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Original Article

Study on the effects of IL-33/ST2 signaling pathway on spinal cord ischemia-reperfusion injury of rats

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Abstract: Objective: To explore the effects and mechanisms of IL-33/ST2 signaling pathway on spinal cord ischemia reperfusion injury (SCIRI). Methods: A rat SCIRI model was established based on the abdominal aorta blocking method. According to the random number table method, 60 healthy male SD rats were randomly divided into five groups: 12 in the sham operated control group (C Group), 12 in the 3 h post SCIRI group, 12 in the 6 h post SCIRI group, 12 in the 24 h post SCIRI group, and 12 in the 72 h post SCIRI group. The water content of spinal cord tissues was measured to evaluate the degree of injury of spinal cord, and modified Tarlov score was applied to appraise the post-operation neurological function. Quantitative fluorescence PCR was adopted to test the expression of IL-33 and its receptor ST2 in the spinal cord tissues, and ELISA was used to test the release of IL-33 in the serum. Peritoneal macrophages of rats were taken to build an *in vitro* ischemia reperfusion model. After given IL-33, the expression of iNOS and Arg1 in the macrophages was tested via quantitative fluorescence PCR to determine whether phenotype changes of M1/M2 macrophages play a part in the process. Results: The water content of spinal cord tissues increased 3 hours after ischemia reperfusion injury ($P<0.05$) and the neurological score dropped evidently, indicating an obvious spinal cord injury. The expression of IL-33 and its receptor ST2 increased 3 hours after ischemia perfusion injury ($P<0.05$; $P<0.001$ respectively). It gradually dropped 72 hours after ischemia reperfusion injury, but still higher than that of the sham operated control group. The level of IL-33 in the serum also remarkably increased 3 hours after ischemia reperfusion injury ($P<0.001$), revealing that IL-33/ST2 played an important role in SCIRI. While the expression of iNOS in macrophages given IL-33 after SCIRI decreased sharply ($P<0.001$), the expression of Arg1 increased tremendously ($P<0.01$). It indicates that IL-33 was able to promote the polarization of M2 macrophages. Conclusion: IL-33/ST2 signaling pathway plays a part in SCIRI and may facilitate the injury repair through accelerating the polarization of M2 macrophages.

Keywords: Ischemia reperfusion injury, IL-33, spinal cord, M2, iNOS, Arg1

Introduction

As one of the common complications in spinal surgery and neuroscience, ischemia reperfusion (I/R) injury has an incidence rate of 1%~32% [1]. After the factor of spinal cord ischemia was removed and blood flow in spinal cord was restored in spinal cord ischemia reperfusion injury (SCIRI), curiously, the neurological function was not improved, but further impaired; irreversible delayed neuronal death in the spinal cord may occur, which would result in acute and delayed paraplegia [2, 3]. The injury mechanisms of SCIR are extremely complicated. As revealed by current researches, major injury mechanisms of SCIR are microcirculation disorder mechanism, inflammation mechanism and cell apoptosis [4-7]. Since inflammation mechanism is an important pathophysiological basis for SCIRI, the control of inflammatory reaction of the spinal cord is vitally significant to the treatment of SCIRI.

Interleukin-33 (IL-33) is a new addition to the IL-1 family and a multi-functional cytokines recently discovered. It can activate NF-κB and MAPK signaling pathways through receptor ST2 and thus boost the generation of cytokine Th2; as a nuclear protein, IL-33 also participates in the regulation of genetic transcription [9, 10]. Serving as an alarming as well, IL-33 can be released from necrotic cells to alert the tissue damage or stress in the immunity system.

IL-33 is considered a key activation factor in Th2 immunoreaction, the biological activity of which depends on the transmembrane ST2 receptor (ST2L) binding. The receptor of IL-33 is a heterodimer complex that mainly consists of ST2 (IL-1RL1) and IL-1 receptor accessory proteins (IL-lRAcP); while all receptors of the IL family including IL-lRAcP, transmembrane ST2 (ST2L) are selectively expressed on the surface of Th2 cells and innate immunocytes [11-14]. IL-33 has diversified regulating effects of on immunoreactions, and recent discoveries reveal that it plays an important part in many diseases and models [15, 16].

