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Intravenous immunoglobulin suppresses NLRP1 and NLRP3 inflammasome-mediated neuronal death in ischemic stroke

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Multi-protein complexes called inflammasomes have recently been identified and shown to contribute to cell death in tissue injury. Intravenous immunoglobulin (IVIg) is an FDA-approved therapeutic modality used for various inflammatory diseases. The objective of this study is to investigate dynamic responses of the NLRP1 and NLRP3 inflammasomes in stroke and to determine whether the NLRP1 and NLRP3 inflammasomes can be targeted with IVIg for therapeutic intervention. Primary cortical neurons were subjected to glucose deprivation (GD), oxygen–glucose deprivation (OGD) or simulated ischemia-reperfusion (I/R). Ischemic stroke was induced in C57BL/6J mice by middle cerebral artery occlusion, followed by reperfusion. Neurological assessment was performed, brain tissue damage was quantified, and NLRP1 and NLRP3 inflammasome protein levels were evaluated. NLRP1 and NLRP3 inflammasome components were also analyzed in postmortem brain tissue samples from stroke patients. Ischemia-like conditions increased the levels of NLRP1 and NLRP3 inflammasome proteins, and IL-1β and IL-18, in primary cortical neurons. Similarly, levels of NLRP1 and NLRP3 inflammasome proteins, IL-1β and IL-18 were elevated in ipsilateral brain tissues of cerebral I/R mice and stroke patients. Caspase-1 inhibitor treatment protected cultured cortical neurons and brain cells *in vivo* in experimental stroke models. IVIg treatment protected neurons in experimental stroke models by a mechanism involving suppression of NLRP1 and NLRP3 inflammasome activity. Our findings provide evidence that the NLRP1 and NLRP3 inflammasomes have a major role in neuronal cell death and behavioral deficits in stroke. We also identified NLRP1 and NLRP3 inflammasome inhibition as a novel mechanism by which IVIg can protect brain cells against ischemic damage, suggesting a potential clinical benefit of therapeutic interventions that target inflammasome assembly and activity.
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Stroke is the second leading cause of death worldwide and a major cause of permanent disability. The molecular and cellular mechanisms responsible for the degeneration of neurons affected by stroke are complex and poorly understood, but involve bioenergetic failure, acidosis, excitotoxicity, oxidative stress and inflammation, resulting in necrotic or apoptotic cell death.[1–3] Post-stroke inflammation is a complex process involving activation of innate local immune responses in glial cells and recruitment of circulating leukocytes into the affected brain tissue.[4,5] Activated glia and leukocytes produce multiple pro-inflammatory mediators including complement anaphylatoxins, cytokines, chemokines and prostaglandins.[4,5] Recent findings have provided insight into a newly discovered inflammatory mechanism that contributes to neuronal and glial cell death in cerebral ischemia mediated by multi-protein complexes called inflammasomes. Studies of the inflammasome complex in peripheral tissues have shown that it amplifies the production and secretion of pro-inflammatory cytokines, and apoptotic and pyroptotic cell death.[6] It was recently reported that the nucleotide-binding

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Abbreviations: ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; Bcl-2, B-cell lymphoma 2; DAMPs, damage-associated molecular patterns; GD, glucose deprivation; IL-1, interleukin 1; I/R, ischemia-reperfusion; IVIg, intravenous immunoglobulin; MAPK, mitogen-activated protein kinases; MCAO, middle cerebral artery occlusion; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLRP, nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) pyrin domain-containing; OGD, oxygen-glucose deprivation; PRRs, pattern recognition receptors; RAGE, receptor for advanced glycation end products; TLRs, toll-like receptors; XIAP, X-linked inhibitor of apoptosis
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oligomerization domain (NOD)-like receptor (NLR) pyrin domain-containing (NLRP) inflammasomes has a role in the inflammatory response during ischemic stroke.[7]

The NLRP1 and NLRP3 inflammasomes are cytosolic macromolecular complexes composed of the NLRP1/3 receptor, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), precursor caspase-1, precursor caspase-11 (homologous to precursor caspase-4 or 5 in humans) and/or XIAP (X-linked inhibitor of apoptosis).[8–10] Activation and homo-oligomerization of NLRP1 and NLRP3 receptors induces formation of the NLRP1 and NLRP3 inflammasomes, respectively, which convert precursor caspase-1 into cleaved caspase-1 via proximity-induced autoactivation.[6,10] Cleaved caspase-1 converts precursors of both IL-1β and IL-18 into biologically active mature pro-inflammatory cytokines that are then released into the extracellular environment.[7,11,12] Moreover, increased cleaved caspase-1 can initiate cell death directly via pyroptosis, or indirectly via apoptosis.[13,14] In stroke-related studies, reduced brain expression of mature IL-1β and IL-18 was shown in mice following cerebral ischemia, using an anti-NLRP1 antibody.[7] Moreover, in caspase-1 knockout mice there was a reduction in mature IL-1β and IL-18 levels in association with a smaller infarct.[15] Furthermore, administration of an IL-1β-neutralizing antibody or IL-1 receptor antagonist reduced subarachnoid hemorrhagic injury.[16] However, the specific pathophysiological role of the NLRP1 and NLRP3 inflammasome in neuronal cell death following ischemic stroke remains to be established.

