Neuroprotective effect of astrocyte-derived IL-33 in neonatal hypoxic-ischemic brain injury

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Abstract

Background: Interleukin-33 (IL-33) is a well-recognized pleiotropic cytokine which plays crucial roles in immune regulation and inflammatory responses. Recent studies suggest that IL-33 and its receptor ST2 are involved in the pathogenesis of neurological diseases. Here, we explore the effect of IL-33/ST2 signaling in neonatal hypoxic-ischemic (HI) brain injury and elucidate the underlying mechanisms of action.

Methods: The brain HI model was established in neonatal C57BL/6 mice by left common carotid artery occlusion with 90 min hypoxia, and treated with IL-33 at a dose of 0.2 μg/day i.p. for three days. TTC staining and neurobehavioral observation were used to evaluate the HI brain injury. Immunofluorescence and flow cytometry were applied to determine the expression of IL-33 and its receptor ST2 on brain CNS cells, cell proliferation and apoptosis. OGD experiment was used to assay the viability of astrocytes and neurons. RT-qPCR was used to measure the expression of neurotrophic factor-associated genes.

Results: The expression level of IL-33 was markedly enhanced in astrocytes 24 h after cerebral HI in neonatal mice. Exogenous delivery of IL-33 significantly alleviated brain injury 7 d after HI, whereas ST2 deficiency exacerbated brain infarction and neurological deficits post HI. Flow cytometry analyses demonstrated high levels of ST2 expression on astrocytes, and the expression of ST2 was further elevated after HI. Intriguingly, IL-33 treatment apparently improved astrocyte response and attenuated HI-induced astrocyte apoptosis through ST2 signaling pathways. Further in vitro studies revealed that IL-33-activated astrocytes released a series of neurotrophic factors, which are critical for raising neuronal survival against oxygen glucose deprivation.

Conclusions: The activation of IL-33/ST2 signaling in the ischemic brain improves astrocyte response, which in turn affords protection to ischemic neurons in a glial-derived
Background

Perinatal hypoxic-ischemic encephalopathy (HIE) is a common and devastating disease that is a primary cause of neonatal mortality and long-term neurological deficits in children [1-3]. HIE in the newborn often results from a hypoxic event and subsequent insufficient cerebral blood flow to the brain, leading to millions of neonatal death or long-term disabilities every year [4]. Although there have been major advances in modern technology and an increased understanding of fetal and neonatal pathologies, the therapeutic interventions for HIE in the clinical setting are limited [5, 6]. Thus, it is urgently needed to develop safe and effective therapies for the treatment of neonates suffering HIE.

As a novel member of the IL-1 family of cytokines, IL-33 has been increasingly implicated in a variety of innate and adaptive immune response [7, 8]. IL-33 is constitutively expressed in the nuclei of endothelial and epithelial cells with barrier function, or to be released into the extracellular space, as an alarmin, after tissue damage to alert the immune system [9]. In the cell nucleus, IL-33 may act as a transcriptional repressor and modulate NF-κB-mediated gene expression. The extracellular IL-33 exerts its biological functions by binding to its receptor complex, which is composed of ST2 and IL-1 receptor accessory protein (IL-1RAcP) [10]. It has been recently observed that both IL-33 and its receptor ST2 mRNA are expressed at high levels in the central nervous system (CNS) [11]. Moreover, several studies also reported the involvement of 33/ST2 signaling in the pathogenesis of neurological diseases including Alzheimer's disease [12], experimental autoimmune encephalomyelitis (EAE) [13], experimental cerebral malaria [14] and stroke [15]. However, the effect of IL-33/ST2 axis in neonatal hypoxic-ischemic brain injury remains unclear.
In the present study, we found that exogenous delivery of IL-33 significantly ameliorated HI-mediated neonatal brain damage, whereas genetic deficiency of ST2 exacerbated brain infarction and behavioral disorders. Further in vivo and in vitro studies showed the importance of the IL-33/ST2 signaling in post-HI astrocyte responses and subsequent protection of ischemic neurons. Mechanistic studies revealed that IL-33/ST2 engagement stimulates the production of neurotrophic factors from astrocytes, which in turn enhances neuronal survival in neonatal HI brain injury.

Methods

Mice and IL-33

Seven-day-old (P7) ST2-deficient mouse pups on a C57BL/6 background and their wild-type littermates were obtained from Cyagen Biosciences Inc., Guangzhou, China. All animals were housed in specific pathogen-free conditions, and experiments were approved by the Animal Care and Use Committee of Guangdong Pharmaceutical University. Recombinant mouse IL-33 protein was prepared in our laboratory as described previously [16]. Mice were injected i.p. with IL-33 (0.2 μg per mouse) or PBS (control) for three days after hypoxic-ischaemic (HI) operation.

Neonatal hypoxic-ischemic brain injury

The modified Rice-Vannucci model was used to establish hypoxic-ischemic (HI) brain injury [17, 18]. P7 pups were anesthetized with isoflurane and underwent a unilateral left common carotid artery occlusion using bipolar electrical coagulation (Vetroson). The incision was closed using tissue adhesive (3M Vetbond). After a recovery period of 2 h, pups were placed in a hypoxia chamber (8% O₂, balanced nitrogen) maintained at 37°C for 90 min. Pups were then recovered and returned to their dams. A subset of control animals were mock-treated with a small incision in their neck without artery occlusion and placed
in a chamber at normal air temperature (sham control).