Of great importance in the injury and repair of the immune system, macrophages have pro-inflammatory effects of M1, and give play to anti-inflammatory effects through polarizing to M2 [20]. Research findings also reveal that IL-33 may be the chief culprit of liver diseases induced by schistosomiasis through M2 polarization induction of macrophages [17]. IL-33 has high expression levels in multiple organs, especially in the brain and spinal cord, which indicates that IL-3 may play an important role in the pathophysiology of central nervous system regulation [9]. How IL-33 and other signaling paths affects central nervous system related diseases has been one of the most researched neuroimmunologic topics in recent years.

Materials and methods

Laboratory animals and main reagents

Sixty 8-week-old healthy male Sprague-Dawley (SD) rats weighing approximately 220 g to 260 g were bought from Shanghai SLRC Laboratory Animal LLC. All animals were kept in animal rooms at a temperature of 20°C to 22°C, for 12 h in the light and 12 h in the darkness, and provided with adequate feeds and water. DMEM and fetal bovine serum (Gibico Company); Trizol reagents (Ambion, Life Technologies, USA); reverse transcription kit, PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Japan); s Rat IL-33 Quantikine ELISA Kit (Bio-Techne China Co. Ltd).

Rat I/R model preparation

The rats were anesthetized with 2.5% sodium pentobarbital intra-peritoneal injection at a dose of 40 mg/kg. After aesthesia, the rats were put on the bench with a supine position, and connected to the heating board to keep them warm. After shearing and disinfection on their abdomen, an incision was made on the middle of their abdomen. The abdominal aorta was occluded with a non-invasive arterial clip from the far end of the initial part of left renal artery.

It was ascertained that the occluded abdominal aorta had no pulse. The time was written down and the clip was removed after 30 min to restore the blood flow. The abdomen was sewed up layer-by-layer and erythromycin ointment was applied on the injury. The modeling process was conducted at a temperature of 22°C in a well-ventilated room. The rats were executed by cervical vertebra luxation after 3 h, 6 h, 24 h, and 72 h of I/R respectively. Then the rats were laparotomized and 1.5 ml of inferior vena cava blood was taken out. When the serum was precipitated from the blood after 1 h of settling, it was centrifuged at a rate of 4000 rpm for 10 min. The supernatant serum was then taken and stored at a temperature of -80°C for testing of inflammatory factor in the blood. The skin and muscles of the back were separated. Rongeurs were used to crack the spinal canal and expose the spinal cord. The spinal card under L4 section was quickly taken out and frozen at -80°C for subsequent testing.

Separation and culture of rat peritoneal macrophages

After executed by cervical vertebra luxation, the rats were soaked with 75% alcohol for 5 min. They were then taken out upside down. After an injection of 10 ml of serum-free DMEM into the lower part of peritoneal cavity on one side, the rats were put in a supine position. The abdomen of the rats was gently rubbed for 2 min. After settling for 5 min, the abdomen was cut open in a sterile environment.

After repeated flushing with the injector, the peritoneal macrophages were taken out and centrifuged at a rate of 1000 rpm for 5 min. The cell concentration in regular DMEM (including 10% fetal bovine serum, 100 $U*mL^{-1}$ penicillin and 100 $\mu g*mL^{-1}$ streptomycin) was adjusted. The peritoneal macrophages were then inoculated on a 12-well plate with $1*10^6$/well, and cultured at a 5% CO_2 incubator at a tem-

Table 1. Specific primers (5'->3')

	Forward primer	Reverse primer
IL-33	CCTTTAAGACCAGCTATCTCCC	TTGTGATTTTGCAAGGCGG
ST2	AATCGTCCTGGGGTCT	GGCGGCTTTTTATGTA
iNOS	ATGAACCACCCGACTGAAGC	GTCGTTGGGAGTGGACGAAG
Arg1	ATATCTGCCAAGGACATCGTG	AGGTCTCTTCCATCACTTTGC
β-actin	AGGGAAATCGTGCGTGCGTGAC	CGCTCATTGCCGATAGTG

