S100A9-containing serum exosomes obtained from patients with burn injuries promote myocardial cell pyroptosis through NLRP3

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Abstract. S100 calcium-binding protein A9 (S100A9) is highly expressed in the serum exosomes of patients with burn injuries. The present study aimed to investigate the underlying mechanisms of burn injury-associated exosomes in the regulation of myocardial cell pyroptosis. Reverse transcription-quantitative PCR and western blotting were used to examine relative mRNA and protein levels. The morphology of exosomes was visualized using transmission electron microscopy. The expression levels of IL-1β and IL-18 in cells were examined using ELISA kits. Finally, cell pyroptosis was examined using flow cytometry. When AC16 cells were treated with the serum exosomes obtained from patients with burn injuries, pyroptosis was significantly promoted and the expression levels of IL-1β and IL-18 were increased. NLR family pyrin domain containing 3 (NLRP3), S100A9, caspase-1 and Gasdermin D (GSDMD)-N expression levels were also upregulated. However, these were significantly reversed by anti-S100A9 antibodies. Thereafter, CY-09, an NLRP3 inhibitor, was revealed to restore the increase in pyroptosis and IL-18, IL-1β, caspase-1, NLRP3 and GSDMD-N expression levels caused by recombinant S100A9 to be similar to the control. These findings suggested that burn injury-associated exosomes containing S100A9 can affect AC16 cell pyroptosis through NLRP3.

Introduction

Burn injuries are a severe form of trauma, and they are a global public health problem that causes an estimated 265,000 deaths each year in China (1). Burn injuries are not a single pathophysiological event; they are destructive injuries that lead to structural and functional deficits in various organ systems (2). Cardiac dysfunction induced by burn injury, such as cardiogenic shock, which often appears early after a burn injury, contributes to multiple organ failure, sepsis and death (3,4). Despite the significant advances in the treatment of patients with burn injuries, systemic and burn wound-related complications are still common (5-7). Therefore, it is important to further explore the pathogenesis of burn complications and to find therapeutic targets and pathways for burn injury treatment.

Exosomes are small extracellular vesicles that can serve as carriers of proteins, DNA and RNA. Exosomes, as important mediators of cell-to-cell communication, have attracted much attention (8). Growing evidence has revealed that substances carried by exosomes can be transported into target cells (9,10). They have notable roles in multiple biological processes and signaling pathways, including cell proliferation, migration, invasion and apoptosis (11). A previous study has demonstrated that microRNA-181c in exosomes derived from human umbilical cord mesenchymal stem cells reduces burn-induced inflammation (12). A previous study also indicated that S100 calcium-binding protein A9 (S100A9) is highly expressed in the serum exosomes isolated from patients with burn injuries (13). S100A9 is a small calcium-binding protein that is hypothesized to be an alarmin released by stressed cells. It is also an endogenous danger signal that promotes and exacerbates inflammatory responses (14). However, the detailed function of S100A9 in burn injury progression remains unclear.

Inflammation, as a self-protective mechanism of the host organ from pathogens, plays a notable role in infectious and non-infectious burn injuries (15). Inflammatory responses are necessary to initiate tissue repair and immune response modulation to improve the recovery of patients with severe burn injuries (16). Pyroptosis is a type of programmed cell death mediated by the gasdermin family. It is accompanied by inflammatory and immune responses (17), and can also regulate cell death depending on the enzymatic activity of inflammatory proteases (18,19), which are members of the cysteine-dependent aspartate-specific protease (caspase) family. Gasdermin D (GSDMD) is an important pyroptosis substrate. Caspase-1, which belongs to the caspase family, has been reported to be widely expressed in humans and mice, and it is closely associated with cell pyroptosis (20-22).

