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Scutellarin inhibits caspase-11 activation and pyroptosis in macrophages *via* regulating PKA signaling

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Abstract Inflammatory caspase-11 senses and is activated by intracellular lipopolysaccharide (LPS) leading to pyroptosis that has critical role in defensing against bacterial infection, whereas its excess activation under pathogenic circumstances may cause various inflammatory diseases. However, there are few known drugs that can control caspase-11 activation. We report here that scutellarin, a flavonoid from *Erigeron breviscapus*, acted as an inhibitor for caspase-11 activation in macrophages. Scutellarin dose-dependently inhibited intracellular LPS-induced release of caspase-11p26 (indicative of caspase-11 activation) and generation of N-terminal fragment of gasdermin D (GSDMD-NT), leading to reduced pyroptosis. It also suppressed the activation of non-canonical nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3) inflammasome as evidenced by reduced apoptosis-associated speck-like protein containing a CARD (ASC) speck formation and decreased interleukin-1 beta (IL-1β) and caspase-1p10 secretion, whereas the NLRP3-specific inhibitor MCC950 only inhibited IL-1β and caspase-1p10 release and ASC speck formation but not pyroptosis. Scutellarin also suppressed LPS-induced caspase-11 activation and pyroptosis in RAW 264.7 cells lacking ASC expression. Moreover, scutellarin treatment increased Ser/Thr phosphorylation of caspase-11 at protein kinase A (PKA)-specific sites, and its inhibitory action on caspase-11 activation was largely abrogated by PKA

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inhibitor H89 or by adenylyl cyclase inhibitor MDL12330A. Collectively, our data indicate that scutellarin inhibited caspase-11 activation and pyroptosis in macrophages at least partly *via* regulating the PKA signaling pathway.

1. Introduction

Macrophages are critical innate immune cells patrolling various tissues to monitor any microbial infections or tissue injury[1]. They express a wide range of pattern-recognition receptors (PRRs) on their surfaces or in the cytosol to sense the presence of infection or cell injury by receiving signals from pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs)[2-4]. PAMPs, like lipopolysaccharide (LPS), are microbial components released during infections, while DAMPs, including extracellular adenosine triphosphate (ATP), are intracellular components released from injured cells. In response to the stimulation of PAMPs, macrophages can express and secret many kinds of inflammatory cytokines or chemokines to activate and recruit other immune cells into infected or injured sites. Upon PAMPs stimulation, macrophages can also upregulate the expression of inflammasome components such as nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3), which is activated to recruit apoptosis-associated speck-like protein containing a CARD (ASC) resulting in the formation of inflammasomes in the cytoplasm by a second signal including extracellular ATP, bacterial toxin nigericin, or uric acid crystal[5-7]. The assembly of inflammasomes provides a platform for the activation of caspase-1, thereby converting pro-IL-1β into mature IL-1β[8]. The activated caspase-1 can also induce a lytic form of cell death named pyroptosis[9-11], an inflammatory cell death, which also has important roles in the innate defense against microbial infections[12].

Activated caspase-1 is critical for IL-1β maturation and release *via* the canonical inflammasome activation in macrophages in response to various stimuli, thus playing important roles in mediating inflammatory responses to microbial infections or tissue injury[5,6]; however, it has long been known that caspase-11 [an interleukin-1β-converting enzyme (ICE)-interacting protease] is essential for the activation of caspase-1 in macrophages in response to LPS stimulation or infection of *Escherichia coli*[13], but the underlying mechanism(s) was unclear. Recently, it has been demonstrated that caspase-11 mediates such responses named non-canonical inflammasome activation in which the activation of caspase-1 and secretion of IL-1β are dependent on the NLRP3/ASC pathway, but induction of pyroptosis is independent of NLRP3/ASC[14]. Subsequent studies revealed that intracellular LPS can activate caspase-11 leading to the activation of the non-canonical inflammasome independent of Toll-like receptor 4 (TLR4)[15,16]. Caspase-11 is subsequently identified as the direct sensor of intracellular LPS and can be activated directly by binding of LPS[17]. Both caspase-1 and caspase-11 can cleave the gasdermin D (GSDMD) to generate its N-terminal fragment (GSDMD-NT)[18,19], which executes pyroptosis through forming pores on the plasma membrane[20-22]. The pores formed by GSDMD-NT in the plasma membrane confer a conduit for the release of IL-1β[10,23-25]. Only activated caspase-1, but not activated caspase-11, can convert pro-IL-1β into mature IL-1β[14,26]. However, activated caspase-11 can mediate the activation of the non-canonical NLRP3 inflammasome leading to caspase-1 activation and IL-1β maturation which is likely *via* potassium efflux through GSDMD-NT pores[27]. The human analogues of caspase-11 are named caspase-4/-5, which can also be similarly activated by intracellular LPS, culminating in GSDMD-NT-mediated pyroptosis[17,19]. Therefore, caspase-11/-4/-5 has essential roles in defending intracellular bacterial infection, which is upstream of the canonical NLRP3 inflammasome.

Although both canonical and non-canonical inflammasome activation are essential for defensing against bacterial infection, over-activation of such caspases and pyroptosis has pivotal roles in pathogenic processes of many inflammatory diseases[11,28]. It has been regarded that the robust release of inflammatory cytokines, including IL-1β and IL-18, is the major reason for septic shock and septic death[29,30]. Accordingly, several natural products and clinically used drugs have been shown to alleviate the symptoms of inflammatory diseases through inhibition of NLRP3 inflammasome activation and IL-1β release[31-34]. However, many recent studies demonstrated that caspase-11 is likely the critical factor in mediating bacterial sepsis, as loss of caspase-11 but not caspase-1 can protect mice from LPS-induced cell death[13-15]. It is therefore of interest to discover caspase-11 inhibitor, which will have potential application in the treatment of inflammatory diseases associated with caspase-11 activation. At present, there is rarely known drugs that can inhibit the activation of caspase-11. One phytochemical displaying such an effect is wedelolactone, yet it has been shown to inhibit caspase-11 activity by acting as an inhibitor of IκB kinase and thus suppressing caspase-11 expression[35]. Recently, it has been reported that cyclic adenosine monophosphate (cAMP) analogs, such as 8-bromoadenosine-3′,5′-cyclic monophosphate sodium salt and N^6-benzoyladenosine-3′,5′-cyclic monophosphate sodium salt, can inhibit the activation of caspase-11 in macrophages in response to LPS transfection, suggesting that cAMP can act as an endogenous inhibitor of caspase-11 activation[36].