Intravenous immunoglobulin (IVIg) is an FDA-approved therapeutic modality used for various inflammatory and autoimmune diseases such as Kawasaki's disease, immune thrombocytopenia and humoral immunodeficiency.[17,18] Thus, IVIg has potential for diminishing inappropriate inflammatory and immune activation and neuroprotection.[19,20] IVIg can inhibit complement activation and infiltration of leukocytes, modulate the cytokine network and inhibit neuronal apoptosis by cleaved caspase-3 in mouse models of ischemic stroke.[21] The pleiotropic effects of IVIg in inhibiting multiple components of inflammation in different cell types make it an attractive candidate for use in stroke therapy.[21–24] Potential effects and underlying mechanisms of IVIg on inflammasome activation in ischemic stroke-induced neuronal cell death have not been reported. In the present study, we performed a comprehensive investigation of the dynamic expression patterns of the NLRP1 and NLRP3 inflammasome in primary cortical neurons subjected to simulated ischemia, in a mouse model of focal ischemic stroke, and in brain tissue samples from stroke patients. We demonstrate expression and a functional role for the NLRP1 and NLRP3 inflammasome in neuronal cell death, and show that the neuroprotective effect of IVIg in experimental stroke involves suppression of inflammasome activity. Collectively, our findings reveal IVIg as a potential therapeutic modality for targeting ischemic stroke-induced inflammasome assembly and activity.

Results

Ischemia induces increased expression of inflammasome proteins, and IL-1β and IL-18, in primary cortical neurons in simulated ischemia. To determine whether ischemia-like conditions activate the inflammasome in neurons, we evaluated the temporal expression of all NLRP1 and NLRP3 inflammasome components in neurons subjected to simulated ischemia-reperfusion (I/R). The levels of all major inflammasome components and effectors were increased in primary cortical neurons in response to glucose deprivation (GD), oxygen–glucose deprivation (OGD) and simulated I/R conditions including NLRP1, NLRP3, ASC, XIAP, and precursor caspases 1 and 11 (Figures 1a–f; Supplementary Figures 1–3). Levels of the latter proteins increased within 1 h of exposure to simulated ischemia and remained elevated for 12–24 h. Activation and oligomerization of the NLRP1 and NLRP3 receptors individually induces the formation of the NLRP1 and NLRP3 inflammasome, respectively, which then activates both precursor caspase-1 and -11 into biologically active cleaved caspase-1 and 11.[10] Following activation, caspase-1 cleaves both precursors IL-1β and IL-18 into biologically active mature pro-inflammatory cytokines, which are released into the extracellular environment.[25] Consistent with the notion that ischemic conditions increase NLRP1 and NLRP3 inflammasome activation, we observed significantly increased levels of both cleaved caspase-1 and -11 and both mature IL-1β and IL-18 in primary cortical neurons following GD, OGD or simulated I/R conditions over 24 h in comparison with control (Figures 1a–f; Supplementary Figures 1–3).

I/R induces increased expression of NLRP1 and NLRP3 inflammasome proteins and also IL-1β and IL-18 in ipsilateral brain tissues of cerebral I/R mice and stroke patients. The role of the NLRP1 and NLRP3 inflammasomes in ischemic stroke was further investigated by measuring the expression levels of NLRP1 and NLRP3 inflammasome proteins in ipsilateral (i.e., ischemic) brain tissues of cerebral I/R injury mice. It was shown that I/R significantly increased the expression of NLRP1 and NLRP3 inflammasome proteins, including NLRP1, NLRP3, ASC, XIAP, precursor caspases 1 and 11 in ipsilateral brain tissue as early as 1 h, and it remained higher at 12, 24, and 72 h following I/R in comparison with sham controls (Figures 2a–c; Supplementary Figures 4 and 5). An indication of NLRP1 and NLRP3 inflammasome activation was demonstrated by increased levels of cleaved caspases 1 and 11, and both mature IL-1β and IL-18, at all time points following I/R in comparison with sham controls (Figures 2a and b). Furthermore, to determine whether increased NLRP1 and NLRP3 inflammasome protein expression might occur in the human brain following ischemic stroke, we analyzed brain tissues obtained from stroke patients at the University Medical Center Hamburg-Eppendorf (Figures 3a and b) and National Taiwan University Hospital (Supplementary Figures 6a and b). We found evidence that ischemic stroke increased NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18 in comparison with control patients (Figures 3a and b; Supplementary Figures 6a and b). In addition, immunohistochemical analysis in ipsilateral brain tissues from stroke patients confirmed a higher level of cleaved caspase-1 in comparison with control patients, suggesting increased inflammasome activity (Supplementary Figure 6a).