**Infarct volume**

Seven days after the termination of hypoxic insult, brains of pups in HI and sham groups were sliced coronally into 2-mm-thick sections and incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C in the dark for 15 min. The areas of ipsilateral and contralateral hemispheres were measured using Image J software (National institute of Health, Bethesda, MD, USA). After correcting for edema, the volumes of infarction were calculated as follows: Total volume of contralateral hemisphere –(Total volume of ipsilateral hemisphere –Average volume of 3 measurements of infarct).

**Neurobehavioral evaluation**

Pups in each treatment group were subjected to three neurobehavioral tests 1, 3 and 7 days after HI procedure as previously described [19, 20]. (1) Geotaxis reflex is used for diagnosing vestibular and/or proprioceptive functions. Pups were placed with its head downward on a 30° incline and the latency to make a 90° turn was recorded. (2) Cliff avoidance reaction is used for assessing maladaptive impulsive behavior. Pups were placed on the protruding end of the board with their head down and forepaws off the board and the latency to place both their forepaws back on the board was measured. (3) Grip test is used for evaluating grip force and fatigability. Pups were suspended by both forepaws on a metallic wire and the total time to fall was recorded.

**Primary neonatal astrocyte culture**

Primary astrocyte cultures were prepared from neonatal mice as described previously [21]. Briefly, cortices from newborn mouse pups were dissected and pooled, and cells dissociated by exposure to of 2.5 % trypsin for 30 min at 37°C. The cells were centrifuged at 300×g for 5 min and resuspended in DMEM containing 10% FBS and 50 U/mL penicillin-streptomycin (Gibco). The cells were plated onto poly-L-lysine coated 25-cm² flasks at a
density of $6 \times 10^5$ cells/cm$^2$ and cultured for 10-12 d. Nonastrocytic cells, such as microglia and neurons, were detached from the flasks by shaking and removed by changing the medium. Astrocytes were dissociated by trypsinization and then reseeded on uncoated 6- and 24-well plates for the following experiments. The purity of GFAP$^+$ astrocytes examined by flow cytometry was over 96%.

**Lentivirus transduction**

Primary astrocytes were infected with either PUMA$\alpha$ shRNA lentiviral particles (PUMA shRNA LV) or control shRNA LV (Santa Cruz biotechnology) in DMEM with 5 $\mu$g/ml polybrene at multiplicities of infection (MOI) of 15. After 12 h of culturing, the medium was replaced by fresh DMEM with 10% FBS in order to remove debris and inactive lentiviruses. Some wells were then supplemented with IL-33 (50 ng/mL) following oxygen-glucose deprivation/reoxygenation (OGD/R) treatment, and then collected for cell apoptosis and viability analysis.

**Astrocyte OGD experiment**

Primary astrocyte cultures were replaced with the glucose-free DMEM and transferred into an anaerobic chamber flushed with 5% CO$_2$ and 95% N$_2$ (v/v) at 37°C for 6 h. The astrocytes were incubated again in DMEM containing 10% FBS and returned to the normoxic incubator (95% air and 5% CO$_2$) at 37°C for 24 h. For IL-33 treatment, cultured astrocytes ($5 \times 10^5$) were treated with 50 ng/ml IL-33 throughout the whole period of OGD/R (30 h), pretreatment with or without Ly294002 (1 $\mu$M; Selleck) for phosphorylation AKT-S473 analysis or P53 inhibitor Pifithrin-\(\alpha\) (10 $\mu$M; Selleck) for cell apoptosis analysis. Cells without exposure to OGD/R were defined as control group.

**Astrocyte-conditioned media (ACM)**

Primary astrocytes cultures were replaced with the glucose-free DMEM and transferred
into an anaerobic chamber flushed with 5% CO₂ and 95% N₂ at 37°C for 6 h. Cells were then incubated in neurobasal medium (Gibco) containing 2% B27 (Gibco) and returned to the normoxic conditions for an additional 24 h. Some wells were treated with IL-33 (50 ng/ml) throughout the whole period of OGD/R (30 h). Conditioned media were collected and filtered using 0.22-μm filter before use in neuron studies.

**Primary cortical neuronal cell culture**

Cerebral cortices from newborn mouse pups were dissected and cells dissociated by incubation of OPC papain solution (1.54 mg/ml papain, 360 μg/ml L-cysteine and 60 μg/ml DNase I) for 20 min at 37°C. Cells were filtered through a 40 μm cell strainer to obtain a single cell suspension, and then resuspended in neurobasal media containing 2% B27. Cells were cultured in a humidified incubator at 37°C with 5% CO₂ for 7 days before experiments.

**Neuron OGD experiment**

Primary cultured cortical neurons were then exposed to OGD/R experiments as described previously [22]. Briefly, cell cultures were replaced with the glucose-free DMEM and transferred into an anaerobic chamber flushed with 5% CO₂ and 95% N₂ at 37°C for 3 h. The neurons were incubated again in neurobasal medium containing 2% B27 and returned to the normoxic conditions for an additional 24 h. For IL-33 or astrocyte-conditioned media (ACM) treatment, cultured neurons (5 x 10⁵) were treated with different concentrations (25, 50, 75 and 100 ng/ml) of IL-33 or various doses of ACM throughout the whole period of OGD/R, pretreatment with or without 20 μM of Nintedanib (MedChem Express), a tyrosine kinase inhibitor. Cells without exposure to OGD/R were defined as control group.