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NLRs are proteins that elicit an inflammatory response through extracellular and intracellular changes. Among them, NLR family pyrin domain containing 3 (NLRP3) is the most characteristic inflammasome. As a redox-sensitive cytosolic sensor, NLRP3 recruits and triggers the formation of adaptor protein apoptosis-associated speck-like proteins; it also activates pro-caspase-1, which processes pre-IL-18 and pre-IL-1β into maturity (23,24). Furthermore, the biochemical function of NLRP3 is to activate caspase-1, which leads to the maturation of IL-1β and IL-18, thereby inducing cell pyroptosis (25). However, the roles of serum exosomes in cell pyroptosis and its associated underlying mechanisms in burn injuries remain unclear. Therefore, the detailed function of S100A9 in this process needs to be further explored.

In the present study, exosomes were isolated from the serum of patients with burn injuries and were used to treat human myocardial cells. Afterwards, S100A9 antibodies and CY-09, an NLRP3 inhibitor, were introduced to explore the underlying molecular mechanisms. These findings provide evidence towards novel therapeutic targets and pathways for burn injury treatment.

Materials and methods

Isolation and characterization of serum exosomes. Patients with burn injuries (III-degree burns) were recruited from Seventh People's Hospital Affiliated to Shanghai University of Traditional Chinese Medicine (Shanghai, China) from June 2017 to October 2019, including (27 males and 13 females; age range, 37-67 years; mean age, 52.1±6.0 years) and 5 ml of venous blood was collected from each patient. The present research protocol was approved by the Ethics Committee of Shanghai Seventh People's Hospital (approval no. 2018-HIRB-046), and written informed consent was obtained from all patients. The venous blood was centrifuged at 1,409 x g for 8 min at 4˚C and the serum was obtained. Differential centrifugation was used to isolate exosomes from the serum. First, the serum was centrifuged at 10,000 x g for 30 min at 4˚C. Afterwards, the supernatant was transferred to a new 5-ml ultrahigh speed centrifuge tube and centrifuged at 17,000 x g for 2 h at 4˚C. This step was repeated three times. After removing the supernatant, the sediment was resuspended in 200 µl of phosphate-buffered saline, filtered using a 0.22-um filter and stored at -80˚C for further analysis.

The concentration of exosomes was measured using a BCA Protein Concentration Assay kit (Wuhan Boster Biological Technology, Ltd.), following the manufacturer's instructions. The morphology of exosomes was visualized using transmission electron microscopy (TEM; FEI Tecnai 12; Philips Healthcare), as previously described (26). Afterwards, based on the method of Yin *et al* (27), the protein expression levels of CD63, CD9 and TSG101, which are specific proteins, were determined using western blotting with their corresponding antibodies as described below.

Cell culture. Human myocardial cell AC16 cells were obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. The cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin in an incubator set at 37˚C with 5% CO_2.

Cellular uptake of exosomes in AC16 cells. Serum exosomes were labeled with PKH67 using a PKH67 Green Fluorescent Cell Linker kit (Sigma-Aldrich; Merck KGaA), following the manufacturer's protocols. Briefly, exosomes diluted in 1.5 ml of Diluent C were incubated with 6 µl of PKH67 dye at room temperature. After being incubated for 5 min, the exosomes were incubated with 3 ml of ultracentrifuged FBS for 1 min at 37˚C to allow the binding of the excess dye. After being washed with 15 ml of keratinocyte serum-free medium (KSFM; Invitrogen; Thermo Fisher Scientific, Inc.), the mixture was centrifuged at 100,000 x g for 75 min at 4˚C, and the sediment (PKH67-labeled exosomes) was resuspended with KSFM for use.

The AC16 cells were seeded into a 24-well plate at a density of $3x10^4$ per well and were cultured overnight at 37˚C. Thereafter, 10 µg/ml PKH67-labeled exosomes were added to the cells. After 24 h of incubation at 37˚C, the AC16 cells were fixed with 4% paraformaldehyde for 10 min at 37˚C. After washing, the cells were stained with 4,6-diamidino-2-phenylindole and were observed under a fluorescence microscope (magnification, x400; Olympus IX71; Olympus Corporation).