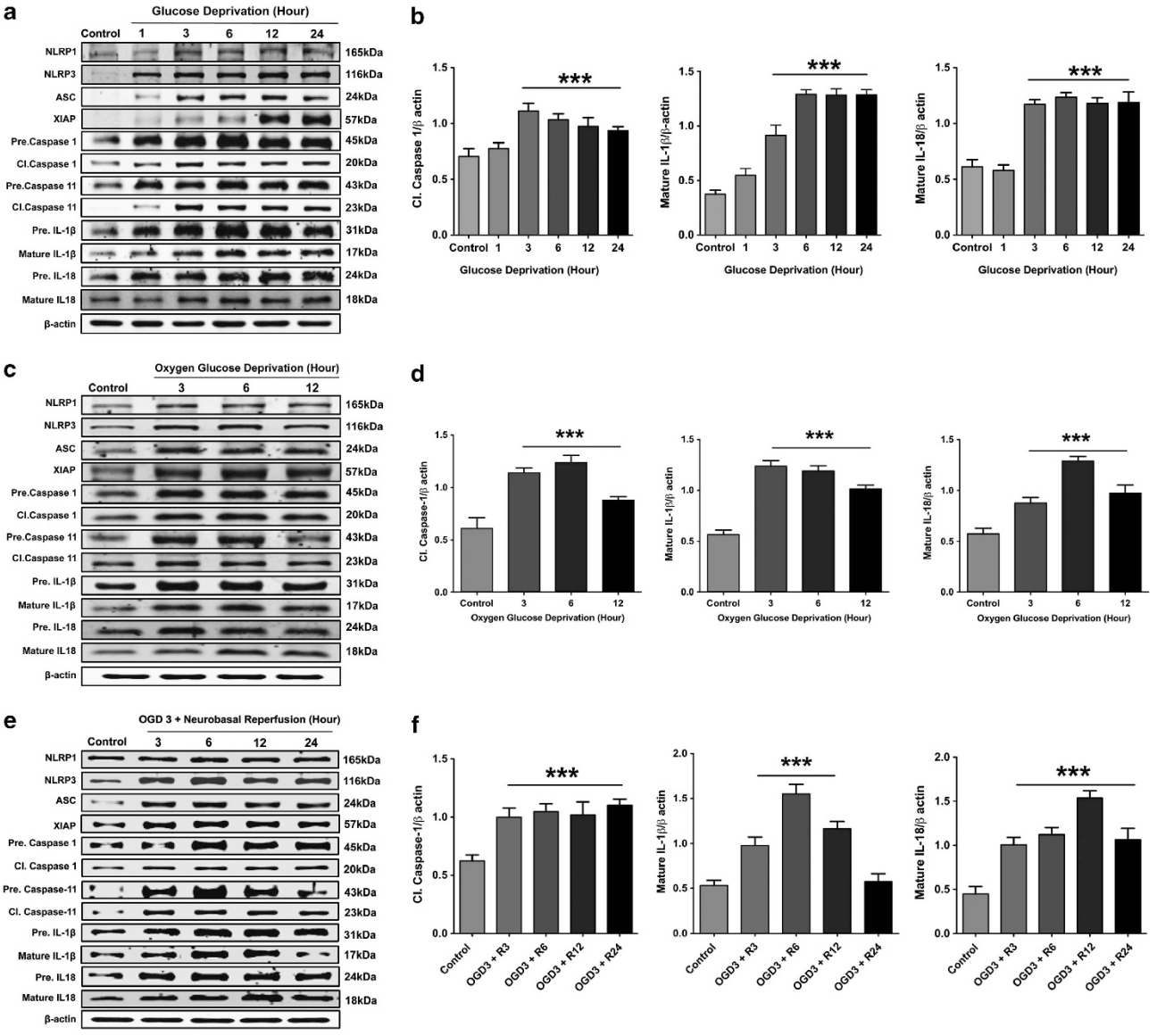

Figure 1 Simulated ischemia increases the levels of multiple inflammasome proteins in primary cortical neurons. (**a** and **b**) Representative immunoblots and quantification of inflammasome proteins, and IL-1β and IL-18, in lysates of cortical neurons at the indicated time points during GD. (**c** and **d**) Representative immunoblots and quantification of inflammasome proteins, IL-1β and IL-18 in lysates of cortical neurons at the indicated time points during OGD. (**e** and **f**) Representative immunoblots and quantification of inflammasome proteins, IL-1β and IL-18 in lysates of cortical neurons after simulated I/R. β-actin was used as a loading control. Data are represented as mean ± S.E.M. $n = 5$ cultures. $^{***}P < 0.001$ compared with control

Caspase-1 inhibitor treatment protects cultured cortical neurons and brain cells *in vivo* in experimental stroke models. In light of the increased expression of NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18 in primary cortical neurons, we next determined the functional role of inflammasomes in the degeneration of neurons subjected to ischemia-like conditions. We tested the efficacy of a caspase-1 inhibitor in primary cortical neurons under ischemic conditions. Increasing concentrations of a caspase-1 inhibitor Ac-YVAD-CMK (1–100 μM) were applied and neurons were then analyzed for cleaved caspase-1, an indicator of inflammasome activation. Caspase-1 inhibitor Ac-YVAD-CMK concentrations above 30 μM were effective in reducing levels of cleaved caspase-1, as well as of cleaved caspase-3, a marker of apoptosis (Figures 4a and b). Mouse primary cortical neurons treated with a caspase-1 inhibitor (30 and 100 μM) were less vulnerable to apoptotic cell death under GD and OGD conditions (Figures 4a and b; Figures 4d and e). The results of a cell viability assay showed that caspase-1 inhibitor (30–100 μM) treatment reduced neuronal cell death under GD conditions (Figures 4a and c). In addition, we investigated the effect of caspase-1 inhibitor Ac-YVAD-CMK (30 and 100 μM) on the expression levels of NLRP1 and NLRP3 inflammasome proteins and both precursor and mature forms of IL-1β and IL-18 during a 6-h period of OGD. Caspase-1 inhibition had no effect on levels of NLRP1, NLRP3, ASC, XIAP, cleaved XIAP, precursor caspase-1, caspase-11, and IL-1β and IL-18 precursors in comparison