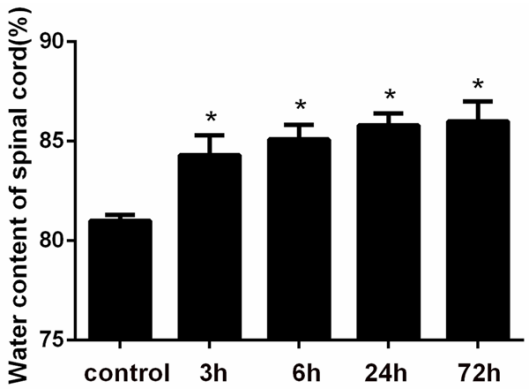

Figure 1. Water content of spinal cord in each group. *P<0.05.

perature of 37°C. After 12 h, the DMEM was replaced to remove the small number of non-adherent cells.

Oxygen glucose deprivation/reperfusion (OGD/R)

After the peritoneal macrophages were cultured for 24 h, the serum-free DMEM was used and the 12-well plate was sealed. DMEM with 10% fetal bovine serum was again used after 30 min. The experimental groups were given IL-33 (100 ng/ml) and the control group was given PBS of the same amount. The cells were taken for testing after regular culture for 5 h.

Test items

Neurological score after I/R: The movement of the rats' hind legs was observed and recorded at respective time points. The standard [18] of modified Tarlov score was used to evaluate the scores. Here was the grading standard: 0 point, the hind legs had no movement and couldn't bear weight; 1 point, the hind legs could move, but couldn't bear weight; 2 points, the hind legs moved frequently or vigorously, but couldn't bear weight; 3 points, the hind legs could support body weight and the rat could take 1 to 2 steps; 4 points, the rat could walk, but was slightly disabled; 5 points, the rat could walk normally.

Measurement of water content of spinal cord: The wet and dry weight method was adopted to measure the water content of spinal cord tissues. After execution, the spinal cord tissues were immediately separated and weighed. The reading was recorded as wet weight. The spinal cord tissues were then put into an oven at a temperature of 100°C. The reading after baking 48 h was recorded as dry weight. The computational formula of water content was: (1- dry weigh/wet weight)*100%.

Measurement of quantitative fluorescence PCR: The spinal cord tissues were taken out of the fridge at a temperature of -80°C. The total RNA of spinal cord tissues was then extracted through Trizol. The Takara reverse transcription kit was used to obtain cDNA. Thin wall PCR tubes were numbered. The templates (cDNA)/ ddH_2O 1.0 ul, 10 uM primer F/R 0.5 ul, 2*qPCR Mix 12.5 ul, ddH_2O 11.0 ul were respectively put into the tubes and mixed. They are then put on the ABI PRISM 7500 PCR System (Applied Biosystems, USA) for quantitative measurement. Specific primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai), as shown in **Table 1**. Amplification conditions were as follows: Pre-denaturation at a temperature of 95°C for 20 s, then at a temperature of 95°C for 3 s, at a temperature of 60°C for 30 s, with 40 cycles in total. The Ct value of each sample was calculated. Relative quantitative was calculated based on $2^{-\Delta\Delta Ct}$ after a comparison of Ct values of internal reference gene β-actin.

ELISA measurement of serum IL-33: The serum IL-33 level of each group was measured via ELISA in strict accordance with the instructions of ELISA kit (Rat IL-33 Quantikine). The operational process was briefly described as follows: The samples were incubated for 1 h at a temperature of 37°C and then washed for 3 times; after adding the enzyme-labeled antibody, the samples were incubated for 0.5 h at a temperature of 37°C and then washed for 3 times; after adding the substrate solution, the samples were incubated for 10 min at a temperature of 37°C. After reaction termination, OD value was measured with enzyme immunoassay analyzer

Table 2. Tarlov scores in each group (Mean ± SD)

Group	Before I/R	After I/R			
		3 h	6 h	24 h	72 h
Control	5±0	5±0	5±0	5±0	5±0
I/R	5±0	0.18±0.27***	0.52±0.73***	1.31±0.61***	1.17±0.72***

Note: ***P<0.001 compared with control group.