Enzyme-linked immunosorbent assay (ELISA). The expression levels of IL-1β and IL-18 in the cells were examined using a rat IL-18 ELISA kit (cat. no. E-EL-R0567c) and rat IL-1β ELISA kit (cat. no. E-EL-R0012c) (both from Elabscience Biotechnology, Inc.), following the manufacturer's protocol. Briefly, IL-1β and IL-18 antibodies were applied to the AC16 cells at 37˚C for 2 h. After rinsing off the washing solution, secondary antibodies were applied. Subsequently, the stop solution was added, A microplate absorbance reader (Tecan Group, Ltd.) was used to measure the absorbance at 450 nm. Standard curves were applied to calculate the concentrations of analytes. Each reaction was carried out three times.

Evaluation of pyroptosis by flow cytometry. The AC16 cells in different treatments as indicated below were harvested and washed with ice-cold PBS. Afterwards, the AC16 cells were stained with activated caspase-1 antibodies (FLICA® 660 Caspase-1 Assay; cat. no. 9122; Bio-Rad Laboratories, Inc.) and propidium iodide (PI; Thermo Fisher Scientific, Inc.). Cell pyroptosis (activated capase-1 and PI double-positive) was analyzed using flow cytometry (BD Biosciences).

Western blotting. Total AC16 cell lysates were prepared using a radioimmunoprecipitation buffer containing a proteinase inhibitor (Beyotime Institute of Biotechnology). Protein levels were quantified using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Protein samples (20 µg per lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to nitrocellulose membranes (MilliporeSigma). After being blocked with 5% skim milk at room temperature for 2 h, the membranes were incubated with primary antibodies overnight

at 4˚C (Table SI). After being washed with PBST (0.05% Tween) for three times, the membranes were incubated with HRP-conjugated rabbit secondary antibodies (1:10,000; cat. no. ZB-2301; OriGene Technologies, Inc.) at room temperature for 1 h. Finally, band intensity was semi-quantified via densitometry analysis using Quantity-One software v4.62 (Bio-Rad Laboratories, Inc.).

Statistical analysis. Statistical analyses were performed using Prism 7.0 (GraphPad Software, Inc.). Data are presented as the mean ± standard deviation of the three replicates. Comparisons among multiple independent groups were analyzed using Kruskal-Wallis test followed by Dunn's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of exosomes. To identify the exosomes isolated from the serum of patients with burn injuries, TEM and western blotting were performed. TEM results revealed that exosomes exhibited a cup-shaped morphology with a diameter of ~100-nm (Fig. 1A). Western blotting revealed that exosome markers CD9, CD63 and TSG101 were all expressed in the serum exosomes (Fig. 1B). These results indicated that exosomes were successfully extracted from the serum of patients with burn injuries using a differential centrifugation method.

Serum exosomes were also labeled with PKH67 (green fluorescence) and were co-cultured with AC16 cells for 24 h. Most AC16 cells exhibited a green fluorescence (Fig. 1C). These results indicated that the serum exosomes extracted from patients with burn injuries could be taken up by human myocardial AC16 cells.

Serum exosomes from patients with burn injuries promote AC16 cell pyroptosis. To further investigate the effects of serum exosomes from patients with burn injuries on AC16 cell pyroptosis, active caspase-1 levels were measured. The pyroptosis of AC16 cells with exosome treatment was significantly increased with increasing culture duration (P<0.01; Fig. 2A). Increasing culture duration also significantly elevated the IL-18 and IL-1β contents in exosome-treated AC16 cells (P<0.01; Fig. 2B). Furthermore, western blotting revealed that S100A9, active caspase-1, NLRP3, pro-caspase-1 and GSDMD-N expression levels were all markedly upregulated in AC16 cells treated with serum exosomes after being cultured for 12, 24 and 48 h compared with those in cells treated with exosomes for 0 h (Fig. 2C). These results indicated that the serum exosomes from patients with burn injuries might promote the pyroptosis of human myocardial cells by upregulating the expression levels of S100A9, NLRP3, caspase-1 and GSDMD-N.

Exosome-induced pyroptosis is reversed by anti-S100A9 antibodies. To explore the role of S100A9 in burn injuries, anti-S100A9 antibodies were used to treat exosome-induced cell injury, after which cell pyroptosis was detected. Relative to the control group, the pyroptosis of cells only treated with exosomes was significantly increased (P<0.001; Fig. 3A).