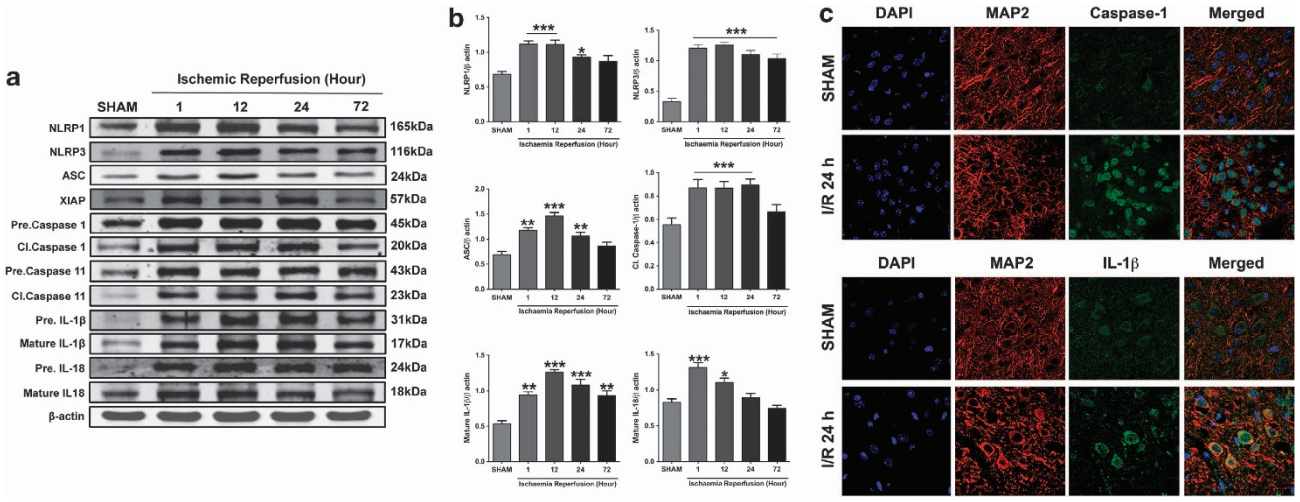

Figure 2 Evidence that focal ischemic stroke activates the inflammasome in cerebral cortical cells. (**a** and **b**) Representative immunoblots and quantification of inflammasome proteins, IL-1β and IL-18 in ipsilateral brain lysates at the indicated post-stroke time points. Data are represented as mean ± S.E.M. $n = 3$–6. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with SHAM (control). (**c**) Immunohistochemical analysis on caspase-1 and IL-1β show localization within the cytoplasm of cortical neurons. The levels of inflammasome proteins, IL-1β and IL-18 are upregulated in I/R in comparison with SHAM (control). Magnification, × 1000. Scale bar, 10 μm. Images were taken under identical conditions and exposures

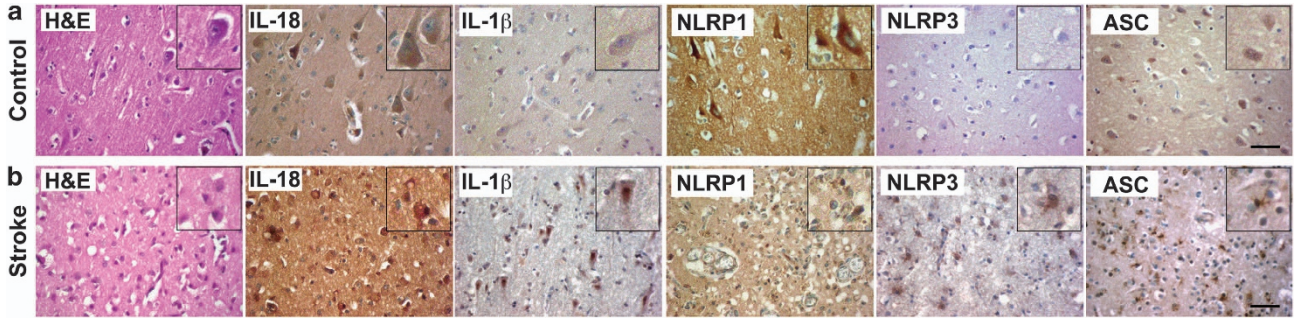

Figure 3 Evidence for inflammasome activation in brain tissue affected by stroke in human patients. (**a** and **b**) Immunohistochemical analysis of NLRP1, NLRP3, ASC, IL-1β, IL-18 show localization within the cytoplasm of cortical neurons. The levels of inflammasome proteins and both IL-1β and IL-18 are elevated in brain tissues of stroke patients in comparison with neurologically normal control subjects. H&E stain was used to distinguish cell types. Images were taken under identical conditions and exposures

