blood pressure induced by Ang II and the pathological damage. See **Table 1**.

#### Concentrations of serum precollagen-type I and III and their ratio among groups

Concentrations of mice serum precollagen-type I and III and their ratio in WT + Ang II group were higher than those of WT + PBS group. Differences were statistically significant ( $P = 0.000 < 0.01$ ). It showed that concentrations

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**Table 3.** Comparison of cytokine levels in spleen cultural supernatant among groups

Group	N	IL-23	IL-10	TGF- $\beta$
WT + PBS group	8	87.67 $\pm$ 7.35	0.66 $\pm$ 0.09	395.68 $\pm$ 30.57
WT + Ang II group	8	21.86 $\pm$ 0.12**	1.28 $\pm$ 0.22**	631.45 $\pm$ 36.66**
WT + AngII + IFN- $\gamma$ group	8	68.94 $\pm$ 10.58*##	0.68 $\pm$ 0.05##	474.48 $\pm$ 35.78*##

Note: Compared with WT + PBS group, \*P<0.05, \*\*P<0.01; compared with WT + Ang II group, ##P<0.01.

of mice serum precollagen-type I and III and their ratio in WT + AngII + IFN- $\gamma$  group were lowered after giving IFN- $\gamma$ . Differences were statistically significant (P=0.000<0.01), which declared that IFN- $\gamma$  could decrease the concentration ratio of precollagen-type I and III of mice with hypertension. See **Table 2** and **Figure 1**.

### *Level expression difference of OPN protein in spleen tissue specimens*

Protein of spleen tissue was extracted and western blotting was used to detect the expression of OPN protein. See **Figure 2**: relative expression of OPN in tissue of WT + Ang II group was higher. After treated by IFN- $\gamma$ , relative expression of OPN was decreased than before, which declared that IFN- $\gamma$  could inhibit the high expression of OPN protein in mice spleen tissue induced by Ang II.

### *Comparison of Th17 cell ratios in spleen among groups*

Results of Th17 cell ratio in spleen detected by flow cytometry could see **Figure 3A** and **3B**. The results showed that Th17 cell ratio in mice spleen of WT + Ang II group was higher than that of WT + PBS group. Differences were statistically significant (P=0.000<0.01). After giving IFN- $\gamma$ , Th17 cell ratio in mice spleen of WT + AngII + IFN- $\gamma$  group was decreased. Differences were statistically significant (P=0.000<0.01), which declared that IFN- $\gamma$  could inhibit the rising of Th17 cell ratio in mice induced by Ang II.

### *Expression levels of ROR $\alpha$ and ROR $\gamma$ t mRNA in spleen Th17 cell*

Results from agarose gel electrophoresis showed that 18 s and 28 s stripe could be seen clearly in total RNA among groups. A260/A280 value measured by ultraviolet spectrophotometer was 1.8-2.0 which declared that RNA sample retention quality was better without obvious degradation of purity and could meet the exper-

imental requirement of PCR. Expressions of ROR $\alpha$  and ROR $\gamma$ t mRNA in Th17 cell of WT + Ang II group was higher than those of WT + PBS group (P=0.000<0.01). Differences were statistically significant. After treated with IFN- $\gamma$ , Expressions of ROR $\alpha$  and ROR $\gamma$ t mRNA in Th17 cell were decreased (P=0.000<0.01). Differences were statistically significant. See **Figure 4**.

### *Comparison of cytokine levels in spleen cultural supernatant among groups*

IL-23 level in mice spleen cultural supernatant of WT + Ang II group was lower than that of WT + PBS group, while IL-10 and TGF- $\beta$  higher than that of WT + PBS group. Differences were statistically significant (P=0.000<0.01). After treated with IFN- $\gamma$ , mice IL-23 level in WT + AngII + IFN- $\gamma$  group was increased, while IL-10 and TGF- $\beta$  decreased. Differences were statistically significant (P=0.000<0.01), which declared that IFN- $\gamma$  could improve the secretion of inflammatory cytokines. See **Table 3**.

## Discussion

In recent years, more and more researches show that the happenings of almost all the diseases, such as arteriosclerosis, myocardial ischemia, cerebral apoplexy and renal failure, are closely related to the vascular remodeling of patients with hypertension [10]. It has become a research hotspot on hypertensive vascular remodeling to explore the new treatment and look for more effective treatment model [11]. IFN- $\gamma$  is an active protein generated from mononuclear cell and lymphocyte with multiple functions, like cytokines, etc. It also has a variety of biological activity, for example, regulating immune function, anti-virus, influencing the cellular growth differentiation, etc. [12]. Research findings of Leskinen et al show that inflammatory cytokines IFN- $\gamma$  generated from macrophage and lymphocyte can induce the vascular smooth muscle cell to apoptosis

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[13]. Findings in this research show that after treated with IFN- $\gamma$ , systolic pressure, percentages of wall thickness/lumen diameter and collagen area, as well as the concentration ratios of precollagen-type I and III of hypertensive mice models induced by Ang II are decreased, which declared that IFN- $\gamma$  can improve the rising of mice systolic pressure induced by Ang II, pathological damage of collagen vascular remodeling and collagen tissue synthesis.