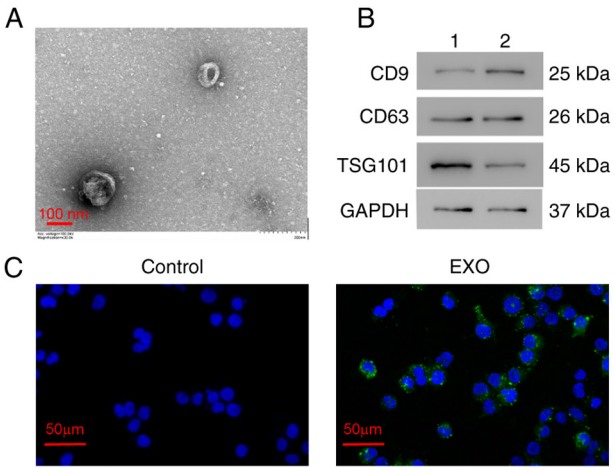

Figure 1. Identification of serum exosomes. (A) Transmission electron microscope observation of whole-mounted purified serum exosomes extracted from patients with burn injuries. Scale bar, 100 nm. (B) CD9, CD63 and TSG101 expression levels in serum exosomes based on western blotting in three independent experiments with three parallel samples. (C) PKH67-labeled serum exosomes could be taken up by AC16 cells when cocultured in three independent experiments with three parallel samples. Blue, DAPI; green, PKH67-labeled serum exosomes. Scale bar, 50 μm. EXO, exosomes.

However, after being treated with different concentrations of anti-S100A9, pyroptosis was significantly lower and gradually decreased with increasing anti-S100A9 concentrations (P<0.001; Fig. 3A). The changes in IL-18 and IL-1β contents of AC16 cells under different treatments presented a similar trend with those of cell pyroptosis in different groups (Fig. 3B). Western blotting results revealed that active caspase-1, GSDMD-N, NLRP3 and pro-caspase-1 expression levels were markedly upregulated after exosome treatment compared with the control group, whereas different anti-S100A9 concentrations markedly suppressed their expressions. The inhibitory effects became more obvious with increasing anti-S100A9 antibody concentrations (Fig. 3C). The results suggested that S100A9 inhibition could reverse the pyroptosis of human myocardial cells induced by exosomes isolated from patients with burn injuries.

NLRP3 inflammasome is involved in S100A9-induced AC16 cell pyroptosis. NLRP3 is a key factor in recruiting caspase-1, and this allows the cleavage of pro-IL-1β to its mature form (25). Therefore, the effects of NLRP3 on S100A9-mediated AC16 cell pyroptosis were further studied. It was revealed that recombinant S100A9 significantly enhanced the pyroptosis of AC16 cells (P<0.001), whereas CY-09, an NLRP3 inhibitor, significantly reversed these effects (P<0.001; Fig. 4A). The IL-18 and IL-1β contents were significantly higher in the recombinant S100A9 group compared with in the control group (P<0.001); whereas their contents in the CY-09 group were similar compared with the control group (P>0.05, Fig. 4B). Furthermore, the expression levels of active caspase-1, NLPR3, pro-caspase-1 and GSDMD-N were markedly elevated in the recombinant S100A9 group compared with the control group, while these were markedly reduced after the CY-09 treatment (Fig. 4C). These results revealed that NLRP3 suppression could alleviate the pyroptosis of human myocardial cells induced by S100A9.