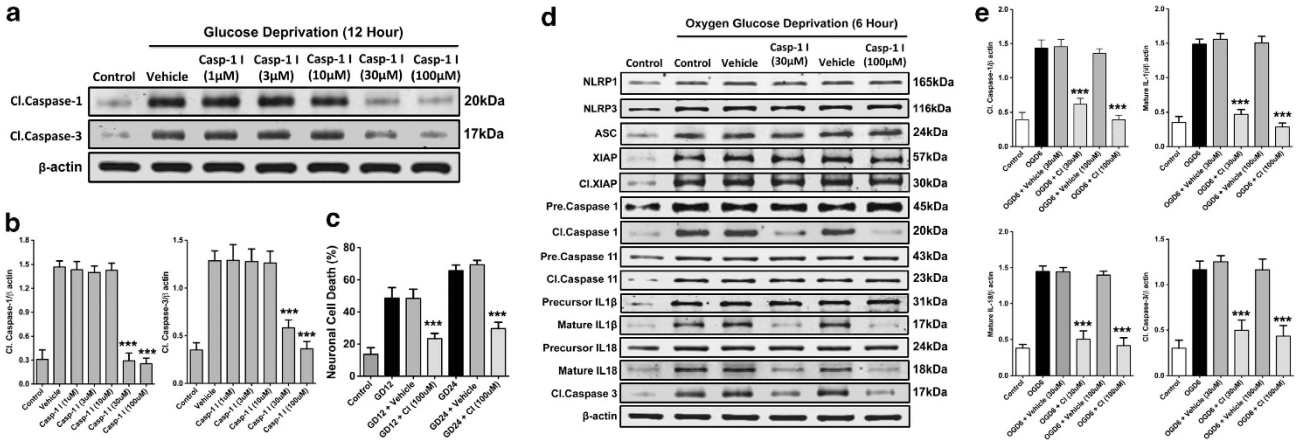

Figure 4 Inhibition of caspase-1 reduces inflammasome activation and cell death in primary cortical neurons subjected to ischemia-like conditions. (**a** and **b**) Representative immunoblots and quantification illustrating the effect of increasing concentrations (μM) of Ac-YVAD.CMK on levels of cleaved caspase-1 and caspase-3 proteins in primary cortical neurons subjected to GD. (**c**) The effect of Ac-YVAD.CMK treatment on cell death (%) in primary cortical neurons subjected to GD. (**d** and **e**) Representative immunoblots and quantification illustrating the effect of 30 μM and 100 μM Ac-YVAD.CMK treatment on inflammasome proteins, IL-1β, IL-18, and cleaved caspase-3 in primary cortical neurons subjected to OGD. β-actin was used as a loading control. Data are represented as mean ± S.E.M. $n = 5$–6 cultures. $^{***}P < 0.001$ compared with control

with vehicle controls under OGD conditions (Figure 4d). However, caspase-1 inhibitor (30 and 100 μM) treatment reduced the levels of cleaved caspase-1 and both mature IL-1β and IL-18 (Figures 4d and e). Furthermore, the levels of cleaved caspase-3 were lower in caspase-1 inhibitor (30 and 100 μM)-treated neurons in comparison with vehicle controls under OGD conditions (Figure 4e).

We next evaluated the potential therapeutic efficacy of a caspase-1 inhibitor in a mouse model of focal ischemic stroke. A dose–response experiment was performed to identify the efficacy of a caspase-1 inhibitor on brain infarct size. It was found that whereas intravenous administration of the two lower doses of the caspase-1 inhibitor (1 and 6 mg/kg) at 3 h after reperfusion had no effect on brain infarct size in comparison with I/R vehicle controls (data not shown), both 10 and 20 mg/kg reduced brain infarct size ($P<0.0001$) and improved functional outcome in comparison with I/R vehicle controls (Figures 5a and b). Cerebral blood flow measurements obtained immediately before and after middle cerebral artery occlusion (MCAO), and at 60, 120, and 180 min after reperfusion, showed a ~90–95% reduction in blood flow in the cerebral cortex supplied by the middle cerebral artery during ischemia, and flow was not significantly different between groups at up to 180 min of reperfusion (data not shown). In addition, we investigated the effect of a caspase-1 inhibitor (10 mg/kg) on levels of the NLRP1 and NLRP3 inflammasome proteins and IL-1β and IL-18 precursor proteins in ipsilateral brain tissues 24 h after I/R. Caspase-1 inhibition had no effect on expression of NLRP1, NLRP3, ASC, XIAP, cleaved XIAP, caspase-1 precursor, caspase-11, and IL-1β and IL-18 precursors in comparison with vehicle controls (Figure 5c). However, the caspase-1 inhibitor at 10 mg/kg significantly reduced the levels of cleaved caspase-1 and both mature IL-1β and IL-18 at 24 h following I/R (Figures 5c and d). Furthermore, levels of cleaved caspase-3 were lower in caspase-1 inhibitor (10 mg/kg)-treated groups in comparison with vehicle controls (Figures 5c and d).

IVIg treatment protects primary cortical neurons and brain tissue by decreasing inflammasome activity under *in vitro* and *in vivo* ischemic conditions. We recently identified IVIg as a potent stroke therapy.[21,24] Specifically, we reported that administration of IVIg to mice subjected to experimental stroke significantly reduced brain infarct size and nearly eliminated mortality. Moreover, there was a reduced volume of infarct, but within the ischemic region neurons were spared and only occasional cell loss was observed. Recently, it was demonstrated that IVIg could decrease the activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases (MAPK) signaling pathways in neurons under ischemic conditions through an unknown mechanism.[24] We therefore investigated the effect of IVIg (5 mg/ml) on levels of the NLRP1 and NLRP3 inflammasome proteins and IL-1β and IL-18 in primary cortical neurons under GD conditions over 6, 12 or 24 h. Indeed, we found that IVIg treatment significantly decreases levels of NLRP1, NLRP3, ASC, XIAP, caspase-1, caspase-11, IL-1β and IL-18 in comparison with vehicle-treated neurons during GD (Figures 6a and b; Supplementary Figure 7). Furthermore, levels of cleaved caspase-3 were significantly lower in IVIg (5 mg/ml)–treated, compared with vehicle-treated neurons during GD (Figures 6a and b). In addition, immunocytochemical analysis indicated that levels of inflammasome proteins and both IL-1β and IL-18 were lower in IVIg-treated neurons compared with vehicle-treated neurons after 12 h of GD (Figure 6c).