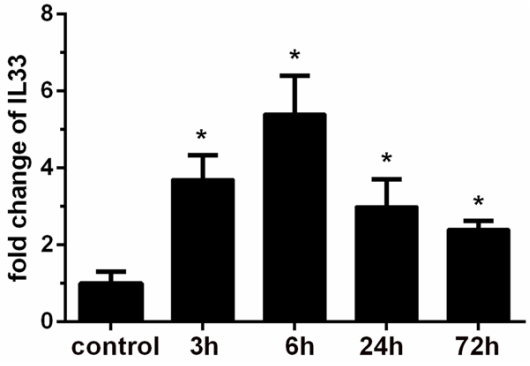

Figure 2. The expression of IL-33 in I/R rats at different time points, *P<0.05 compared with control group.

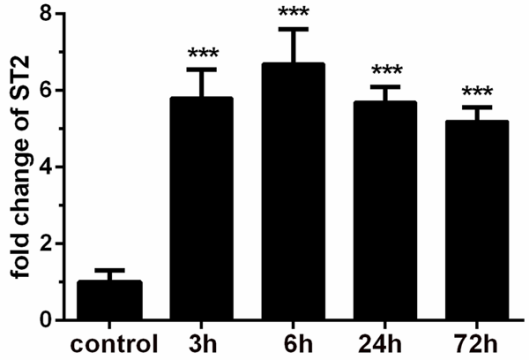

Figure 3. The expression of ST2 in I/R rats at different time points, ***P<0.001 compared with control group.

at 450 nm. The final concentration of IL-33 was calculated.

Statistical methods

All measurement data was represented as Mean ± SD, and analyzed with statistical software SPSS17.0. The neurological score was analyzed through non-parametric rank sum test (Kruskal-Wallis). Single factor variance was adopted for comparison between two groups. If P<0.05, it was considered statistically significant.

Results

Water content of spinal cord after I/R injury

The water content of spinal cord in each group was measured at corresponding time points (**Figure 1**). Results indicated that the water content of spinal cord evidently increased after I/R injury and had statistical significance in comparison to the C Group (P<0.05). It revealed that I/R caused a serious injury to the spinal cord.

Tarlov neurological score after SCIRI

The neurological function of the rats in each group was measured before I/R and after 3 h, 6 h, 24 h and 72 h of I/R. Pre-I/R results revealed that all rats had normal neurological function and a Tarlov score of 5 points. In comparison to the control group, I/R group had an evidently lower Tarlov score, and the difference was statistically significant (P<0.001, **Table 2**).

Expression of IL-33 and its receptor ST2 in the tissues measured by quantitative fluorescence PCR

After 3 h of I/R, the expression of IL-33 in the spinal cord tissues increased. The expression decreased from 6 h to 72 h after I/R, but was still higher than that of the control group, which had statistical difference (P<0.05, **Figure 2**). The expression of IL-33's receptor ST2 also evidently increased after SCIRI, and showed remarkable difference (P<0.01, **Figure 3**).

Release of IL-33 in the serum measured by ELISA

After 3 h of I/R injury, the IL-33 level in the serum evidently increased. It slightly decreased afterwards, but is still higher than the level before I/R. The difference had statistical significance (P<0.001, **Figure 4**).

Effects of IL-33 on the expression of iNOS and Arg1 in OGD/R of peritoneal macrophages

To further study the effects of IL-33 in SCIRI, the peritoneal macrophages were used to establish an *in vitro* OGD/R model. After the experimental group was given with IL-33, the

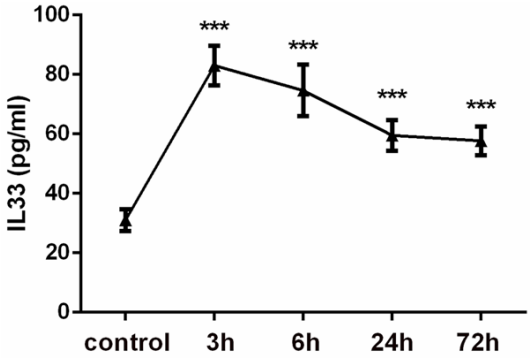

Figure 4. The production of IL-33 in serum of I/R rats at different time points, ***P<0.001 compared with control group.