**Cell viability**

Cell viability was determined by cell counting kit-8 (CCK-8, Dojindo, Japan) assay. Cells
were seeded at a density of $1 \times 10^3$ cells per well in 96-well plates. After underwent OGD, the cells were cultured with IL-33 or ACM for 24 h and subsequently with CCK-8 solution for 2 h at 37 °C. The absorbance at 450 nm was measured using a microplate reader (Model 680, Bio-Rad Laboratory, Hercules, CA).

**Flow cytometry**

To examine the percentages of ST2+ cells, cells were suspended in PBS and incubated with PE-labeled anti-ST2 antibody (Biolegend) for 30 min. Cell apoptosis was measured using the Annexin V apoptosis detection kit (eBioscience) and *in situ* apoptosis detection kit (Roche) according to the manufacturer's directions. For analysis of phosphorylation AKT-S473, cells were fixed and permeabilized with the BD Cytofix/Cytoperm kit (BD Biosciences), and then stained with mouse anti-phospho-AKT-S473 PE or isotype control (eBioscience). For analysis of RET, PUMA or p53 protein levels, cells were fixed in 4% paraformaldehyde and permeabilized in 0.25% saponin. Cells were stained with a primary anti-RET, anti-PUMA or anti-p53 antibody (MultiSciences Biotech Co., Ltd, Hangzhou, China) followed by a secondary donkey anti-rabbit PE antibody (Biolegend). Flow cytometric analysis was performed with FACSCalibur cytometer (BD Biosciences) and CellQuest v3.3 software.

**Cell proliferation**

For analysis of cell proliferation, cells were cultured with 50 ng/ml IL-33 or ACM following OGD/R (30 h) treatment, fixed and permeabilized using the BD Cytofix/Cytoperm kit and stained intracellularly with PE anti-Ki-67 (eBioscience). Cell proliferation was analyzed with a FACSCalibur cytometer.

**Cell-cycle analysis**

Cell were fixed with 75% ethanol at 4°C for 16 h and then treated with 100 μg/mL
ribonuclease A and 50 μg/mL propidium iodide (PI; Sigma, St Louis, MO, USA) at room temperature for 30 min. DNA fluorescence of the stained cells was measured with a FACSCalibur cytometer. The percentages of cells within the G1, S, and G2/M phases of the cell cycle were calculated by use of ModFit software (Verity, Topsham, ME).

**Immunofluorescence**

Mouse pups were anesthetized and transcardially perfused with 4% paraformaldehyde in PBS. The brain tissues were embedded in paraffin, sliced coronally in 5-μm. Tissue sections were dewaxed, quenched with 3% hydrogen peroxide for 10 min, and incubated with 5 % bovine serum albumin (BSA) for 30 min. The sections were stained overnight at 4°C with either anti-ST2 (1:200, ab25877, Abcam), anti-IL-33 (1:500, AF3626, R&D), anti-GFAP (1:500, 104805-T08, Sino Biological Inc.), anti-Olig2 (1:500, EPR2673, Abcam), anti-Iba1(1:50, 20A12.1, Sigma-Aldrich), anti-Neun (1:50, A60, Sigma-Aldrich), or anti-Ki67 (1:500, ab15580, Abcam) followed by PE- or FITC-conjugated secondary antibodies (eBioscience). TUNEL staining was performed with the *in situ* cell death detection kit (Roche). The slides were counterstained with nuclear dye DAPI and observed on an Olympus BX51 fluorescent microscope.

**Western blotting**

Neonatal brains were dissected at the appointed times after HI. Proteins from ipsilateral hemisphere were extracted by tissue homogenization in RIPA buffer containing a proteinase inhibitor cocktail (Santa Cruz). Equal amounts of protein (30 μg/well) were separated by gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes. Blots were probed with anti-IL-33 (AF3626, R&D), anti-cleaved caspase-3 (9661, Cell Signaling Technology, Inc.), anti-Bax (ab32503, Abcam), and anti-Bcl-2 (ab196495, Abcam) antibodies, and then detected using HRP-conjugated secondary antibody (Abcam). β-actin (100166-MM10, Sino Biological Inc., China) was used as a
loading control. Images were analyzed using ImageJ and normalized to β-actin.