We also investigated the effect of IVIg (5 mg/ml) on levels of NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18 in primary cortical neurons subjected to transient OGD (simulated I/R) conditions. We found that IVIg treatment inhibited the simulated I/R-induced elevation of NLRP1, NLRP3, IL-1β and IL-18 levels (Figures 6d and e; Supplementary Figure 8). Furthermore, IVIg treatment significantly attenuated the simulated I/R-induced increase in levels of NLRP1, NLRP3, ASC, XIAP, cleaved XIAP, caspase-1, caspase-11, IL-1β, and IL-18 and cleaved caspase-3 (Figures 6d and e).

We also tested the effect of IVIg treatment on inflammasome activity *in vivo* following experimental stroke. Intravenous administration of 1 g/kg IVIg at 3 h following reperfusion was previously reported to reduce brain infarct size and improve neurological outcome in rodent stroke

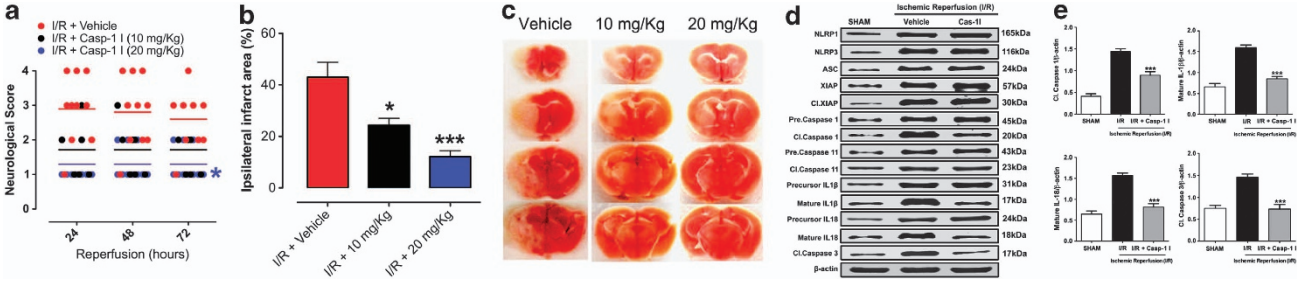

Figure 5 A caspase-1 inhibitor improves neurological outcome, reduces infarct size and suppresses the inflammasome in a mouse model of focal ischemic stroke. (**a**) The effect of Ac-YVAD.CMK (10 mg/kg and 20 mg/kg) treatment on neurological scores of C57BL/6J mice following MCAO (1 h) and reperfusion at indicated times. *$P<0.05$. (**b**) The effect of Ac-YVAD.CMK (10 mg/kg and 20 mg/kg) treatment on ipsilateral infarct area (%) of C57BL/6J mice. $n=9$–11 in each group. *$P<0.05$, ***$P<0.001$. (**c**) Representative images of brains from each treatment group. (**d**) Representative immunoblots and quantification illustrating the effect of Ac-YVAD.CMK (30 and 100 μM) treatment on the expression levels of inflammasome proteins, IL-1β, IL-18, and cleaved caspase-3 following MCAO (1 h) and reperfusion (24 h) in ipsilateral brain tissues of C57BL/6J mice. (**e**) Administration of Ac-YVAD.CMK significantly reduces the levels of inflammasome proteins, IL-1β, IL-18, and cleaved caspase-3. Data are represented as mean ± S.E.M. $n=5$–6 animals. ***$P<0.001$ compared with I/R