Scutellarin (4′,5,6-hydroxy-flavone-7-glucuronide, Fig. 1A), a flavonoid glucuronide isolated from *Erigeron breviscapus* (Vant.) Hand.-Mazz., has been shown to have a wide range of pharmacological activities, including anti-ischemic stroke, cardiovascular protection, anti-cancer effect, protection against neuro-degeneration, protection against diabetic complications, and anti-glaucoma effect[37]. Such pharmacologic effects have been proposed to be mediated by its anti-oxidative, anti-apoptosis, anti-thrombosis and anti-coagulation, as well as anti-inflammatory actions[37]. As for the action mechanisms of the anti-inflammation activity, it has been proposed that scutellarin
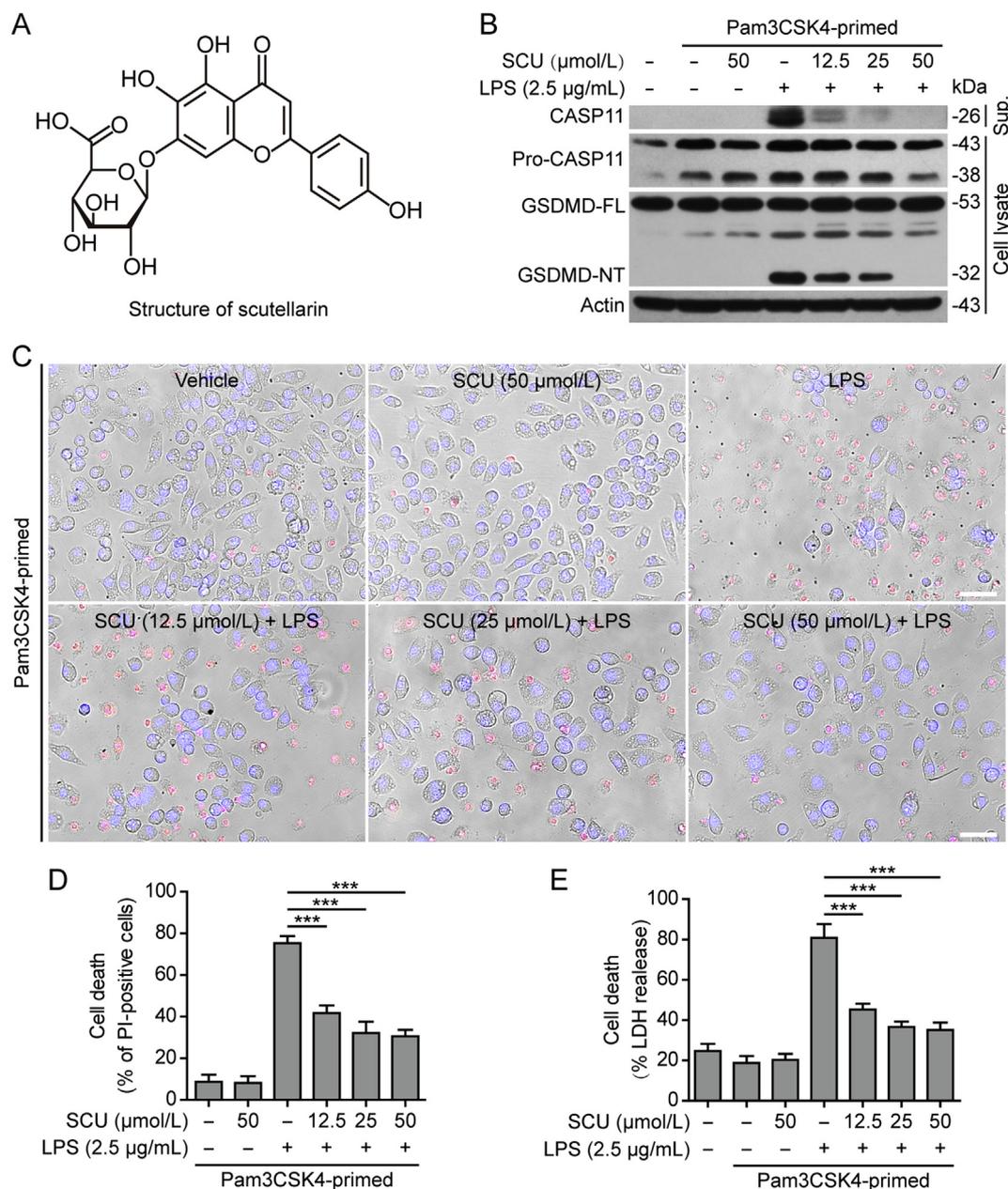
Figure 1 Scutellarin inhibited caspase-11 activation and lytic cell death in J774A.1 macrophages. Cells were primed with Pam3CSK4 (1 μg/mL) for 4 h, and then treated with indicated concentrations of scutellarin for 1 h without Pam3CSK4, followed by transfection with 2.5 μg/mL lipopolysaccharide (LPS) plus 0.25% FuGENE HD for 16 h. (A) Structure of scutellarin. (B) Western blot assay was used to determine the expression levels of indicated proteins in culture supernatants and cell lysates, respectively. Actin was adopted as a loading control for cell lysates. (C) Lytic cell death was assayed by staining with propidium iodide (PI, red) for 10 min. Images show bright-field merged with PI and Hoechst 33342 (blue, staining all nuclei) fluorescence. Scale bar, 50 μm. (D) PI-positive cells were quantified in 5 randomly chosen fields and ratios of PI-positive over all cells (revealed by Hoechst 33342) are used to show the percent of lytic cell death. (E) Lytic cell death was also measured by lactic acid dehydrogenase (LDH) release in culture supernatants. Data are shown as mean ± SD ($n = 5$); ***$P < 0.001$. SCU, scutellarin; Sup., supernatant; CASP11, caspase-11; GSDMD-FL, full-length gasdermin D (GSDMD); GSDMD-NT, N-terminal fragment of GSDMD.

probably mediates such effects by inhibiting inflammatory cytokine expression *via* dampening the nuclear factor κ-light-chain-enhancer of activated B (NF-κB), mitogen-activated protein kinase P38 and c-Jun N-terminal kinase (JNK), and the Notch signaling in macrophages[38–43]. *In vivo* studies also showed that it ameliorates cartilage destruction in a mouse model of osteoarthritis[41] and that it alleviates cognitive deficits in rats treated with LPS[42]. We have previously found that scutellarin can suppress the canonical

NLRP3 inflammasome activation, thus alleviating bacterial sepsis in mice[44]. In view of the critical role of caspase-11 in bacterial sepsis[13,14], it is of great interest to explore whether scutellarin can inhibit caspase-11 activation in macrophages.

In this study, we provide evidence indicating that scutellarin is able to inhibit caspase-11 activation and pyroptosis in macrophages upon intracellular LPS stimulation. Scutellarin dose-dependently inhibited LPS-induced activation of caspase-11 in

macrophages, leading to reduced ASC speck formation, caspase-1 activation, mature IL-1β release, as well as decreased pyroptosis. Scutellarin-induced inhibition of caspase-11 activation was likely mediated by the protein kinase A (PKA) signaling but was independent of the NLRP3/ASC pathway.

2. Materials and methods

2.1. Reagents and antibodies

Scutellarin (#B21478, with >98% purity), was obtained from Shanghai Yuanye Bio-Technology Co. (Shanghai, China) which structure is shown in Fig. 1A. Dulbecco's modified Eagle's medium (DMEM) with high glucose, fetal bovine serum (FBS), Opti-MEM, streptomycin and penicillin, Lipofectamine 2000 (#11668−030), and Pierce classic IP kit (#26146) were bought from Thermo Fisher Scientific (Waltham, MA, USA). LPS (*E. coli* O111:B4, #L4391), fluorescein isothiocyanate (FITC)-LPS (*E. coli* O111:B4, #F3665), propidium iodide (PI, #P4170) and Hoechst 33342 (#B2261), and dimethyl sulfoxide (DMSO, #D8418), were obtained from Sigma−Aldrich (St. Louis, MO, USA). Cell lysis buffer for Western blot and IP (#P0013), phenylmethanesulfonyl fluoride (PMSF, #ST505), and H89 (#S1643) were purchased from Beyotime (Shanghai, China). Pam3CysSerLys4 (Pam3CSK4, #tlrl-pms), flagellin (#tlrl-pafla), polydA:dT) (#tlrl-patn), and nigericin (#tlrl-nig) were purchased from InvivoGen (San Diego, CA, USA). FuGENE HD transfection reagent (#E2311) and CytoTox 96 non-radioactive cytotoxicity assay kit (#G1780) were obtained from Promega (Madison, WI, USA). Antibodies against caspase-11 (#14340, for detection, and immunoprecipitation staining of caspase-11), IL-1β (#12242), ASC (#67824), phospho-protein kinase A catalytic subunit (p-PKA C) (Thr197, #4781), PKA C-α (#4782), ASC AlexaFluor488-conjugated (#17507), p-(Ser/Thr) PKA substrate (an antibody detects proteins containing a phospho-serine/threonine residue with arginine at the −3 position, #9621), horse-radish peroxidase (HRP)-conjugated goat-anti-rabbit IgG (#7074), and HRP-conjugated horse anti-mouse IgG (#7076) were bought from Cell Signaling Technology (Danvers, MA, USA). Antibodies recognized pro-caspase1+p10+p12, GSDMD (#ab209845), and caspase-11 (#ab180673, for immunofluorescence and detecting caspase-11p26 in culture supernatant and immuno-precipitated caspase-11) were obtained from Abcam (Cambridge, UK). CF568-conjugated goat-anti-rabbit IgG highly cross-adsorbed (#20103) and CF488-conjugated goat-anti-mouse IgG highly cross-adsorbed (#20018) were bought from Biotium (Hayward, CA, USA). The antibody against NLRP3 (Cryo-2, #AG-20B-0014) was bought from Adipogen AG (Liestal, Switzerland). The antibody specific for actin (#sc-1616-R) was obtained from Santa Cruz (Dallas, TX, USA). Scutellarin was freshly dissolved in DMSO at 100 mmol/L before experiments.

2.2. Animals

C57BL/6 mice (6−8 weeks old, female) were bought from the Experimental Animal Center of Southern Medical University (Guangzhou, China). The mice were maintained under a 12 h/12 h dark/light cycle condition with free access to food and water and acclimatized for one week before experiments. All animal experiments were performed according to the guidelines for the care and use of animals approved by the Committee on the Ethics of Animal Experiments of Jinan University (Guangzhou, China).

2.3. Cell culture

Murine J774A.1 macrophage cell line was obtained from the Kunming Cell Bank of Type Culture Collection Chinese Academy of Sciences (Kunming, China), while RAW 264.7 cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). J774A.1 and RAW 264.7 cells were maintained in DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (complete DMEM medium) at 37 °C in a humidified incubator of 5% CO_2 and sub-cultured every 2−3 days by using a cell scraper (#541070, Greiner, Frickenhausen, Germany). J774A.1 cells were cultured in complete DMEM medium overnight in 96-well plates at 0.9×10^4 cells/well (0.2 mL) or in 6-well plates at 3.2×10^5 cells/well (2.0 mL), respectively, while RAW 264.7 cells were in 96-well plates at 1.0×10^4 cells/well (0.2 mL) or in 6-well plates at 5.2×10^5 cells/well (2.0 mL), respectively.

2.4. Bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDMs) were induced as previously reported[45], with minor modification. Briefly, bone marrow cells were collected from hind femora and tibias of mice and differentiated in DMEM supplemented with 10% FBS and 20% macrophage colony-stimulating factor (M-CSF)-conditioned medium from L929 cells (BM-Mac medium) at 37 °C in a humidified incubator of 5% CO_2. The culture was replenished with 5 mL BM-Mac medium after 3 days, and BMDM cells were ready for experiments after 6 days. After being collected with cell-scraper, BMDMs were cultured overnight in complete DMEM medium in 96-well plates at 4.5×10^4 cells/well (0.2 mL) or in 6-well plates at 1.6×10^6 cells/well (2.0 mL), respectively.