**Quantitative PCR assay**

RNA from primary astrocytes was purified using TRIzol reagent (Invitrogen) and transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen). Quantitative PCR (qPCR) was performed to detect mRNA expression using SYBR Green qPCR kit (Invitrogen) and primers for PCR amplification included: p53 forward 5'-TCA CAG TCG GAT ATC AGC CT-3', reverse 5'-ACA CTC GGA GGG CTT CAC TT-3'; PUMA forward 5'- CCT CCT TCC TCC GGA GTG TTC A-3', reverse 5'- ATA CAG CGG AGG GCA TCA GG-3'; GDNF forward 5'-CTC TAG CTC TTG GGG GAA TC-3', reverse 5'-ACG ACC GAG ACA TCA GAG AG-3'; ARTN forward 5'-TAC TGC ATT GTC CCA CTG CCT CC-3', reverse 5'-TCG CAG GGT TCT TTC GCT GCA CA-3'; PSPN forward 5'- TGT CAC AAT GGC TGC AGG AAG ACT T-3', reverse 5'-AGC TCA GCC ACT GGT AGG GTC AGG-3'; NRTN forward 5'- CAG CGG AGG CGC GTG CGC AGA GA-3', reverse 5'- CGG CTG TGC ACG TCC AGG AAG GA-3' and GAPDH forward 5'-TTC ACC ACC ATG GAG AAG GC-3', reverse 5'-GGC ATG GAC TGT GGT CAT GA-3'. All qPCR reactions were performed with an ABI PRISM® 7000 Sequence Detector Systems (Applied Biosystems, Foster City, CA), and expression values were normalized to the housekeeping gene GAPDH using the comparative threshold cycle (CT) method.

**Statistical analyses**

Statistical analyses were performed using SPSS version 13.0 (SPSS, Chicago, IL, USA). All the data are presented as the mean ± standard error of the mean (SEM). Statistical differences between groups were evaluated by the Student's t test or one way ANOVA. P values less than 0.05 were considered significant.

**Results**

**HI injury induces an elevation of IL-33 in the neonatal brain**

To identify the involvement of IL-33 in post-HI neuroprotection, we firstly examined the
expression of IL-33 on the brain after HI injury in neonatal mice. A dramatic increase in IL-33 protein was observed at the lesion site 1 d after HI and then decreased in the following days (Fig. 1a). The phenotype of IL-33-expressing cells was identified by immunofluorescent staining, which showed that IL-33 readily detected from astrocytes (IL-33^+GFAP^+) in the periinfarct areas, but less or none was present in the microglia and neurons (Fig. 1b and c). Furthermore, flow cytometry analysis confirmed distinctly increased counts of IL-33^+ astrocytes at 1 d after HI (Fig. 1d). Together, these results suggest that astrocyte-derived IL-33 is a specific factor up-regulated after brain HI and that astrocytes are a major source of IL-33 in the CNS.

**IL-33 administration alleviates HI-induced neonatal brain injury**

Next, we explored whether IL-33 can ameliorate brain damage *in vivo* using a neonatal mouse HI brain injury model. TTC staining of coronal sections of mouse brains was used for evaluating the infarct volume. We observed that treatment with IL-33 at a dose of 0.2 μg/dose/day for three days significantly reduced brain infarct sizes 7 d after HI (Fig. 2a). Exogenous IL-33 markedly reduced the levels of apoptosis-related proteins including cleaved caspases-3 and bax in brain tissue 7 d after HI challenge (Fig. 2b). The protein ratio of Bcl-2/Bax was obviously restored in IL-33-treated group compared to the vehicle-treated group (Fig. 2c). Furthermore, Neun and TUNEL double staining confirmed a significantly decrease in neuronal death (Neun^+ TUNEL^+) 7 d after HI in IL-33-treated mice (Fig. 2d).

Neurological functions after HI injury were assessed in sham, vehicle-treated and IL-33-treated mice. Neurobehavioral tests evaluating the geotaxis reflex, cliff avoidance and grip were performed 1, 3 and 7 days after HI in the three groups. Compared with pups in the sham group, neurobehavioral deficits of pups in the vehicle-treated HI group were
clearly observed 1, 3 and 7 days after HI (Fig. 2 e-g). However, IL-33 treatment significantly improved these neurological defects (Fig. 2 e-g). Therefore, these data imply that IL-33 not only reduces brain damage but also improves behavioral performance after HI challenge.

**ST2 deficiency exacerbates neonatal brain injury after HI**

To demonstrate an endogenous role and specificity of IL-33, we performed an equal HI injury in WT and ST2-deficient ($ST2^{-/-}$) neonatal mice. Infarct volumes were quantified at 7 d after HI using TTC staining. As shown in figure 3a, lack of ST2 expression aggravated the brain infarct sizes. Moreover, treatment with IL-33 failed to reduce the ischemic brain damage in neonatal $ST2^{-/-}$ mice. $ST2^{-/-}$ mice displayed significantly increased cerebral accumulations of TUNEL$^+$ Neun$^+$ cells after HI compared with their WT littermates (Fig. 3b). Meantime, sensorimotor tests such as the geotaxis reflex (Fig. 3c), cliff avoidance (Fig. 3d) and grip (Fig. 3e) consistently revealed worse neurological performance in $ST2^{-/-}$ mice up to 7 d after HI. The sham WT and sham $ST2^{-/-}$ mice exhibited no significant differences in any behavioral tests, suggesting equivalent baselines in both genotypes (data not shown). Together, these data indicate that the receptor ST2 is crucial for IL-33 exerting the neuroprotective effect after brain HI injury.

**ST2 is highly expressed on astrocytes under physiological and pathological conditions**

To determine the cellular targets of IL-33/ST2 signaling in the brain, we used flow cytometry to analyze the expression of the ST2 receptor on different CNS cells in the normal and the ischemic brain at 3 d after HI induction. In normal brains, ST2 was mainly expressed in GFAP$^+$ astrocytes. After brain HI, the expression of ST2 was dramatically increased in this population (Fig. 4 a-e). Immunohistochemical staining confirmed the
expression of ST2 on GFAP⁺ astrocytes 3 d after HI (Fig. 4f). There was no expression of
ST2 in neurons before or after HI (Fig. 4). Together, the data gathered thus far suggest
that HI increases the expression of the ST2 receptor on brain astrocytes, which may
modulate the astrocyte activity in an autocrine manner during HI injury.