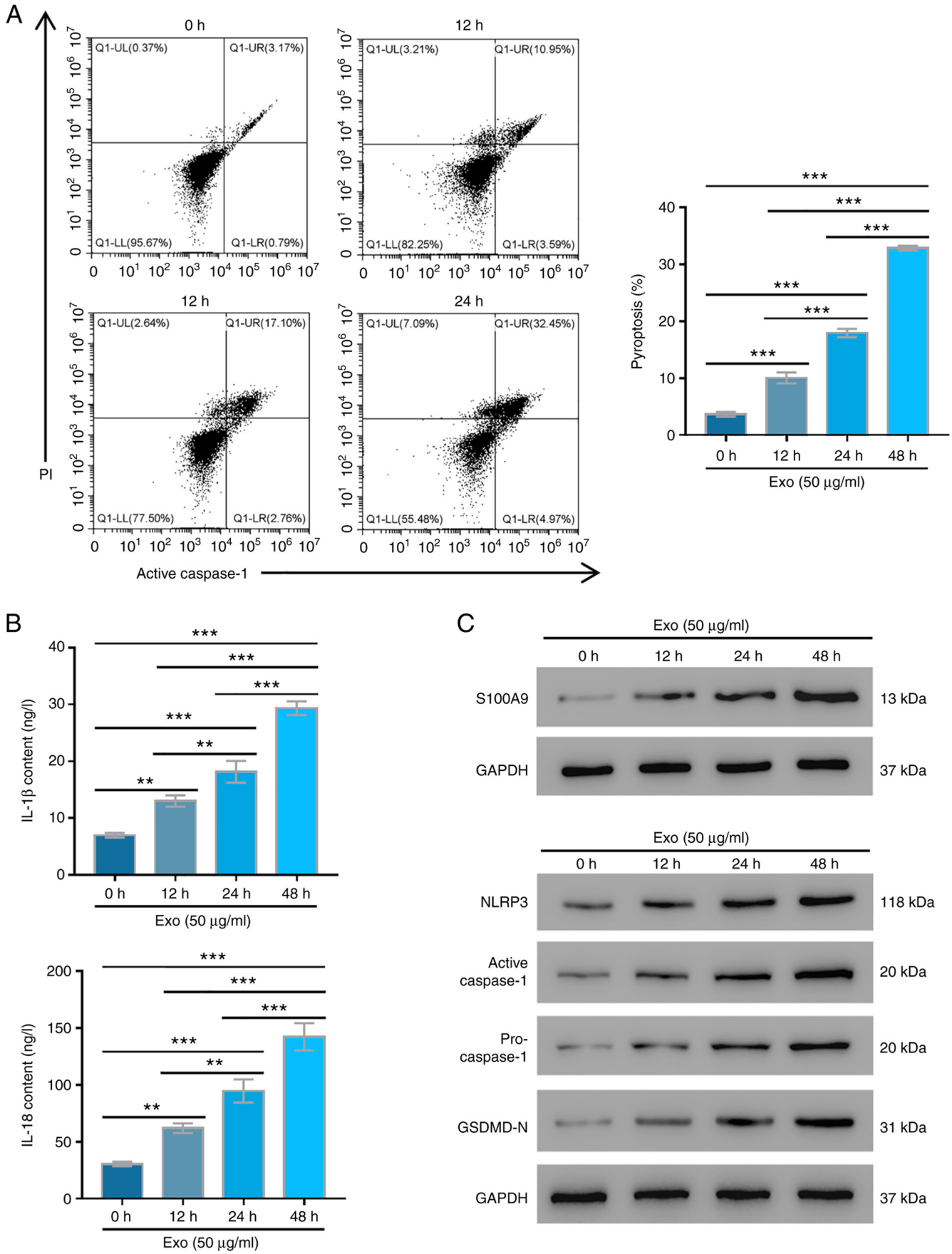

Figure 2. Serum exosomes extracted from patients with burn injuries promote pyroptosis and S100A9 expression. AC16 cells were treated with 50 μg/ml exosomes for 0, 12, 24 and 48 h. (A) Cell pyroptosis was tested by flow cytometry in three independent experiments with three parallel samples. (B) IL-1β and IL-18 contents were determined using ELISA kits in three independent experiments with three parallel samples. (C) S100A9, NLRP3, active caspase-1, pro-caspase-1 and GSDMD-N expression levels were measured using western blotting in three independent experiments with three parallel samples. **P<0.01, ***P<0.001. Exo, exosomes; S100A9, S100 calcium-binding protein A9; NLRP3, NLR family pyrin domain containing 3; GSDMD, Gasdermin D.