2.5. Cell death assay

Lytic cell death was determined by PI incorporation or lactic acid dehydrogenase (LDH) release. Briefly, cells were seeded in 96-well plates and primed with 1 µg/mL Pam3CSK4 in Opti-MEM for 4 h, considering that LPS but not Pam3CSK4 can activate caspase-11 during subsequent transfection[15,17]. Subsequently, the cells were treated with various concentrations of scutellarin or inhibitors for specific time periods and then transfected with 2.5 µg/mL LPS plus FuGENE-HD (2.5%, *v/v*) in Opti-MEM for 16 h. To trigger the activation of NLR family CARD domain-containing protein 4 (NLRC4) and absent in melanoma 2 (AIM2) inflammasome, the cells were transfected with 0.5 µg/mL flagellin plus 0.25% (*v/v*) FuGENE HD for 16 h, or 2 µg/mL poly(dA:dT) plus 0.1% (*v/v*) Lipofectamine 2000 for 16 h, respectively. PI (2 µg/mL) solution together with Hoechst 33342 (5 µg/mL) was applied to measure the percentage of lytic cell death[46]. Cells were observed immediately by live imaging with a Zeiss Axio Observer D1 microscope (Carl Zeiss, Gottingen, Germany). Scutellarin's effects on intracellular LPS-induced lytic cell death were also measured by LDH release using CytoTox 96 non-radioactive cytotoxicity assay kit, according to instructions of the manufacturer (Promega).

2.6. Western blot analysis

Protein expression was determined by Western blot analysis as previously described[47]. Briefly, proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked and incubated with indicated primary antibody at 4 °C overnight, followed by incubation with appropriate HRP-conjugated secondary antibody. After washing, X-ray films (Carestream, Xiamen, China) were used to record specific blots on the membranes having been treated with enhanced chemiluminescence solution (BeyoECL Plus, Beyotime). The blot images were captured and quantified by FluorChem8000 imaging system (Alpha Innotech, San Leandro, CA, USA).

2.7. Immunofluorescence microscopy

Immunofluorescence analysis was performed as previously described[48] with minor modification. In brief, cells were seeded in glass-bottomed dish (#801002; NEST, Wuxi, China; 2.2×10^5 cells/dish for J774A.1 and 1.5×10^5 cells/dish for BMDMs) overnight, followed by priming with Pam3CSK4 (1 μg/mL) in Opti-MEM for 4 h. After pre-treatment with scutellarin (50 μmol/L) or MCC950 (2 μmol/L) for 1 h prior to transfection with 2.5 μg/mL LPS plus FuGENE-HD (2.5%, v/v) in Opti-MEM for 16 h, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. After blocking, the cells were incubated with the anti-caspase-11 antibody (#ab180673) overnight, followed by incubating with CF568-conjugated goat-anti-rabbit IgG for 1 h. Subsequently, the cells were incubated with AlexaFluor488-conjugated anti-ASC antibody overnight. In some experiments, Alexa-Fluor488-conjugated anti-ASC antibody was used to reveal ASC expression. After being stained with Hoechst 33342 solution [5 μg/mL in phosphate-buffered saline (PBS)] to reveal the nuclei, the cells were observed under a Zeiss Axio Observer D1 microscope armed with a Zeiss LD Plan-Neofluar 100×/0.6 Korr M27 objective lens (Carl Zeiss).

2.8. Soluble protein precipitation from supernatants

Soluble proteins in culture supernatants (equal volume from each sample) were precipitated as previously described[14,48]. The precipitated proteins were washed with cold acetone and re-dissolved in 2 × SDS-PAGE loading buffer of equal volume, and secreted mature IL-1β, caspase-1p10, and caspase-11p26 were assayed by Western blot analysis.

2.9. Soluble IL-1β determination

Soluble IL-1β in serum and culture supernatants were measured by cytometric bead array (CBA) mouse IL-1β Flex Set (#560232) with mouse/rat soluble protein master buffer kit (#558266) according to the instructions of the manufacturer (BD Biosciences, San Jose, CA, USA). Data were analyzed with a flow cytometer (Attune NxT acoustic focusing cytometer, Thermo Fisher Scientific).

2.10. Immunoprecipitation (IP)

After cells were incubated with medium containing indicated drugs or vehicle, IP was carried out using a Pierce classic IP kit according to the instructions of the supplier. Briefly, rat-anti-caspase-11 antibody (#14340, Cell Signaling Technology) or isotype control IgG was used to precipitate mouse caspase-11 in cell lysates. The protein−antibody complexes binding to beads were finally boiled in 100 °C for 10 min and subsequently subjected to Western blot analysis.

2.11. Statistical analysis

Experiments were performed three times independently. Data were expressed as mean ± standard deviation (SD) and analyzed for statistical significance using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Bonferroni post hoc test for multiple comparisons, while unpaired Student's t-test was used to analyze the statistical significance between two groups. P-values<0.05 were considered statistically significant.

3. Results

3.1. Scutellarin suppresses caspase-11 activation and pyroptosis in macrophages

To sufficiently activate caspase-11, appropriate PAMP priming is needed to stimulate the expression of pro-caspase-11 protein[15,16], prior to transfection or delivery of LPS into the cytosol[14]. We initially performed this in murine J774A.1 macrophages by using Pam3CSK4 (synthetic ligand for TLR2/1) as the priming reagent, as has been used previously[15,16,19]. Western blot analysis shows that the expression of pro-caspase-11 (both 43 and 38 kDa isoforms)[14] was upregulated in Pam3CSK4-primed macrophages, and that LPS transfection markedly increased the secretion of caspase-11p26 fragment, indicative of the activation of caspase-11[16]. Consistent with the caspase-11 activation, GSDMD was cleaved to generate its N-terminal fragment GSDMD-NT of ～32 kDa (Fig. 1B). Further assays with PI staining and LDH release consistently revealed a lytic form of cell death after LPS transfection (Fig. 1C−E). Interestingly, pre-treatment with scutellarin inhibited all such processes in a dose-dependent manner, whereas it alone did not induce any release of caspase-11p26 and LDH or lytic cell death (Fig. 1B−E). We next sought to verify these results in mouse primary BMDMs. The results show that scutellarin also dose-dependently inhibited intracellular LPS-induced caspase-11 activation (Fig. 2A) and lytic cell death (Fig. 2B−D). Similarly, scutellarin alone did not induce the release of caspase-11p26 or lytic cell death. Together, these data indicate that scutellarin is able to inhibit caspase-11 activation and pyroptotic cell death in macrophages.