**IL-33 enhances astrocyte survival and proliferation after HI injury**

As astrocytes express high levels of ST2 under normal conditions and after HI (Fig. 4), we
assessed the direct effects of IL-33 on astrocyte survival *in vitro*. Primary astrocytes were
subjected to 6 h oxygen-glucose deprivation (OGD) and treated with different
concentrations of IL-33 or vehicle (PBS). Flow cytometry analysis demonstrated high
expression of ST2 on astrocytes, which was further upregulated after OGD for another 24
h culture (Fig. 5a). Astrocyte survival was measured 24 h later using CCK-8 assay. There
was a dramatic decrease in astrocyte viability after OGD insult, which was significantly
rescued by IL-33 after treatment at concentrations ranging from 25 to 100 ng/ml (Fig. 5b).
The maximal efficacy of IL-33 was achieved at 50 ng/ml, which was used for all
subsequent *in vitro* experiments.

PI3K/Akt pathway is known as to be the pro-survival and proliferation cell signaling. Here,
we asked whether IL-33-mediated astrocyte protection involved PI3K/Akt pathway. IL-33
administration of mice for 3 d after brain HI obviously increased counts of Ki-67⁺
astrocytes in the periinfarct areas compared to PBS-treated HI mice (Fig. 5c). In OGD
astrocytes, IL-33 treatment increased AKT phosphorylation corresponded with elevated
cell proliferation (Fig. 5 d and e). Treatment of OGD astrocytes with Ly294002, a PI3K
inhibitor, blocked IL-33-mediated AKT phosphorylation and inhibited cell proliferation in
response to IL-33 (Fig. 5 d and e). OGD astrocytes treated with IL-33 and Ly294002 also
showed apparently reduced cell cycling compared to astrocytes treated with IL-33 alone
(Fig. 5f). All together, these data suggest that IL-33 treatment facilitates astrocyte cycling and cell proliferation, which is related to, at least in part, activation of the PI3K-AKT pathway.

**IL-33 inhibits astrocyte apoptosis by repression of PUMA**

We further investigated whether the beneficial effect of IL-33 on astrocyte survival is associated with inhibition of cell apoptosis after HI injury. At 24 h after OGD challenge, IL-33-treated cultures contained twofold decreased numbers of annexin-positive astrocytes compared to vehicle cultures (Fig. 6a). C57BL/6 mice subjected to brain HI and then treated with IL-33 for 3 d exhibited more than twofold decreased numbers of TUNEL-positive astrocytes compared to PBS-treated controls (Fig. 6b).

Growing evidence showed that PUMA is involved in ischemia/reperfusion-induced cerebral astrocyte apoptosis [23]. Because IL-33 improved astrocyte survival after HI, we explored whether IL-33 causes such effects through repression of PUMA. An increase in p53 mRNA and protein was observed in astrocytes subjected to OGD, which was markedly decreased by IL-33 treatment (Fig. 6c). IL-33 repressed OGD-induced astrocyte apoptosis but had no effect on those pretreated with Pifithrin-α (PFTα), a p53 inhibitor (Fig. 6d). PUMA expression elevated remarkably in astrocytes subjected to OGD, where PUMA mRNA and protein expression did not change in PFTα-pretreated OGD astrocytes, indicating that PUMA induction in astrocytes is p53 dependent (Fig. 6e). IL-33 treatment inhibited OGD-induced PUMA expression in astrocytes but had no effect on PFTα-pretreated astrocytes (Fig. 6e).

Primary astrocytes with specific PUMAα knockdown showed a lower percentage of apoptotic cells and increased cell survival at 24 h after OGD compared to PUMA-expressing astrocytes (Fig. 6 f and g). IL-33 treatment increased astrocyte survival and proliferation in PUMA-expressing astrocyte cultures after OGD but had no effect on
astrocyte survival or proliferation in PUMA deficient astrocyte cultures after OGD (Fig. 6 f and g). All together, these data suggest that IL-33 treatment inhibits OGD-induced astrocyte apoptosis, which is dependent on suppression of PUMA.

**ST2 signaling in astrocytes is essential for their neuroprotective effect *in vitro***

Finally, we used neuron cultures to explore the potential mechanisms of IL-33-afforded neuroprotection. Primary neurons were subjected to 3 h OGD and then treated with different concentrations of IL-33 or vehicle (PBS). As shown in figure 7a, neuronal viability, as measured by the CCK-8 assay, was significantly decreased 24 h after OGD challenge. However, treatment with IL-33 offered no protection to OGD neurons compared with vehicle treatment.

We sought to investigate whether activation of IL-33/ST2 signaling in astrocytes preserves ischemic neurons *in vitro*. Astrocytes were subjected to 6 h OGD in the presence or absence of IL-33, and then conditioned media (CM) from OGD astrocytes were collected after 24 h normoxic culture. These CM were added to the neuron-enriched cultures that had been exposed to 3 h OGD. Intriguingly, treatment with IL-33-CM obviously enhanced neuronal survival and abrogated neuronal apoptosis when compared with PBS-CM treatment 24 h after OGD challenge, as demonstrated by CCK-8 and TUNEL assay (Fig. 7 b and c).