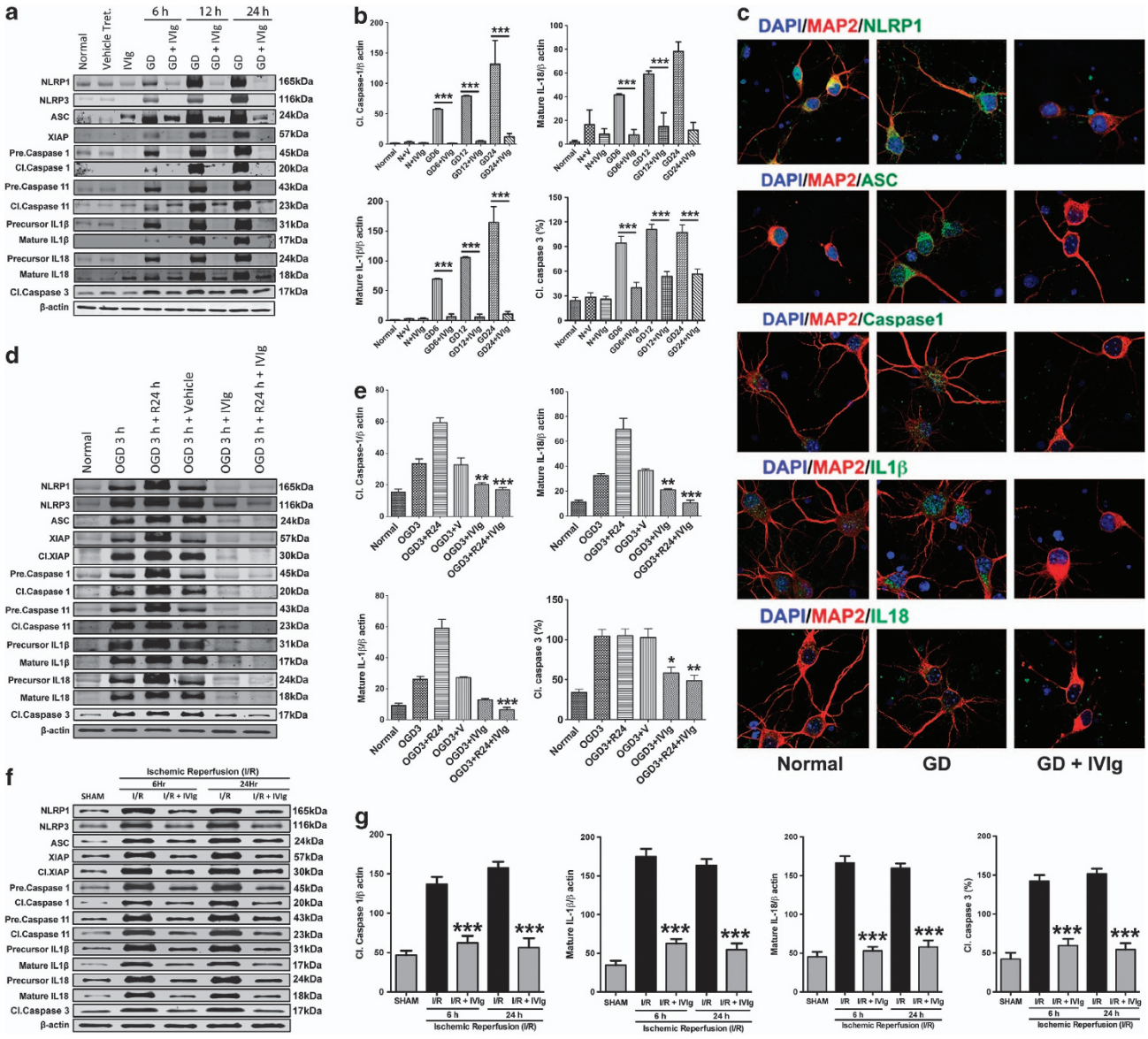

Figure 6 IVIg treatment inhibits the inflammasome in cultured cortical neurons subjected to simulated ischemia, and in a mouse model of focal ischemic stroke. (**a** and **b**) Representative immunoblots and quantification illustrating increases in the levels of inflammasome proteins, and both IL-1β and IL-18 in primary cortical neurons at indicated times during GD. Administration of IVIg (5 mg/ml) significantly reduces the levels of inflammasome proteins and both IL-1β and IL-18. Data are represented as mean ± S.E.M. $n = 6$ cultures. ***$P < 0.001$ in comparison with GD. (**c**) Immunocytochemical analysis of NLRP1, ASC, caspase-1, IL-1β, and IL-18 show localization within the cytoplasm of primary cortical neurons. The levels of inflammasome proteins and both IL-1β and IL-18 are elevated in neurons subjected to GD. Treatment with IVIg (5 mg/ml) significantly reduced the levels of inflammasome proteins and both IL-1β and IL-18 in neurons subjected to GD. Magnification $\times$ 1000. Scale bar, 10 μm. Images were taken under identical conditions and exposures. (**d** and **e**) Representative immunoblots and quantification illustrating increases in the levels of inflammasome proteins and both IL-1β and IL-18 in primary cortical neurons subjected to simulated I/R. Administration of IVIg (5 mg/ml) significantly reduces the levels of inflammasome proteins and both IL-1β and IL-18. Data are represented as mean ± S.E.M. $n = 6$ cultures. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ in comparison with cultures not treated with IVIg. (**f** and **g**) Representative immunoblots and quantification illustrating increase in the levels of inflammasome proteins and both IL-1β and IL-18 in ipsilateral brain tissues of C57BL/6J mice following MCAO (1 h) and reperfusion (6 and 24 h). β-actin was used as a loading control. Administration of IVIg (1 g/kg) significantly reduces the levels of inflammasome proteins and both IL-1β and IL-18. Data are represented as mean ± S.E.M. $n = 5$–6 animals in each group. ***$P < 0.001$ in comparison with I/R (6 and 24 h)

models.[21,24] Here, we investigated the effect of IVIg (1 g/kg) on levels of NLRP1 and NLRP3 inflammasome proteins, and IL-1β and IL-18, in ipsilateral brain tissue at 6 and 24 h of I/R. IVIg treatment significantly decreased levels of NLRP1 and NLRP3 inflammasome proteins, and IL-1β and IL-18, in comparison with vehicle-treated mice (Figures 6f and g; Supplementary Figure 9). Furthermore, levels of cleaved caspase-3 were significantly lower in IVIg (1 g/kg)-treated groups in comparison with vehicle controls (Figures 6f and g).