3.2. Scutellarin suppresses non-canonical NLRP3 inflammasome activation in macrophages

As intracellular LPS-induced caspase-11 activation can further trigger the non-canonical NLRP3 inflammasome activation[17,19], we next explored the influence of scutellarin on this process. As shown in Fig. 3A−D, transfected LPS induced not only the release of caspase-1p10 (Fig. 3A and B), but also the secretion of IL-1β (Fig. 3C and D) into the culture supernatants of both J774A.1 and BMDMs. LPS transfection also induced the formation of ASC specks in macrophages (Fig. 4). Consistent with its inhibitory activity on caspase-11 activation, scutellarin strikingly suppressed the release of caspase-1p10 and IL-1β in a dose-dependent manner (Fig. 3A−D). Scutellarin also markedly suppressed ASC speck formation in macrophages transfected with LPS (Fig. 4). Although scutellarin is able to suppress canonical NLRP3 inflammasome activation upon ATP or nigericin stimulation in our previous study[44], it had no overt effect on NLRC4 (stimulated by flagellin) and AIM2 [by poly(dA:dT)] inflammasome activation (Supporting
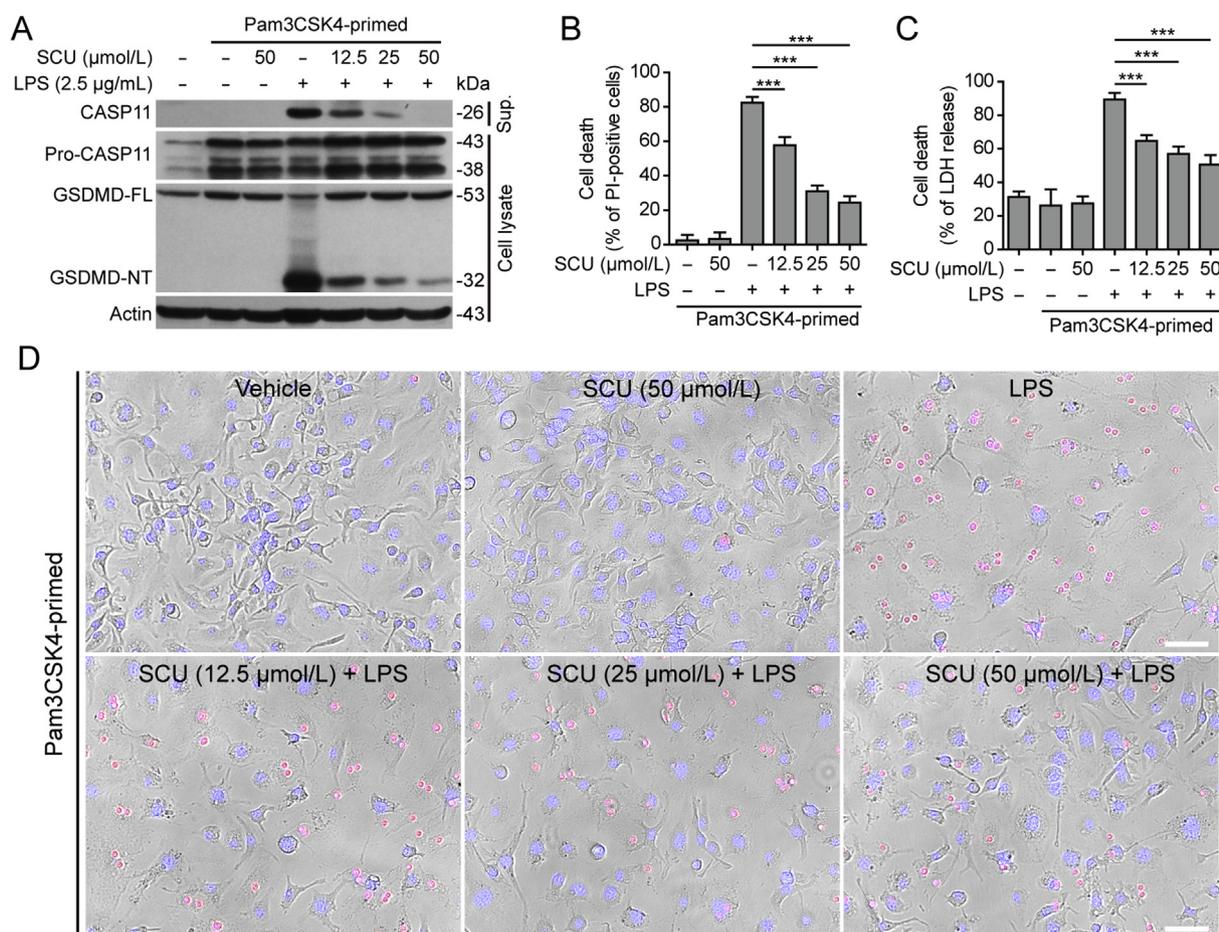
Figure 2   Scutellarin suppressed caspase-11 activation and lytic cell death in bone marrow-derived macrophages (BMDMs). Macrophages were primed with 1 μg/mL Pam3CSK4 for 4 h, pre-treated with indicated concentrations of scutellarin for 1 h without Pam3CSK4, and transfected with 2.5 μg/mL LPS for 16 h. (A) Western blot analysis showed the expression levels of indicated proteins in cell lysates and culture supernatants. Actin was adopted as a loading control for cell lysates. (B) PI-positive cells in 5 randomly chosen fields were quantified and percentage of lytic cell death is defined as the ratio of PI-positive over all cells (revealed by Hoechst 33342). (C) Lytic cell death was measured by LDH release in culture supernatants. Data are shown as mean $\pm$ SD ($n = 5$); $^{***}P < 0.001$. (D) Lytic cell death was measured by staining with PI (red, staining dead cells) for 10 min. Images captured by fluorescence microscopy show bright-field merged with PI and Hoechst 33342 (blue, staining all cells) fluorescence. Scale bar, 50 μm.

Information Fig. S1). As the non-canonical inflammasome activation is reliant on caspase-11 activation *in vivo*[14], we also assayed whether scutellarin inhibited the serum levels of IL-1β in LPS-treated mice. The results show that scutellarin was able to do so (Fig. 3E), suggestive of inhibition of caspase-11 activation *in vivo*. These results together demonstrate that scutellarin, to certain extent, specifically suppressed both canonical and caspase-11-mediated non-canonical NLRP3 inflammasome activation in macrophages.

3.3.   Scutellarin-mediated suppression of caspase-11 activation and pyroptosis is independent of the NLRP3/ASC inflammasome pathway

As previous studies have revealed that scutellarin can inhibit canonical NLRP3 inflammasome activation in macrophages[44], we sought to discern whether scutellarin-mediated inhibition of caspase-11 activation is dependent on the NLRP3/ASC inflammasome pathway. MCC950, a small molecule inhibitor of NLRP3

by targeting its NACHT [NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from *Podospora anserina*) and TP1 (telomerase-associated protein)] domain[49,50], was used to block the NLRP3 inflammasome activation. As expected, MCC950 markedly blocked the formation of ASC specks in J774A.1 macrophages transfected with LPS (Supporting Information Fig. S2) and strikingly decreased the release of caspase-1p10 and IL-1β (Fig. 5A and B), indicating effective inhibition of the NLRP3 inflammasome activation. However, MCC950 had minimal inhibitory effects on intracellular LPS-induced lytic cell death (Fig. 5C and Fig. S2) and the activation of caspase-11 as indicated by the release of caspase-11p26 (Fig. 5A). In contrast, scutellarin inhibited not only the ASC speck formation (Fig. S2) and IL-1β release (Fig. 5A and B), but also the lytic cell death (Fig. 5C and Fig. S2) as well as caspase-11 activation induced by intracellular LPS (Fig. 5A). Similarly, in BMDMs, MCC950 inhibited ASC speck formation but not lytic cell death as revealed by loss of cytoplasmic components and shrinkage of nuclei
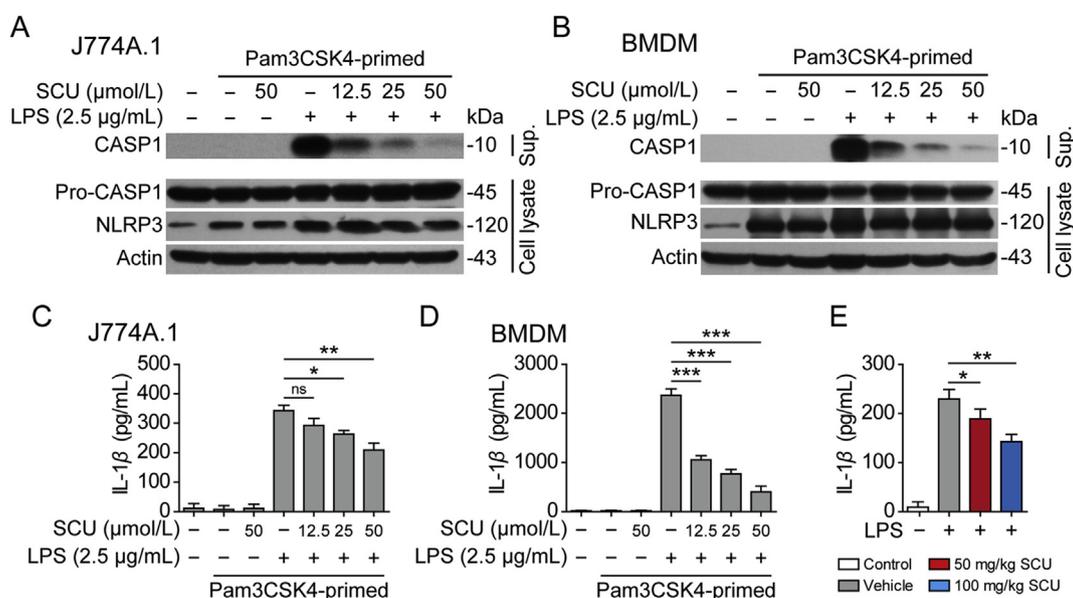
Figure 3   Scutellarin inhibited the non-canonical NLRP3 inflammasome activation in macrophages. J774A.1 cells and BMDMs were primed with Pam3CSK4 for 4 h, pre-treated with or without scutellarin for 1 h and then transfected with LPS (2.5 μg/mL) for 16 h. (A) and (B) Western blot analysis of caspase-1p10 fragments in culture supernatants and indicated proteins in cell lysates. Actin was adopted as a loading control for cell lysates. (C) and (D) Cytometric bead array (CBA) was used to measure interleukin-1 beta (IL-1β) levels in culture supernatants. (E) Mice were intraperitoneally (i.p.) injected with scutellarin or vehicle 1 h before injection (i.p.) of LPS (10 mg/kg) or PBS. The mice were sacrificed 6 h after LPS injection, and serum levels of IL-1β were measured by CBA. Data are shown as mean ± SD ($n = 3$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ns, not significant. CASP1, caspase-1.

(indicative of dying cells), whereas scutellarin inhibited both ASC speck formation and nucleus shrinkage (Fig. 5D). Furthermore, we also performed experiments in RAW 264.7 macrophage cell line that is lacking of ASC expression thus being defective in the NLRP3/ASC/caspase-1 pathway[51]. As expected, intracellular LPS induced caspase-11 activation as evidenced by caspase-11p26 release and GSDMD-NT generation (32 kDa), and lytic cell death revealed by PI incorporation (Fig. 6). Scutellarin was able to inhibit both caspase-11 activation and lytic cell death in a dose-dependent manner (Fig. 6). Besides, LPS transfection-induced lytic cell death in $ASC^{-/-}$ THP-1-derived macrophages was also inhibited by scutellarin (Supporting Information Fig. S3). Together, these results indicate that scutellarin is capable of inhibiting caspase-11 activation independent of the NLRP3/ASC pathway.