As neurotrophic factors are essential environmental cues to neuronal survival [24], we here assessed whether neurotrophic factors are critical protective mediators released by IL-33-treated astrocytes. The expression of glial-derived neurotrophic factor family ligands (GFL) such as GDNF, ARTN, PSPN and NRTN were measured by real-time PCR. There was a decrease in the levels of astrocyte GFL after 6 h OGD, which was significantly upregulated after IL-33 treatment (Fig. 7d). Moreover, flow cytometry showed that IL-33-CM reversed the decreased expression of the GFL receptor, namely the tyrosine kinase receptor RET,
on neurons caused by 3 h OGD challenge (Fig. 7e). Treatment with IL-33-CM apparently increased neuronal survival and proliferation in RET-expressing neuron cultures after OGD but had no effect on neuronal survival or proliferation in RET-inhibited neurons after OGD challenge (Fig. 7 f and g). Together, these studies strongly suggest that activation of the IL-33/ST2 axis in astrocytes promotes the production of neurotrophic factors, which in turn protect neurons from ischemic injury.

Discussion

There is a series of studies that IL-33 is released from CNS cells quickly after damage and subsequently triggers the activation of immune responses in lesion areas [25, 26]. In line with these observations, the current study identified that the levels of IL-33 were rapidly elevated in the lesion site 1 d after neonatal HI brain injury. And enhanced IL-33 expression was predominantly localized to astrocytes after HI insult. These findings prompted us to explore the effect of the IL-33/ST2 signaling in neonatal HI brain injury. We found that systemic delivery of IL-33 remarkably alleviated HI-mediated cerebral damage, whereas loss of ST2 aggravated brain infarction and neurological deficits. The activation of IL-33/ST2 signaling in the ischemic brain improved astrocyte response, which in turn afforded protection to ischemic neurons in a glial-derived neurotrophic factor-dependent manner.

Astrocytes are the most abundant type of glial cell within the mammalian brain, which activity is crucial for neuroprotection during brain ischemic damage [27]. Increment studies show that ischemia can induce cell-cycle arrest or apoptotic cell death of cortical astrocytes in infarct area [28]. Ischemia-induced growth arrest of astrocytes can be overridden by inhibition of regulator of G protein signaling 2 (RGS2) and treatment with cytokine erythropoietin (EPO) [28, 29], and cytokine-mediated induction of astrocyte survival early post ischemia may contribute to neuroprotective effect [30]. Although the
precise mechanism behind these effects remains unclear, these treatments may induce synchronous entry of cells into late S phase that is associated with apoptotic resistance [28, 31]. In nonhematopoietic tissues, IL-33 receptor ST2 has been reported to modulate cell proliferation through activation of the PI3K-AKT pathway [32]. Consistent with these findings, we here observe that cerebral astrocytes express high levels of ST2 under normal conditions and even higher levels upon HI challenge. Exogenous administration of IL-33 apparently improves astrocyte survival and proliferation after HI damage. Interestingly, IL-33 attenuates HI-induced growth arrest by promoting astrocyte cycling and that this effect is dependent on activation of the PI3K-AKT signaling. These results suggest that IL-33-mediated upregulation of astrocyte viability provides beneficial neuroprotection against neonatal HI injury.

The p53 upregulated modulator of apoptosis (PUMA) was discovered as a transcriptional target of p53 and exerted a powerful apoptosis-promoting activity [33]. PUMA activates the mitochondrial pathway via the Bcl-2 family member Bax/Bak following neutralizing all members of antiapoptotic Bcl-2 like molecules [34]. Recent studies show that blocking of PUMA signaling protects astrocytes against ischemic-caused death and may confer a significant neuroprotection to brain ischemic/reperfusion injury [35]. In this study, we demonstrate that PUMA is a powerful executor of p53-mediated apoptosis in cerebral astrocytes after HI insult. IL-33 treatment represses HI-induced upregulation of PUMA and downstream apoptotic protein in reactive astrocytes. Furthermore, the effects of IL-33, mediating apoptotic resistance in astrocytes, are dependent largely on repression of PUMA transcription. These findings are consistent with the recently published results showing that inhibition of aberrant p53-PUMA feedback loop activation by treatment with PUMA-specific siRNA and inhibitor pifithrin-α prevented ischemia reperfusion-induced neuroapoptosis and inflammatory responses [36].
It has become increasingly evident that astrocytes play a crucial role in neuron survival during brain ischemia/reperfusion injury [37]. Ischemic lesion in the brain stimulates enhanced expression and release of neurotrophic factors from reactive astrocytes supplying the neurons with appropriate mediators necessary for their survival and maintenance [38]. Therefore, it is critical to determine the specific effectors or functions of the IL-33-stimulated astrocyte response. In line with this goal, our mechanistic studies have identified the glial-derived neurotrophic factor (GDNF) family ligands (GFL) as key molecules released by IL-33-stimulated astrocytes and the major neuroprotective factors in the ischemic brain. The conditioned media derived from IL-33-stimulated astrocytes significantly activate the GFL receptor, a tyrosine kinase receptor RET, and protect neuron survival against ischemic insult. On the contrary, inhibition of the RET receptor almost completely abrogated the protective effects of IL-33 in ischemic neuron culture. In agreement with these data, recent studies demonstrated that neurotrophic factors play a critical role in the survival, differentiation, and maintenance of neurons [39]. Exogenous delivery of neurotrophic factor artemin can have protective effects in animal models of experimental nerve-injury, as measured by increased regeneration and functional recovery, and transient reductions in hyperalgesia following nerve-injury [40, 41]. Therefore, the capacity of IL-33 to induce neurotrophic factor production in astrocytes may serve as an important mediator of IL-33-afforded CNS protection.