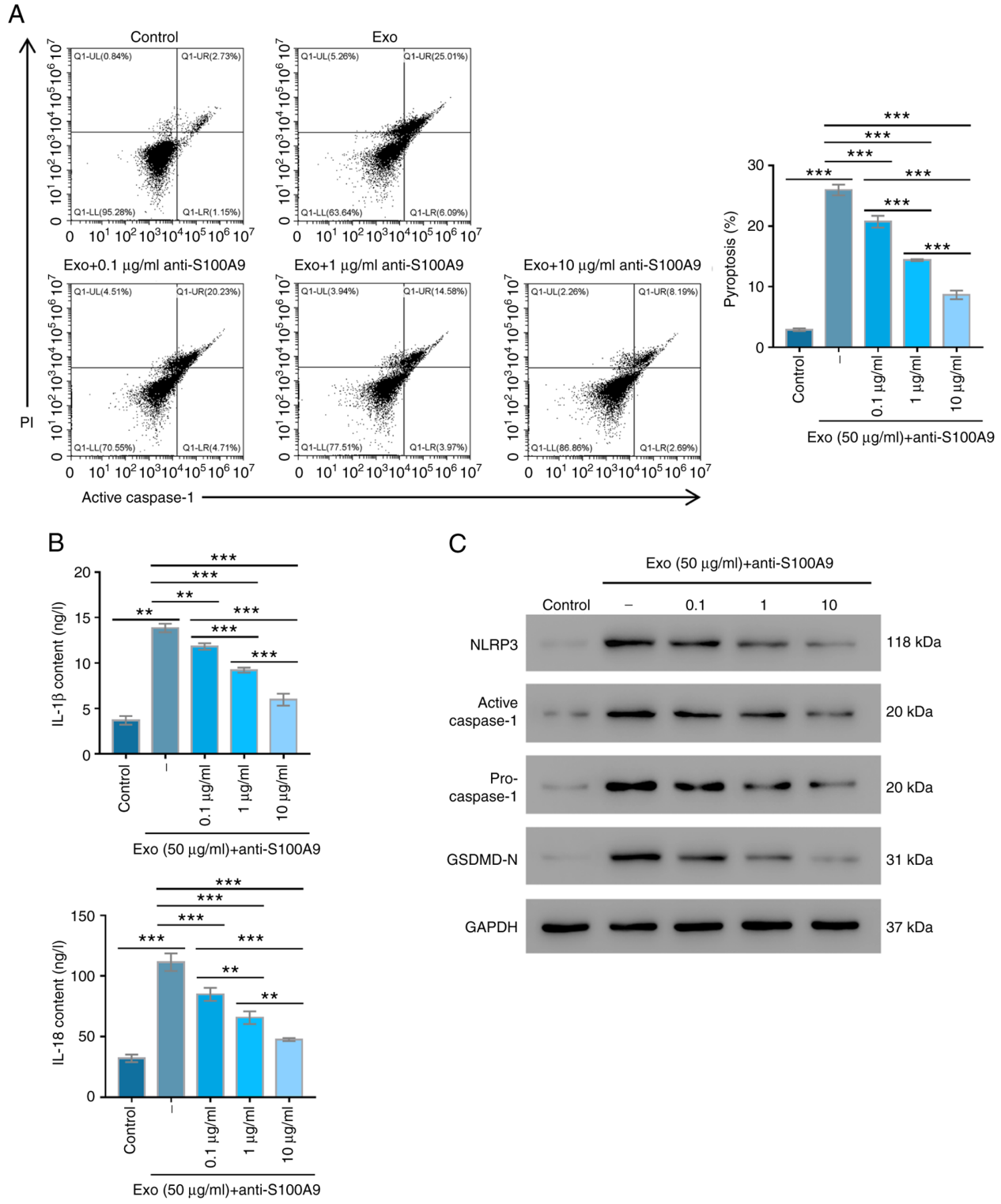

Figure 3. Anti-S100A9 protect AC16 cells from serum exosome-induced pyroptosis. (A) Pyroptosis was tested in AC16 cells treated with 50 μg/ml exosomes supplemented with 0.1, 1, or 10 μg/ml anti-S100A9 antibodies by flow cytometry in three independent experiments with three parallel samples. (B) IL-1β and IL-18 contents of AC16 cells under different treatments were determined by ELISA in three independent experiments with three parallel samples. (C) S100A9, NLRP3, active caspase-1, pro-caspase-1 and GSDMD-N expression levels were measured using western blotting in three independent experiments with three parallel samples. **P<0.01, ***P<0.001. Exo, exosomes; S100A9, S100 calcium-binding protein A9; NLRP3, NLR family pyrin domain containing 3; GSDMD, Gasdermin D.

Discussion

Burn injuries are one of the most debilitating traumas that can inflict humans, seriously affecting the individuals' health and mental health, and puts a huge burden on healthcare systems (2). Exosomes, as carriers of bioactive substances, have been reported to play important roles in various diseases, including cardiovascular and central nervous system-related