3.4.   LPS-induced co-localization of aggregated caspase-11 with ASC speck is blocked by scutellarin

Immunofluorescence microscopy was next used to explore the sub-cellular distribution of caspase-11 in relation to ASC specks in macrophages upon intracellular LPS stimulation. In unstimulated macrophages, caspase-11 was distributed diffusely both in the nucleus and in the cytoplasm; upon LPS stimulation; however, the caspase-11 began to aggregate and finally form puncta with one punctum being co-localized with the ASC speck beside the nucleus (Fig. 7). Consistent with the data shown in Fig. 5A, MCC950 suppressed the ASC speck formation but did not prevent the aggregation of caspase-11, whereas scutellarin blocked both processes (Fig. 7). Interestingly, after MCC950 treatment, the caspase-11 aggregation seemed to be limited within the nuclei. These results corroborate that scutellarin inhibited LPS-induced caspase-11 activation likely *via* blocking its aggregation and

suggest that caspase-11 aggregation outside the nuclei may play a role in inducing the non-canonical NLRP3 activation, yet further work is needed to elucidate this hypothesis and the underlying mechanism.

3.5.   Scutellarin inhibits caspase-11 activation likely via the PKA signaling pathway

We sought to explore the mechanism underlying scutellarin-mediated inhibition of caspase-11 activation. One possibility is that scutellarin may directly inhibit LPS entry into the cytoplasm thus attenuating caspase-11 activation. To this end, we detected the entry into cells of FITC-LPS by transfection. The results showed that scutellarin did not significantly influence the entry of FITC-LPS into macrophages (Supporting Information Fig. S4), suggesting other mechanism being involved in. As cAMP has recently been demonstrated to regulate caspase-11 activation[36] while scutellarin has been shown to influence the cAMP/PKA signaling[44,52], we next explored whether PKA signaling was involved in scutellarin-mediated suppression of caspase-11 activation. As cAMP is an inducer of PKA signaling which is regulated by adenylyl cyclase (AC), both PKA inhibitor H89 and AC inhibitor MDL12330A were used respectively to block the PKA signaling. Western blot analysis shows that scutellarin induced increased phosphorylation of PKA by LPS transfection (Supporting Information Fig. S5), suggesting that PKA signaling was activated by scutellarin. Interestingly, H89 antagonized scutellarin-induced suppression of caspase-11 activation and GSDMD cleavage in BMDMs treated with intracellular LPS (Fig. 8A). Similarly, MDL12330A partly attenuated such processes (Fig. 8B). Furthermore, scutellarin-mediated inhibition of LPS-induced lytic cell death was markedly reversed by pre-treatment of H89 or MDL12330A (Fig. 8C−F). Concomitantly,
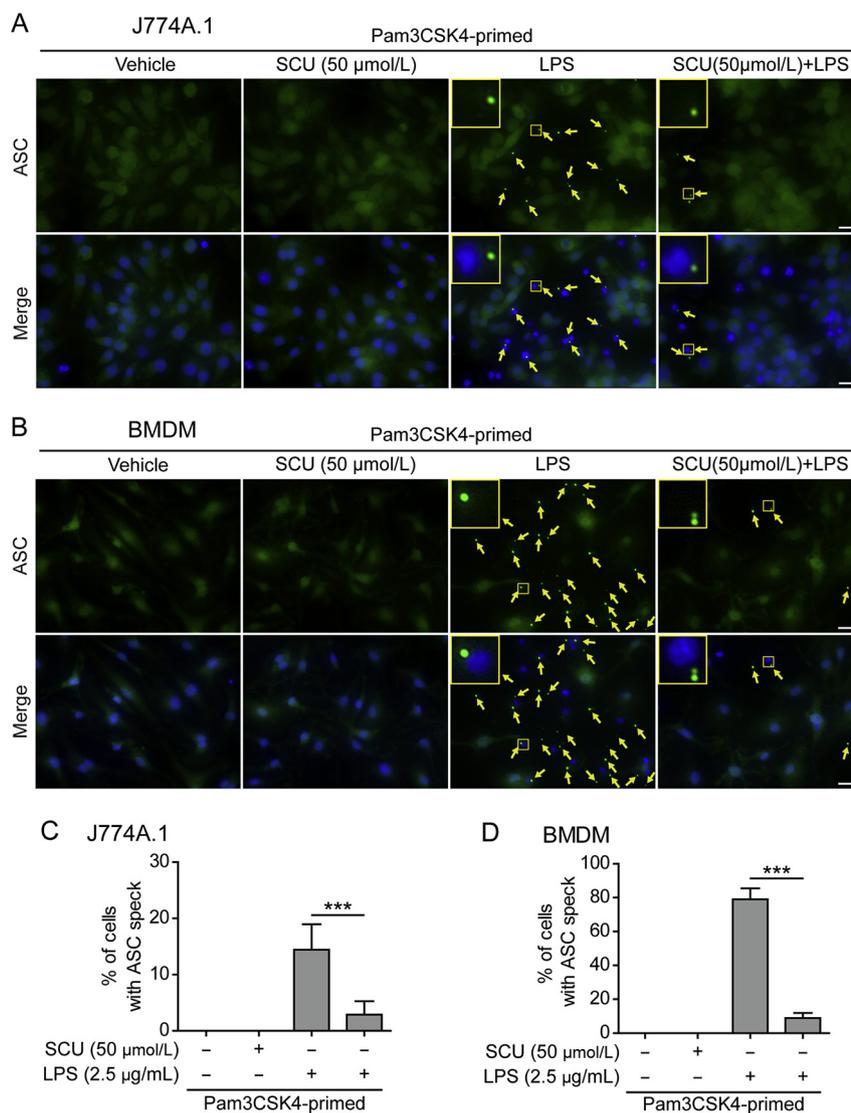
Figure 4 Scutellarin suppressed apoptosis-associated speck-like protein containing a CARD (ASC) speck formation in macrophages. (A) and (B) Cells were primed with Pam3CSK4 for 4 h, pre-treated with or without scutellarin for 1 h, and then transfected with LPS for 16 h. Immunofluorescence microscopy was used to reveal ASC (green) distribution and nuclei (blue, stained by Hoechst 33342). Yellow arrows indicate ASC specks and enlarged insets showing one cell with an ASC speck. Scale bar, 20 µm. (C) and (D) Percentages of cells with an ASC speck over total cells from 5 random fields. Data are shown as mean $\pm$ SD ($n = 5$); $^{***}P < 0.001$.

increased PKA phosphorylation by scutellarin was counteracted by either H89 or MDL12330A (Fig. S5). Together, these data indicate that scutellarin may exhibit its action on caspase-11 by regulating the cAMP/PKA signaling.

As previous studies have shown that PKA can phosphorylate caspase-11 to suppress its activation[36], whether scutellarin could increase Ser/Thr phosphorylation of caspase-11 at PKA-specific sites needs to be further studied [motifs of consensus RRXS*/T* that can be phosphorylated by PKA on the serine (S*)/threonine (T*) residue with arginine (R) at the -3 position and any amino acid residue (X) at -1 position[53]]. Caspase-11 was precipitated from cell lysates with a rat-anti-mouse caspase-11 antibody and its phosphorylation was detected by using a rabbit monoclonal antibody that can recognize a phospho-Ser/Thr residue with arginine at the -3 position (PKA-specific sites), thus avoiding the interference of antibody heavy chain. As shown in Fig. 8G, scutellarin increased the phosphorylation levels on PKA-specific Ser/Thr

residue of caspase-11 (43 but not 38 kDa, with the underlying reason being unclear). Together with published findings showing the involvement of PKA in regulating caspase-11 activation[36], our results suggest that scutellarin inhibits caspase-11 activation, at least partly, *via* its PKA-mediated phosphorylation.