Conclusions

The current study reveals a previously unknown function of IL-33/ST2 signaling in neuroprotective effect during neonatal HI brain injury. The activation of IL-33/ST2 axis in the ischemic brain improves astrocyte response, which in turn affords protection to ischemic neurons in a neurotrophic factor-dependent manner. Translationally, these findings imply that IL-33/ST2 signaling may represent a potent new immunotherapy for
neonates with hypoxic ischemic encephalopathy.

Declarations

Acknowledgement

Not applicable.

Authors' contributions

Mengya Jiao, Xiangyong Li, Xiaodi Wang, Baohong Yuan and Qun Dong performed the research. Liying Chen and Tao Liu analysed the data. Hanfang Mei and Hui Yin reviewed the data and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The authors declare that all the data supporting the findings of this study are available within the article and that no data sharing is applicable to this article.

Ethics approval and consent to participate

All animal studies were approved by the Animal Care and Use Committee at the Guangdong Pharmaceutical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details
Abbreviations

IL-33: Interleukine-33; CNS: Central nervous system; HIE: Hypoxic-ischemic encephalopathy; TTC: 2,3,5-triphenyltetrazolium chloride; OGD/R: Oxygen-glucose deprivation/reoxygenation; PUMA: p53 upregulated modulator of apoptosis; RT-qPCR: Real-time quantitative PCR; MOI: Multiplicities of infection; ACM: Astrocyte-conditioned media; IL-1RAcP: IL-1 receptor accessory protein; GFL: Glial-derived neurotrophic factor family ligands; RGS2: Regulator of G protein signaling 2; EPO: Erythropoietin; GDNF: glial-derived neurotrophic factor

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Figures
Astrocytic IL-33 is robustly upregulated after hypoxic-ischemia in neonatal mouse brains. A Western blot of IL-33 was performed using protein extracted from the mouse brain at 1, 3, 7 and 14 d after HI or sham operation. Data are mean ± SEM (n = 6 in each group). # P < 0.01 compared to sham. B Representative images of IL-33 (green) and GFAP or Olig2 (red) labeling in the cerebral cortex of WT mice.
at 1 d after HI injury. No IL-33 staining was observed in Neun or Iba1 cells 1 d after HI. Scale bar, 25 μm. Nuclei were stained blue with 4,6-diamidino-2-phenylindole (DAPI). c Semi-quantitative analysis of glia or neuron type-cell contributions to the IL-33-positive cell population in vehicle group 1 d after HI. Data are mean ± SEM (n = 6 in each group). *P < 0.05 compared to sham. d Representative IL-33 expression on astrocytes in WT mice at 1 d after HI injury. The numbers shown indicate the percentage of IL-33 expression on the indicated cell population. Data are mean ± SEM (n = 6 in each group). *P < 0.05 compared to sham.
Figure 2

IL-33 ameliorates HI-induced brain injury in neonatal mice. a Representative photographs of TTC-stained coronal brain sections from IL-33- and PBS-treated mice at 7 days post HI (left). Right, infarct volumes at 7 days post HI (n = 12 per group). b Representative western blot images of cleaved caspase-3 (C-casp-3), Bax and Bcl-2 from IL-33- and PBS-treated mice at 7 days post HI (n = 9 per group). c Densitometric analysis of data is shown in b. d Representative images of NeuN (red) and TUNEL (green) in the cerebral cortex of IL-33- and PBS-treated mice at 7 days post HI (left). Scale bar, 25 μm. Right, quantification of NeuN and TUNEL dual-labeled cells at 7 days post HI (n = 9 per group). e-g Neurobehavioral outcomes of the geotaxis reflex (left), cliff avoidance reaction (middle) and grip test (right) at 1, 3 and 7 days after HI (n = 9 per group). Data are mean ± SEM. * P < 0.05 compared to untreated controls.
Figure 3