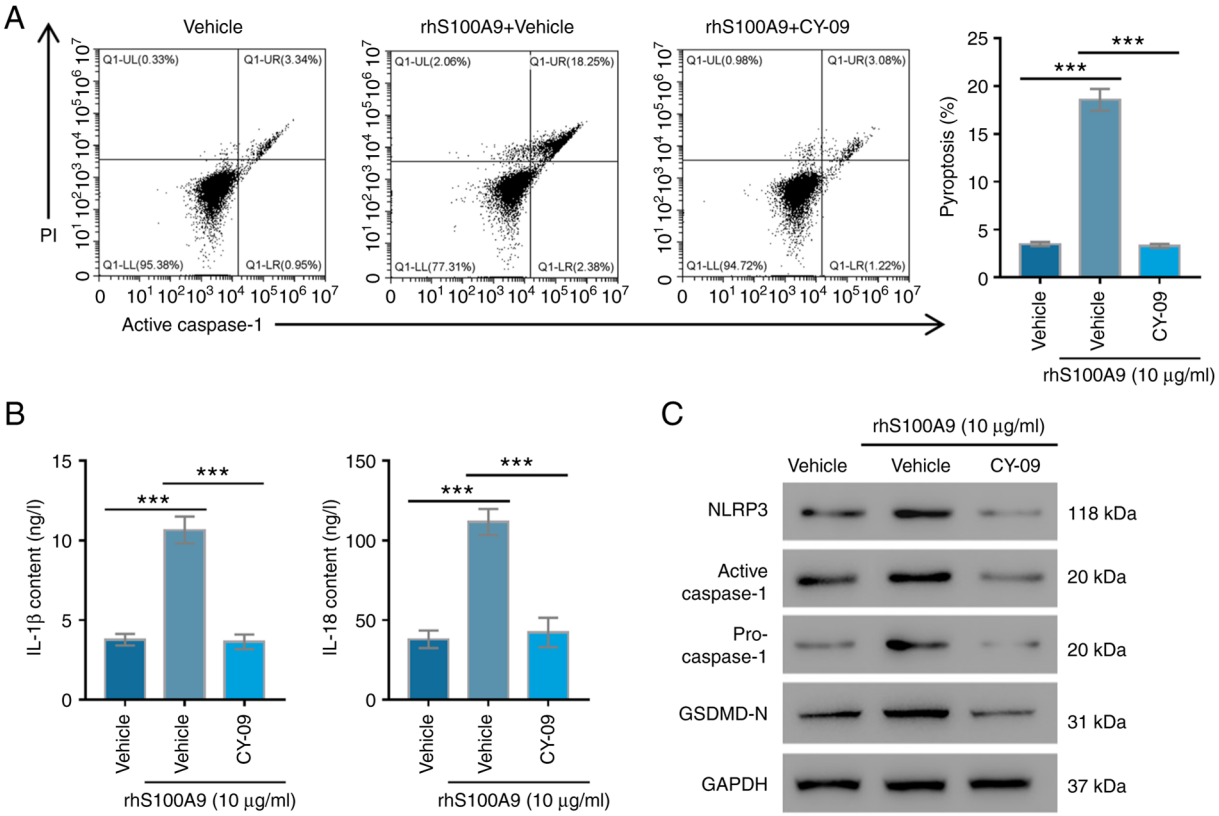

Figure 4. CY-09 protects AC16 cells from S100A9-induced pyroptosis. (A) Pyroptosis was tested in AC16 cells treated with 10 μg/ml recombinant S100A9 proteins supplemented with 1 μM CY-09 and measured using flow cytometry in three independent experiments with three parallel samples. (B) IL-1β and IL-18 contents of AC16 cells under different treatments were determined using ELISA in three independent experiments with three parallel samples. (C) S100A9, NLRP3, active caspase-1, pro-caspase-1 and GSDMD-N expression levels were detected using western blotting in three independent experiments with three parallel samples. ***P<0.001. Exo, exosomes; S100A9, S100 calcium-binding protein A9; NLRP3, NLR family pyrin domain containing 3; GSDMD, Gasdermin D.

diseases, and cancer progression (26-29). S100A9 is highly expressed in the exosomes extracted from the serum of patients with burn injuries, and it may be a potent stimulator of inflammatory responses (30). The present study revealed that burn injury-associated exosomes significantly promoted the pyroptosis of AC16 cells, and that this was significantly reversed by S100A9 antibodies. Moreover, CY-09 could restore the pyroptosis caused by recombinant S100A9. These findings indicated that burn injury-associated exosomes promoted the pyroptosis of AC16 cells by enhancing the activity of the S100A9-NLRP3 axis.

Previous studies have demonstrated that exosomes can serve as a natural carrier system that can transport RNA, DNA and proteins and participate in multiple cellular functions (31,32). In the current study, exosomes were successfully isolated from the serum of patients with burn injuries; these promoted S100A9 expression and AC16 cell pyroptosis. A previous study has indicated that S100A9 activates the NLRP3 inflammasome and promotes IL-1β release (33). Another study has indicated that S100A9 induces the formation of NLRP3 inflammasomes, activates caspase-1 and produces IL-1β and IL-18, leading to pyroptosis in myelodysplastic syndrome cells (34). Thus, it can be concluded that burn injury-associated exosomes promote AC16 cell pyroptosis by regulating the S100A9/NLRP3 pathway.