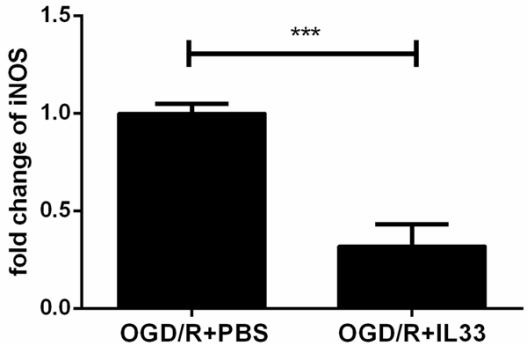

Figure 5. IL-33 inhibited the expression of iNOS in OGD/R macrophages, ***P<0.001.

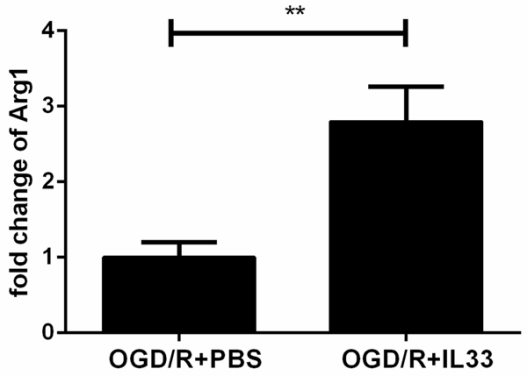

Figure 6. IL-33 increased the expression of Arg1 in OGD/R macrophages, **P<0.01.

expression of OGD/R macrophages evidently decreased (P<0.001, **Figure 5**), but the expression of Arg1 increased noticeably (P<0.01, **Figure 6**). It revealed that the application of IL-33 facilitated the transformation of macrophages to M2.

Discussion

SCIRI may lead to acute and flaccid paralysis, which is detrimental to patients. The pathogenesis and injury mechanism of SCIRI has always been a hot clinical and fundamental research topic. In the early stage of spinal cord I/R, the expression of cytokines and adhesion molecules increase. It facilitates a transition from ischemia and hypoxia injury to inflammatory injury. Cytokines such as TNF-α, IL-6, IL-8, IL-1 and PAF play an important part in the pathological changes of spinal cord injury.

As a new addition to the IL-1 family, IL-33 has many unique features [11-14]. Recent researches indicate that IL-33 can serve as an alarming in central nervous system injury to accelerate the release of chemokines and recruitment of monocytes, and thus play an important part in injury repair [19]. As shown in this study, the expression of IL-33 and its receptor both increased after several hours of I/R. It manifested that it also played a part in the development of SCIRI. Current research reveals that IL-33's receptor ST2L is selectively expressed in the surface of Th2 cells, but can't be expressed in Th1 or Treg cells [12]. IL-33 can be bound with ST2L receptor on the surface of Th2 cells, which induces the release of anti-inflammatory cytokines from Th2 cells and gives play to the Th2 immunological effect. As innate immunocytes, macrophages can monitor the steady state of the tissues, which is vitally important to injury and repair [20].

They have pro-inflammatory effect as M1 macrophages and anti-inflammatory effect as M2 macrophages. In central nervous system injury, microglia and macrophages in the circulation are recruited and activated, differentiated towards M1 or M2 [21]. M1 macrophages can produce reactive oxygen species and various proteases to facilitate secondary injury [20]. On the contrary, M2 macrophages can secrete growth factors, swallow cell debris and accelerate the differentiation of neural stem cells, thus facilitate injury repair [22]. *In vitro* experiments in this study revealed that IL-33 can promote the transformation of OGD/R macrophages towards M2 macrophages. It indicates that IL-33 can not only activate Th2 inflammatory reaction, but also help the transformation of mononuclear macrophages towards M2 macrophages in I/R injury, starting injury repair.

To sum up, this study demonstrated that IL-33/ST2 play an important part in SCIRI, and may help injury repair by facilitating the M2 polarization of macrophages. New drugs based on IL-33 or analogues for human application may be developed in the future to treat spinal cord injury. The injury mechanisms of SCIR are extremely complicated. More researches and explorations are needed to find better treatment methods, prevent spinal cord injury and facilitate the repair of injured spinal cord.

Disclosure of conflict of interest

None.

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