ST2 deficiency aggravates HI-induced brain injury in neonatal mice. a TTC staining and infarct volumes in WT and ST2-/- mice treated with or without IL-33 at 7 days post HI (n = 12 per group). b Representative images of Neun (red) and TUNEL (green) from WT and ST2-/- mice at 7 days post HI (left). Scale bar, 25 μm. Right, quantification of Neun and TUNEL dual-labeled cells at 7 days post HI (n = 9 per group). c-e Neurobehavioral outcomes of the geotaxis reflex (left), cliff avoidance reaction (middle) and grip test (right) at 1, 3 and 7 days after HI (n = 9 per group). Data are mean ± SEM. * P < 0.05 compared to WT. n.s. not significant.
ST2 expression on astrocytes is elevated after HI. a Histograms showing ST2 expression on astrocytes, oligodendrocytes, microglia and neurons in the brain 3 d after HI or sham operation. The blue and red lines represent staining in sham and HI brains, respectively, at 3 d after surgery. The gray area represents isotype control staining. b–e Percentages of ST2 expression on astrocytes (b), oligodendrocytes (c), microglia (d) and neurons (e) in sham brain and HI brain at 3 d after surgery. Data are mean ± SEM (n = 5-7 per group), * P < 0.05 compared to sham. f Representative images of GFAP (green) and ST2 (red) labeling in the brain 3 d after HI. Scale bar, 25 μm. Nuclei were stained blue with DAPI.
IL-33 promotes astrocyte survival and proliferation after HI injury. a Representative FACS analysis of ST2+ astrocytes at 24 h after 6 h OGD. Right, numbers indicate the percentage of ST2+ astrocytes at 24 h after 6 h OGD. Data are mean ± SEM (n = 3 in each group). * P < 0.05 compared to untreated controls. b CCK-8 assay in astrocyte-enriched cultures subjected to 6 h OGD or sham conditions followed by treatment with PBS or a range of concentrations of IL-33 for another 24 h. Data are mean ± SEM (n = 3 in each group). # P < 0.01 compared to untreated controls. c Representative images of Ki-67 (red) and GFAP (green) in the cerebral cortex of IL-33- and PBS-treated mice at 7 days post HI (left). Scale bar, 25 μm. Right, quantification of Ki-67 and GFAP dual-labeled cells at 7 days post HI (n = 9 per group). d and e Percentages of phosphorylated AKT (pAKT) (d) and Ki-67+ (e) in astrocytes at 24 h after 6 h OGD and the culture
IL-33 protects astrocytes from apoptosis after OGD insult. a The percentage of annexin+ astrocytes at 24 h of culture with or without IL-33 and after 6 h OGD and the culture conditions shown. Data are mean ± SEM (n = 3 in each group). * P < 0.05 compared to untreated controls. b The percentage of TUNEL+ astrocytes from IL-33- and PBS-treated mice at 7 days post HI. Data are mean ± SEM (n = 6 in each group). * P < 0.05 compared to untreated controls. c Left, P53 mRNA expression in astrocytes at 12 h of culture with or without IL-33 and after 6 h
OGD and the culture conditions shown. Right, mean percentages of P53 protein expression in astrocytes at 24 h of culture with or without IL-33 and after 6 h OGD and the culture conditions shown. Data are mean ± SEM (n = 3 in each group). * P < 0.05 compared to untreated controls. 

d The percentage of annexin+ astrocytes with or without P53 inhibition at 24 h of culture with or without IL-33 and after 6 h OGD and the culture conditions shown. Data are mean ± SEM (n = 3 in each group). * P < 0.05 compared to untreated controls. n.s. not significant. 

Left, PUMA mRNA expression in astrocytes at 12 h of culture with or without IL-33 and after 6 h OGD and the culture conditions shown. Right, mean percentages of PUMA protein expression in astrocytes at 24 h of culture with or without IL-33 and after 6 h OGD and the culture conditions shown. Data are mean ± SEM (n = 3 in each group). * P < 0.05 compared to untreated controls. n.s. not significant. 

f The percentage of annexin+ astrocytes with or without PUMA knockdown at 24 h of culture with or without IL-33 and after 6 h OGD and the culture conditions shown. Data are mean ± SEM (n = 3 in each group). * P < 0.05 compared to untreated controls. n.s. not significant. 

g Ki-67 assay in astrocytes with or without PUMA knockdown at 24 h of culture with or without IL-33 and after 6 h OGD and the culture conditions shown. Data are mean ± SEM (n = 3 in each group). * P < 0.05 compared to untreated controls. n.s. not significant.
Figure 7

ST2 signaling in astrocytes is essential for their neuroprotective effects in vitro. a CCK-8 assay in neuron-enriched cultures subjected to 3 h OGD or sham conditions followed by treatment with PBS or a range of concentrations of IL-33 for another 24 h. Data are mean ± SEM (n = 3 in each group). n.s. not significant. b-c CCK-8 assay (b) and TUNEL measure (c) in neurons at 24 h of culture with CM from untreated OGD astrocytes (PBS-CM) or CM from IL-33-treated OGD astrocytes (IL-33-CM) and after 3 h OGD and the culture conditions shown. Data are mean ± SEM (n = 3 in each group). * P < 0.05 compared to untreated controls. d mRNA expression of glial-derived neurotrophic factors GDNF, ARTN, PSPN and NRTN in astrocytes at 24 h of culture with or without IL-33 and after 6 h OGD and the culture conditions shown. Data are mean ± SEM (n = 3 in each group). * P < 0.05 compared to untreated controls. e Mean percentages of RET protein expression in
neurons at 24 h of culture with PBS-CM or IL-33-CM and after 3 h OGD and the culture conditions shown. Data are mean ± SEM (n = 3 in each group). * P < 0.05 compared to untreated controls. f-g Percentages of phosphorylated AKT (pAKT) (f) and Ki67+ (g) in neurons with or without RET inhibition at 24 h of culture with PBS-CM or IL-33-CM and after 3 h OGD and the culture conditions shown. Data are mean ± SEM (n = 3 in each group). * P < 0.05.