S100A9, a member of the S100 protein family, can increase the metabolism of the cytoskeleton, enhance the migration of phagocytes and inhibit the development of microbes (35-37). S100A9 overexpression was observed in a number of inflammation-related diseases, including sepsis, acute pancreatitis, inflammatory bowel disease and myocardial infraction (38). Isaacs *et al* (39) indicated that tasquinimod can bind with S100A9, inhibiting the interaction between S100A9 and Toll-like receptor 4 and repressing the TNF-α release. Furthermore, S100A9 has been identified to be a novel clinical target for cardiovascular diseases, including aneurysms and acute coronary syndrome (40). To the best of our knowledge, this is the first study to show that S100A9 secreted by burn injury-associated exosomes could regulate the progression of human myocardial cell pyroptosis and its potential value as a novel target for burn injury therapy. However, the lack of data to obtain in *in vivo* assays limited the significance of these findings. Therefore, further examination should be conducted *in vivo*. Nevertheless, the present data illustrated the notable role of S100A9 and its potential role as a target for burn injury treatment.

In conclusion, burn injury-associated exosomes containing S100A9 can promote myocardial cell pyroptosis by upregulating the expression levels of NLRP3, caspase-1 and GSDMD-N involved in cardiogenic shock after a burn injury. These findings could help improve understanding of

pyroptosis after a burn injury and provide novel insights for the S100A9/NLRP3 pathway as a novel therapeutic approach.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SX and AW designed the experiments. AW, NZ, YC and QJ performed the experiments. AW and YC confirm the authenticity of all the raw data. AW, NZ and SX wrote and edited the manuscript. All authors read and approved the final the manuscript.

Ethics approval and consent to participate

The present research protocol was approved by the Ethics Committee of Shanghai Seventh People's Hospital (approval no. 2018-HIRB-046), and written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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