4. Discussion

It has long been believed that the robust release of inflammatory cytokines (including IL-1β) plays a critical role in the pathogenesis of sepsis[29,30]. However, recent studies indicated that caspase-11-mediated pyroptosis, but not caspase-1-dependent IL-1β and IL-18 secretion, is likely responsible for sepsis of mice treated with a lethal dose of LPS[13,14]. Recent studies revealed that activated caspase-11 induces pyroptosis in macrophages through cleavage of GSDMD to generate the pore-forming fragment
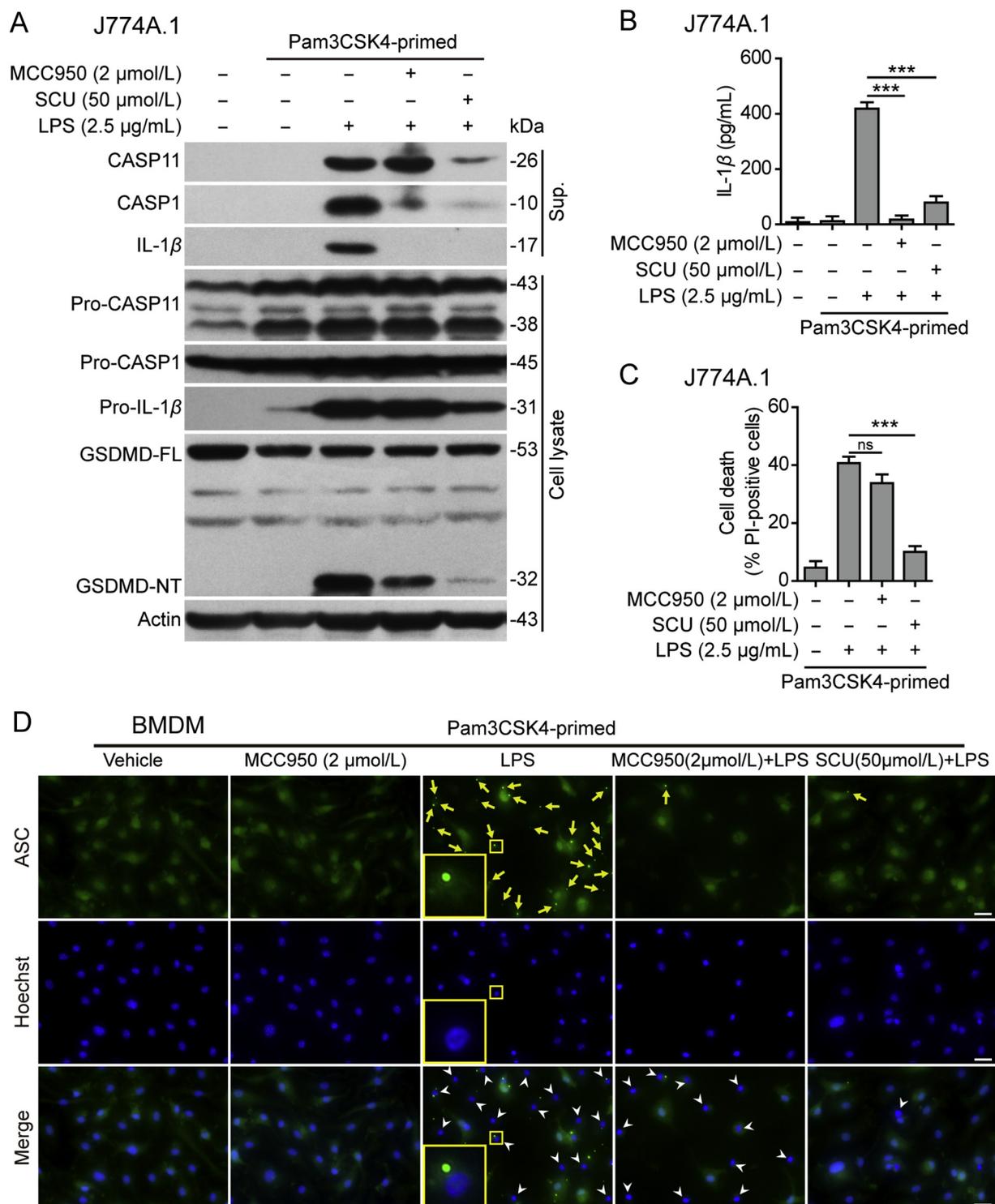
Figure 5 Scutellarin but not MCC950 suppressed both ASC speck formation and lytic cell death in macrophages. J774A.1 cells and BMDMs were first primed with Pam3CSK4, pre-treated with scutellarin or MCC950 for 1 h, and then transfected with LPS for 16 h. (A) Western blot assay was used to determine expression levels of indicated proteins in cell lysates and culture supernatants (Sup.). Actin was detected as a loading control for cell lysates. (B) Cytometric bead array was used to measure IL-1β levels in culture supernatants. Data are shown as mean $\pm$ SD ($n = 3$). (C) Lytic cell death was measured by PI staining. PI-positive cells in 5 randomly chosen fields were quantified. The percentage of lytic cell death is determined by the ratio of PI-positive over all (revealed by Hoechst 33342 staining) cells. (D) Immunofluorescence microscopy was used to demonstrate ASC distribution (green) and nucleus (blue) morphology (revealed by Hoechst 33342 staining). The enlarged insets show one cell with an ASC speck. Yellow arrows indicate ASC specks. White arrow heads indicate dying cells with a shrunk nucleus and loss of ASC fluorescence. Scale bar, 20 μm. Data are shown as mean $\pm$ SD ($n = 5$); $^{***}P < 0.001$; ns, not significant.
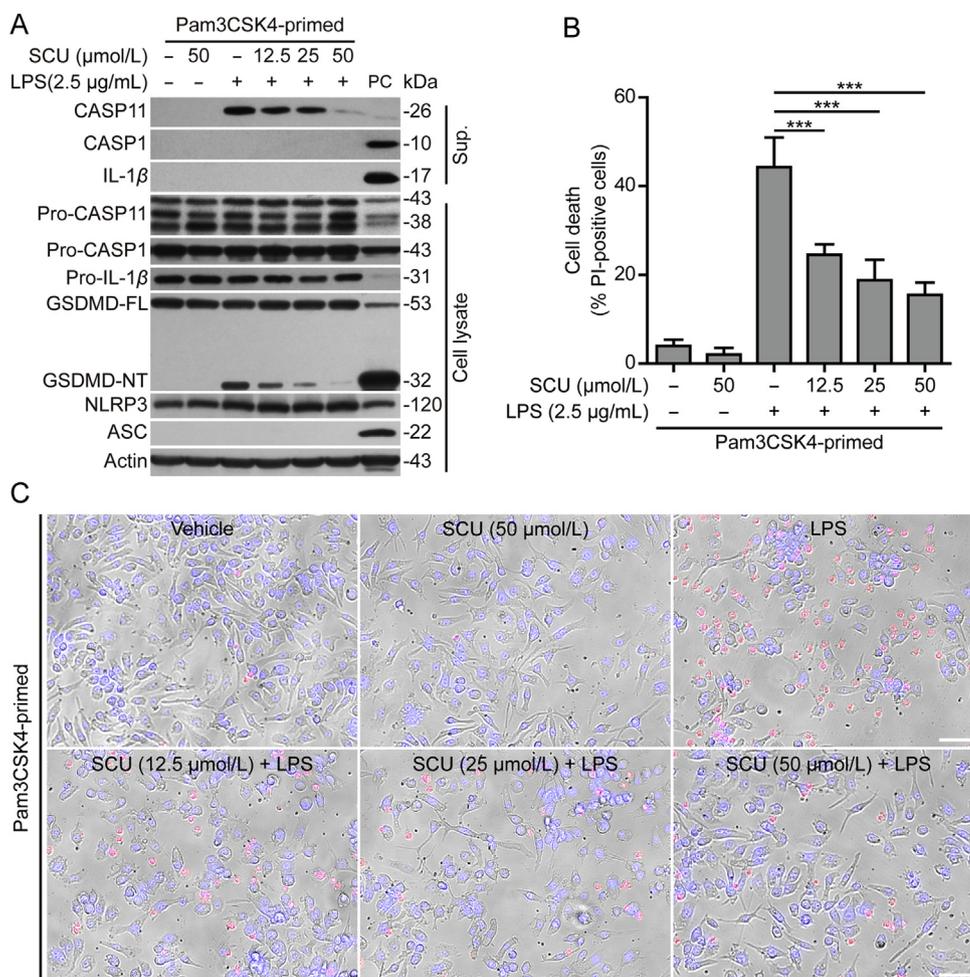
Figure 6 Scutellarin-mediated suppression of caspase-11 activation and lytic cell death was independent of ASC expression. RAW 264.7 cells (lacking ASC) were primed with Pam3CSK4 for 4 h, followed by pre-treatment with indicated concentrations of scutellarin for 1 h without Pam3CSK4, and then transfected with LPS for 16 h. (A) Western blot analysis was used to assess expression levels of indicated proteins in cell lysates and culture supernatants (Sup.), respectively. Positive control (PC) was a cell lysate from BMDMs primed with LPS (500 ng/mL) for 4 h and then incubated with ATP (3 mmol/L) for 30 min without LPS. Actin was assayed as a loading control for cell lysates. (B) PI-positive cells in 5 randomly chosen fields were quantified to show the percentage of lytic cell death defined as the ratio of PI-positive relative to all cells (revealed by Hoechst 33342). Data are shown as mean $\pm$ SD ($n = 5$); $^{***}P < 0.001$; ns, not significant. (C) Lytic cell death was measured by staining with PI for 10 min. Fluorescence microscopy was used to capture images with bright-field merged with PI (red) and Hoechst 33342 fluorescence (blue; showing all nuclei). One set of representative images are shown. Scale bar, 50 μm.

GSDMD-NT[18−20]. Targeting both caspase-11 and GSDMD may therefore represent a new avenue for preventing Gram-negative bacterial-induced sepsis. Here we showed that scutellarin can not only dose-dependently inhibit intracellular LPS-induced caspase-11 activation and pyroptosis, but also suppress the non-canonical NLRP3 inflammasome activation, which may add another layer of action mechanism accounting for previous findings that scutellarin ameliorated systemic inflammatory responses and increased survival of mice with bacterial sepsis[44].