Widespread expression of OPN in many tissues is a kind of pleiotropic cytokine and inflammatory factor by inducing self-reactive T cell to regulate immune response. Researches show that OPN is participated in the development and progression of hypertensive vascular remodeling [14]. As an inductor of immune response, OPN can affect the balance of Th1/Th2 and regulate the release of cytokines, such as IL-10, IL-12, etc. [15]. OPN in this research in mice spleen tissue induced by Ang II is over expressed. After treated with IFN- $\gamma$ , the relative expression of OPN in tissue is decreased, which shows that IFN- $\gamma$  can be used to treat hypertensive vascular remodeling induced by Ang II by inhibiting OPN.

Findings in recent years show that Th17 cell is a kind of new cell subset in hypertension and exerts important inflammatory reaction during vascular remodeling. Th17 cell is participated in body immune response by mediating of pro-inflammatory cytokine and chemokine and generates by secreting monocyte/macrophage induced IL-17 [16]. Researches show that TGF- $\beta$  and IL-6 can induce Th17 cell to differentiate retinoin orphan nuclear receptor family. This receptor family is the main transcription factor of Th17 cell and can regulate the expression and secretion of its specific effector IL-17 by promoting the differentiation of Th17 cell [17]. Th17 cell ratio in mice spleen of WT + Ang II group in this research is increased, while decreased after treated with IFN- $\gamma$ , which shows that IFN- $\gamma$  can inhibit the rising of Th17 cell ratio in mice induced by Ang II and regulate the disorder of Th17/Treg.

ROR $\gamma$ t is necessary to the survival of lymphoid cell and the developing of lymph node in specific immune system [18]. It has been verified in vitro study that ROR $\gamma$ t is the key nuclear factor of Th17 cell differentiation [19]. Don't express IL-17 if ROR $\gamma$ t in CD4<sup>+</sup>T cell gene is knocked out.

Do express IL-17 if there is no need of foreign cytokines for over-expression of ROR $\gamma$ t gene vector. ROR $\gamma$ t transcriptional regulation region is positioned on non-coding sequence 2 saved by assumption enhancer. There are two specific DNA response elements (ROREs), which can be directly bond ROR and mediate transcriptional activation IL-17 target gene regulated by ROR $\alpha$  and ROR $\gamma$ t [20]. ROR $\alpha$  is also confirmed to be the important transcription factors which can regulate the Th17 cell differentiation. Induction of both IL-6 and TGF- $\beta$  is relying on the ROR $\alpha$  activation with STAT3 form. IL-17 genetic transcription will be activated to promote the differentiation of Th17 cell. Researches of Delerive, et al [21] show that ROR $\alpha$  is the negative regulator of inflammation, which can affect the expression of negative-regulatory factors IKBa and positive regulation NF-kB signal pathway. Researches show that gene deletion of ROR $\alpha$  and ROR $\gamma$ t will frustrate the process of Thprecursor cell in differentiating into Th17 cell [22]. The influence of ROR $\gamma$ t deletion on IL-17 gene expression is more serious than that of ROR $\alpha$  deletion. Therefore, the influence of ROR $\gamma$ t is more important. FoxP3 is one of the members of transcription factors in Fox family and has the action on controlling and regulating the development and function of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells. After treated with IFN- $\gamma$ , the high expression of ROR $\alpha$  and ROR $\gamma$ t mRNA in Th17 cell of WT + Ang II group has decreased, which shows that IFN- $\gamma$  can affect the differentiation of Th17 cell in mice model induced by Ang II.

TGF- $\beta$  is important cytokine for mediating the Th17 cell differentiation. IL-17 can promote the activation of myloid dendritic cell and mononuclear cell, enhance DC to produce IL-1 $\beta$ , IL-6, IL-23 and TGF- $\beta$  and exert the cytokines regulating effect during the process of inducing Th17 differentiation [23]. IL-23 level in mice spleen cultural supernatant of WT + Ang II group is low, while levels of IL-10 and TGF- $\beta$  are high. After treated with IFN- $\gamma$ , it shows that IL-23 level is increased and levels of IL-10 and TGF- $\beta$  are decreased, which shows that IFN- $\gamma$  may regulate the immunologic balance of Th17 cell by improving the secretion of inflammatory cytokines.

In conclusion, IFN- $\gamma$  can regulate the hypertensive vascular collagen remodeling induced by

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Ang II, lower the rising of systolic pressure, improve the pathological damage of vascular collagen remodeling and reduce the synthesis of collagen tissue. The mechanism of IFN- $\gamma$  is to regulate the secretion of inflammatory cytokines by inhibiting the OPN expression, thereby, to inhibit Th17 differentiation and hint that OPN/Th17 pathway has important effect on the development and progression of hypertensive vascular collagen remodeling. We research the action mechanism from the point of immune regulatory function to provide theoretical foundation to clinic.

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### Disclosure of conflict of interest

None.

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