We provided several lines of evidence showing that scutellarin can inhibit caspase-11 activation in macrophages. Firstly, transfection of LPS into cells is a commonly used approach to activate caspase-11[15,16], leading to GSDMD-mediated pyroptosis and the non-canonical NLRP3 inflammasome activation. In such a circumstance, scutellarin rather than MCC950 (NLRP3-specific inhibitor[49]) inhibited LPS-induced release of caspase-11p26 (indicating caspase-11 activation[16]). Secondly, scutellarin

inhibited not only the formation of ASC specks and the release of IL-1β and caspase-1p10 (indicative of caspase-1 activation), but also pyroptosis, whereas MCC950 could only suppress ASC speck formation and IL-1β and caspase-1p10 release, but not pyroptosis. Thirdly, scutellarin inhibited LPS-induced caspase-11p26 release and pyroptosis in RAW 264.7 cells lacking ASC expression[51]. Lastly, scutellarin could inhibit intracellular LPS-induced pyroptosis in *ASC*$^{-/-}$ THP-1-derived macrophages. These data indicate that scutellarin inhibited intracellular LPS-induced activation of caspase-11 inflammasome independently of the NLRP3/ASC pathway. Together with our previous study[44], scutellarin can not only inhibit the NLRP3 inflammasome activation, but also suppress the caspase-11 inflammasome activation (Supporting Information Fig. S6), thereby attenuating bacterial sepsis in mice[44].

As a sensor for cell stress and injury, the NLRP3 inflammasome activation is tightly regulated by many different pathways and its full activation requires both a priming signal and a second
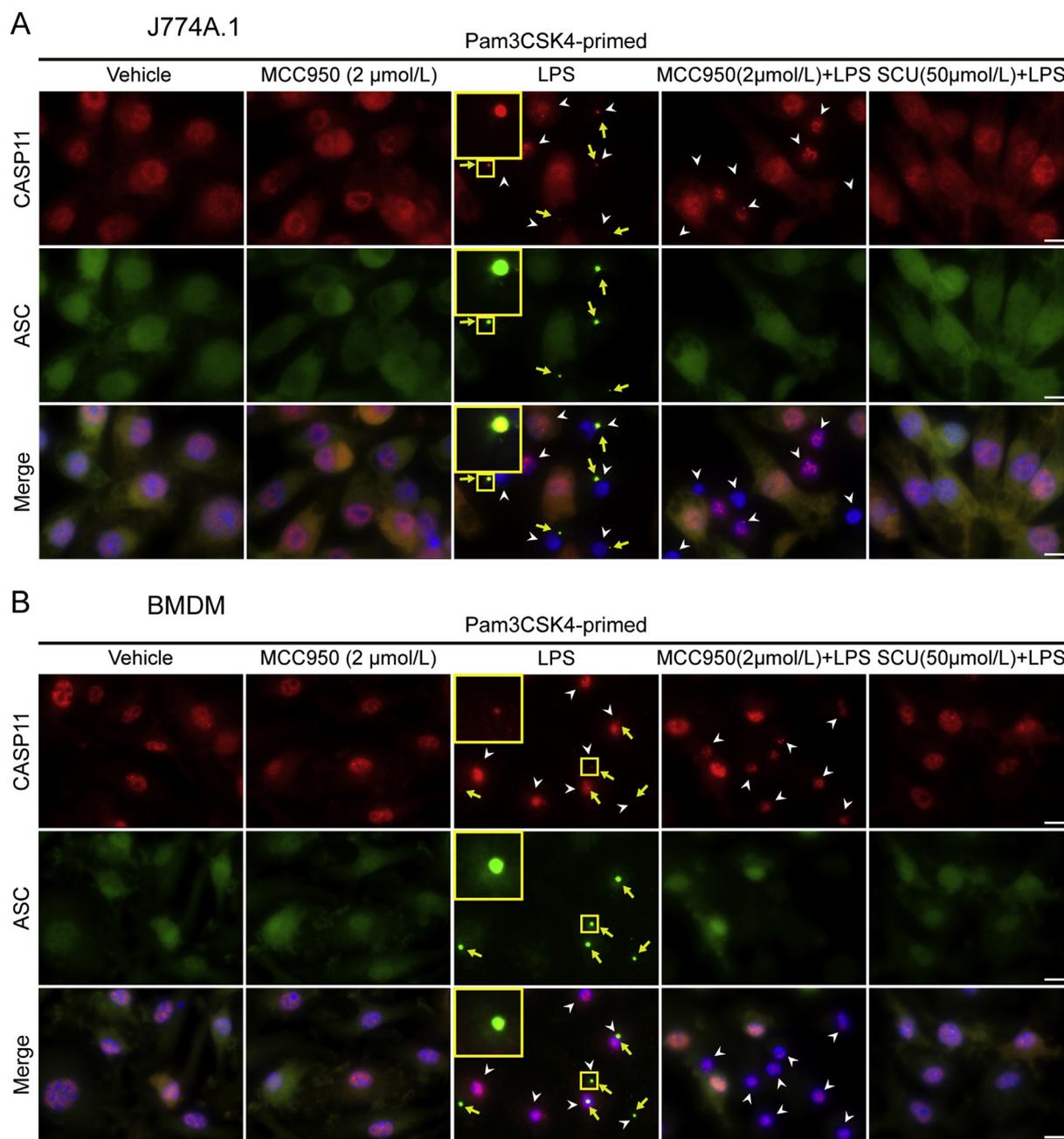
Figure 7 LPS-induced co-localization of ASC specks with aggregated caspase-11 was blocked by scutellarin. J774A.1 cells (A) and BMDMs (B) were primed with Pam3CSK4 for 4 h, pre-treated with scutellarin or MCC950 for 1 h, and then transfected with LPS for 16 h. Immuno-fluorescence microscopy was adopted to reveal the distribution of ASC (green) and caspase-11 (red) while nuclei (blue) being revealed by Hoechst 33342 staining. The insets show a magnified area of one cell with an ASC speck co-localized with a caspase-11 punctum as being revealed as a yellow dot in the merged image. Yellow arrows indicate ASC specks which were co-localized with aggregated caspase-11 dots. White arrow heads indicate dying cells that lost cytoplasmic components with a shrunk nucleus. Scale bar, 10 μm.

triggering signal[54,55]. Caspase-11 acting as a sensor of intracellular LPS is expressed at low levels in resting macrophages and is markedly upregulated by a priming signaling[15,16]. However, the regulation of caspase-11 activation is largely elusive. One recent study demonstrated that cAMP metabolism can regulate caspase-11 activation, indicating the involvement of PKA signaling in this process[36]. In view of the finding that scutellarin can regulate the cAMP/PKA signaling[44,52], we also explored the role of PKA signaling in mediating scutellarin's inhibitory action on caspase-11 activation. Two pieces of evidence support the involvement of PKA

signaling: first, scutellarin increased not only the phosphorylation levels of PKA but also Ser/Thr phosphorylation on caspase-11 at PKA-specific sites; second, both adenylyl cyclase inhibitor MDL12330A and PKA inhibitor H89 abrogated the inhibitory action of scutellarin on caspase-11 activation and pyroptosis. Besides, scutellarin seems to have no significant effect on the entry of LPS by transfection method, but it is still unclear whether scutellarin can influence the high mobility group box 1 (HMGB1)-mediated delivery of LPS *in vivo*[56]. It also remains to be explored whether scutellarin affects the release of LPS from the bacteria in the endosome into the cytosol of macrophages.
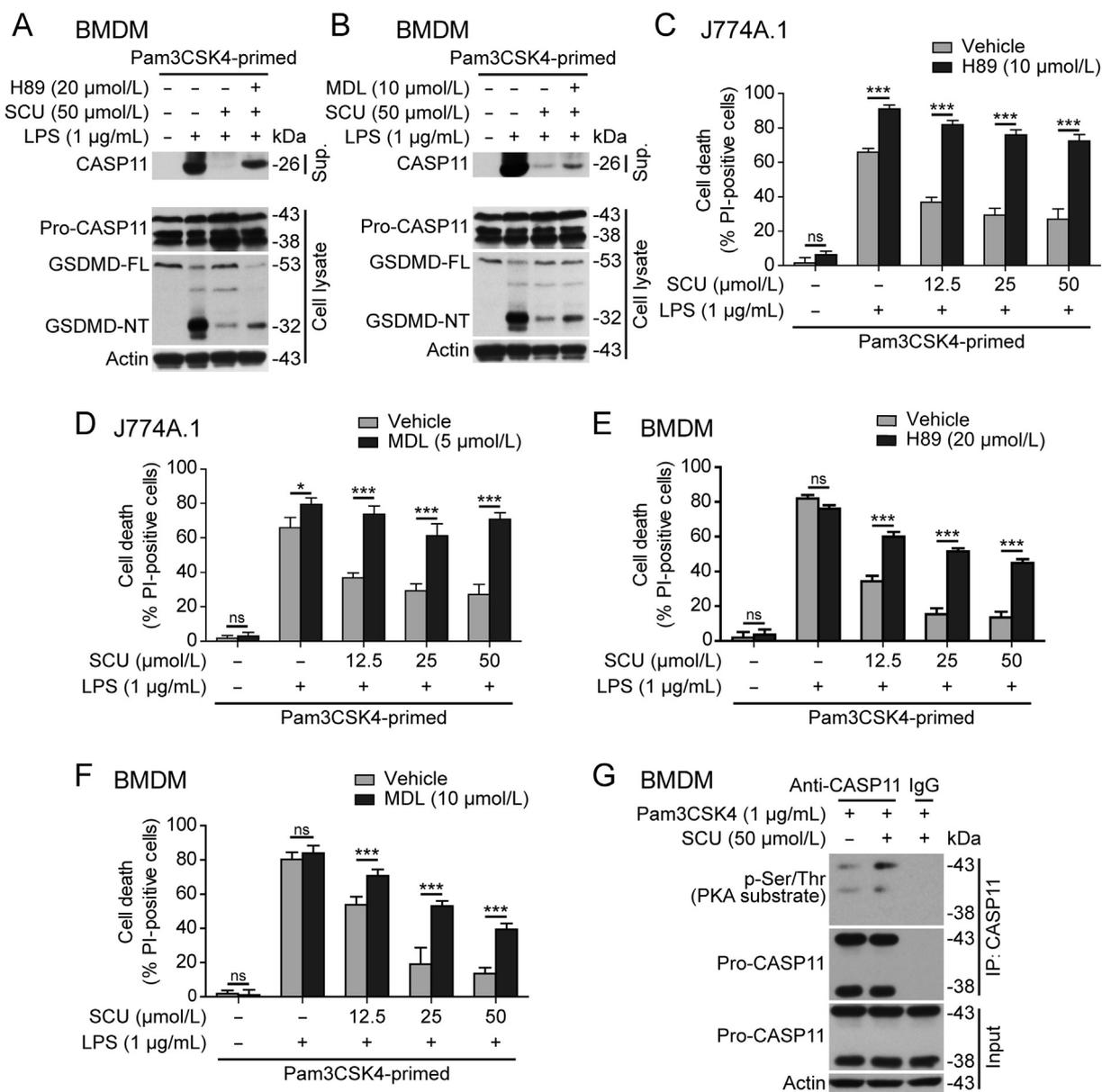
Figure 8   Scutellarin inhibits caspase-11 activation likely *via* the protein kinase A (PKA) signaling pathway. Pam3CSK4-primed cells were pretreated with PKA inhibitor H89 or adenylyl cyclase inhibitor MDL12330A (MDL) for 30 min, and then incubation with indicated doses of scutellarin for 1 h, followed by transfection with LPS for 16 h (A)−(F). (A) and (B) Western blot analysis was used to detect the expression and secretion of caspase-11p26 in culture supernatants and other indicated proteins in cell lysates. Actin was determined as a loading control for cell lysates. (C)−(F) PI-positive cells in 5 randomly chosen fields were quantified. The percentages of lytic cell death were calculated as the ratio PI-positive over all cells. Data are shown as mean ± SD ($n = 5$); $^{*}P < 0.05$, $^{***}P < 0.001$; ns, not significant. (G) BMDMs were primed with Pam3CSK4 for 4 h, followed by treatment with scutellarin for 1 h. Caspase-11 was immunoprecipitated from cell lysates and analyzed for its Ser/Thr phosphorylation of caspase-11 at PKA-specific sites.

Although we cannot exclude such pathways involving in these processes and further study is still needed to elucidate the underlying details, our data highlight an important role of PKA signaling in mediating scutellarin's action on caspase-11 activation.

Notably, it has recently been demonstrated that caspase-11 activation in macrophages requires advanced glycosylation end-product specific receptor (AGER/RAGE)-mediated lipid peroxidation *via* arachidonate 5-lipoxygenase (ALOX5)[57]. Consistently, it has also been revealed that glutathione peroxidase 4 (GPX4)

serves a protective role in mice undergoing septic shock and that myeloid-specific deficiency of *Gpx4* increased lipid peroxidation-dependent caspase-11 activation and gasdermin D-mediated pyroptosis during polymicrobial sepsis[58]. These studies have identified a critical regulatory signaling pathway for caspase-11 activation, which provides potential therapeutic targets for sepsis. As a flavonoid with anti-oxidative activity[37], scutellarin may also possibly display its inhibitory effects on caspase-11 by regulating lipid peroxidation. Besides, scutellarin is likely able to

regulate PKA signaling by modulating cellular oxidation and reduction (redox) status, considering that PKA activation is regulated by redox[59]. Yet these hypotheses remain to be clarified in further investigation.

Our results also showed that scutellarin inhibited the non-canonical NLRP3 inflammasome activation downstream of caspase-11 activation in macrophages transfected with LPS. As such non-canonical inflammasome activation is likely mediated by potassium efflux through GSDMD-NT-formed pores on the plasma membrane[20,21,27,60], it is possibly because scutellarin inhibited caspase-11 activation thereby reducing GSDMD-NT generation and perforation of the membrane, culminating in decreased potassium efflux. Concomitantly, scutellarin can also directly regulate NLRP3 activation *via* PKA-mediated phosphorylation[44]. Thus scutellarin acts simultaneously as both caspase-11 and NLRP3 inhibitors leading to reduced NLRP3 inflammasome activation.

Previous studies have shown that scutellarin can not only inhibit the NF-κB signaling *in vitro* to decrease inflammatory cytokine expression, but also suppress this signaling pathway *in vivo* to protect mice against LPS-induced lung injury[38,61]. In our study, we used TLR2/1 ligand Pam3CSK4 as the first step to prime macrophages for upregulating caspase-11 expression and then transfected LPS into the cells as the second step to activate caspase-11 according to the previous publications[15,17]. The effects of scutellarin on caspase-11 activation had been tested during the second step in the current study. Our results show that scutellarin dose-dependently suppressed LPS-transfection-induced caspase-11 activation and pyroptotic cell death, but barely affected the protein level of caspase-11, suggesting that this flavonoid inhibited caspase-11 activation not by affecting its protein expression in our experimental settings. However, we still do not exclude the potential involvement of the NF-κB pathway in scutellarin's anti-inflammatory effects, particularly in its *in vivo* actions, the latter of which awaits further research.

It has been demonstrated that murine caspase-11 and human caspase-4/-5 are innate immune sensors of intracellular LPS, and LPS can directly bind to caspase-11/-4/-5 to induce their activation[17]. Moreover, the activation of caspase-11 is independent of NLRP3 and upstream of the NLRP3/ASC inflammasome assembly[14]. Consistent with these findings, we found in this study that NLRP3 inhibitor MCC950 suppressed LPS-transfection-induced caspase-1 activation and IL-1β maturation but not caspase-11 activation and pyroptotic cell death, confirming that caspase-11 activation is independent of NLRP3 and ASC[14]. Our observation is in line with previous findings that MCC950 was unable to suppress LPS-transfection-induced lytic cell death even though IL-1β release was attenuated[49]. Interestingly, we found that LPS transfection could induce caspase-11 aggregation and that some aggregated caspase-11 was co-localized with ASC specks near the nuclei; ASC speck formation was inhibited by MCC950, whereas caspase-11 aggregation was not, again verifying that caspase-11 activation is upstream of NLRP3/ASC inflammasome assembly. The co-localization of aggregated caspase-11 with ASC specks suggests that caspase-11 might have a role in subsequent induction of NLRP3/ASC assembly, which remains to be verified. Taken together, previous studies and our current data indicate that MCC950 can only block NLRP3 inflammasome activation but not LPS-induced caspase-11/-4/-5 activation.

Different from MCC950, however, scutellarin not only inhibited non-canonical NLRP3 inflammasome activation but also suppressed caspase-11 activation and caspase-11-mediated pyroptosis. Given previous findings that caspase-11-mediated pyroptosis, but not caspase-1-mediated IL-1β and IL-18 release, is critical for LPS-induced sepsis in mice[13,14], our results suggest that scutellarin may possess some advantages over MCC950 in combating Gram-negative bacterial sepsis. However, considering that caspase-11 has an essential role in defensing against cytosolic bacterial infection[62], long-term suppression of caspase-11 by scutellarin may to some extent weaken the innate defense mechanism. The doses and durations of scutellarin administration should therefore be carefully balanced in clinical applications.

It is of interest to learn whether the concentrations of scutellarin used in cellular models *in vitro* could be reached *in vivo*. One previous study demonstrated that the plasma concentration of scutellarin could reach 5.29 mg/L (about 11.4 μmol/L) 5 h after a single oral dose of 400 mg/kg scutellarin in female rats[63]. Considering that scutellarin is acutely minimally toxic or non-toxic in rodents[64], multiple oral or intraperitoneal/intravenous doses of scutellarin may be administered in order to reach a plasma concentration similar to those used *in vitro* in this study, even though this remains to be verified. Indeed, we observed a significant decrease of LPS-induced IL-1β release in serum of mice treated with one dose of 100 mg/kg scutellarin, suggesting that scutellarin could suppress caspase-11 activation *in vivo*. Future research is needed to explore the precise mechanism underlying the *in vivo* actions.

In conclusion, our data indicate that the natural flavonoid scutellarin isolated from medicinal herbals is a potent inhibitor for caspase-11 activation. Given the critical role of caspase-11-induced pyroptosis in septic pathogenesis but few caspase-11 inhibitors have been found yet[13,14], scutellarin represents a useful candidate for further research and development of drugs against bacterial sepsis-related diseases.

Author contributions

Xianhui He and Dongyun Ouyang conceived and designed the study and wrote the manuscript. Jiezhou Ye, Bo Zeng and Meiyan Zhong performed major experiments and analyzed data. Hongchun Li and Lihui Xu performed Western blotting and CBA assays. Junxiang Shu, Yaofeng Wang and Fan Yang performed animal experiments. Chunsu Zhong and Xunjia Ye performed cell culture and cell death assay experiments. All authors had contributed and approved the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A.   Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2020.07.014.

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