Discussion

Inflammation is a major contributor to the pathogenesis of ischemic stroke. The deleterious effects of the inflammatory response following cerebral ischemia are mediated by

neurons, glial cells, endothelial cells, and infiltrating leukocytes in the brain, which secrete numerous cytokines and chemokines at the site of injury. Studies have shown that pro-inflammatory cytokines such as IL-1β and IL-18 have a significant role in cerebral ischemic damage.[7,9,26,27] A macromolecular complex, termed the inflammasome, in particular the NLRP1 and NLRP3 inflammasomes, regulate the maturation of these pro-inflammatory cytokines. The present study provides strong evidence that the NLRP1 and NLRP3 inflammasomes have a major role in neuronal cell death and behavioral outcome following stroke. The second part of the study investigated the effect of IVIg treatment on ischemic stroke-induced NLRP1 and NLRP3 inflammasome activity. A high concentration of IVIg has been reported to exert protective effects in neurons during ischemic conditions.[21,23,24] The present results demonstrate for the first time that IVIg modulates ischemic stroke-induced NLRP1 and NLRP3 inflammasome levels, with a corresponding down-regulation of the pro-inflammatory cytokines IL-1β and IL-18.

An increase in expression of NLRP1 and NLRP3 inflammasome proteins and IL-1β and IL-18 precursor proteins in neurons under ischemic conditions may be induced by the activation of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), the receptor for advanced glycation end products (RAGE), and the IL-1 receptor 1 (IL-1R1) present on neurons and glial cells, which detect damage-associated molecular patterns (DAMPs) released from necrotic tissue within the infarct core.[28–31] TLR and pro-inflammatory cytokine signaling activate intracellular NF-κB and MAPK signaling pathways resulting in increased production of NLRP1 and NLRP3 inflammasome proteins and IL-1β and IL-18 precursors, a process called 'priming'.[32–35] The activation and oligomerization of the NLRP1 and NLRP3 receptors in response to DAMPs, or irregularities within the cellular microenvironment under ischemic conditions, results in the formation of the NLRP1 and NLRP3 inflammasome, which then activates precursor caspase-1 into cleaved caspase-1 through proximity-induced auto-activation facilitating the cleavage of IL-1β and IL-18 precursors to generate their biologically active mature forms.[7,9] Although previous studies have shown that priming is required for the expression of NLRP1 and NLRP3 inflammasome proteins and IL-1β and IL-18 precursor proteins in immune cells,[25,36] it is not known whether inflammasome priming occurs similarly in neurons during ischemic conditions.

We found that when cultured cortical neurons were subjected to ischemia-like conditions (GD or OGD), NLRP1 and NLRP3 proteins were increased, and this was accompanied by elevated levels of cleaved caspase-1 and cleaved caspase-11, and maturation of IL-1β and IL-18 precursor proteins. Furthermore, immunofluorescence data showed expression and localization of these proteins in the cytoplasm of neurons following ischemia. These findings suggest that the NLRP1 and NLRP3 inflammasome complex is formed in neurons following cerebral ischemic damage. In relation to ischemic stroke, no DAMPs have yet been identified that can directly activate the NLRP1 and NLRP3 receptors in neurons. However, evidence suggests that ATP, released from necrotic neurons in culture, and in the ischemic core *in vivo,* binds to P2X7 receptors on the plasma membrane of neighboring neurons and glial cells to cause the opening of ligand-gated ion channels and increased efflux of K^+.[37,38] A decreased intracellular K^+ ion concentration in neurons and glial cells may induce activation of the NLRP1 and NLRP3 receptors, either directly or indirectly through an unknown mechanism.[39] Recent studies have suggested that stimulation of astrocytes with ATP results in activation of the NLRP2 inflammasome, and that ATP-induced activation of the NLRP2 inflammasome was inhibited by a pannexin 1 inhibitor and a P2X7 receptor antagonist.[40,41] The ATP-dependent oligomerization of NLRPs will then promote cleavage of precursor caspase-1 into cleaved caspase-1, which in turn will cleave IL-1β and IL-18 into their mature forms.[39,40,41]

The current data show an increase in levels of the NLRP1 and NLRP3 receptors and in the products of inflammasome activation in cultured neurons subjected to simulated ischemia. However, whether activation of the NLRP1 and NLRP3 inflammasome in neurons under ischemia-like conditions is a result of ATP release from necrotic neurons remains to be determined. The increase in levels of both mature IL-1β and IL-18 under ischemia-like conditions *in vitro* supports findings in which both IL-1β and IL-18 were implicated in causing autocrine and paracrine effects by binding to their respective receptors on the plasma membrane of neighboring neurons and glial cells, and activating NF-κB and MAPK signaling pathways.[42] Accordingly, increased priming would be expected to lead to increased production of NLRP1 and NLRP3 inflammasome proteins and IL-1β and IL-18 precursor proteins. In addition, these pro-inflammatory cytokines will induce secondary injuries by facilitating leukocyte recruitment, which in turn leads to an increased production of reactive oxygen species and pro-inflammatory cytokines, thus exacerbating neuronal cell death and tissue damage during ischemia.[7,41,43]

Our findings indicate increased levels of not only cleaved caspase-1 but also cleaved caspase-11, in neurons under ischemia-like conditions. Recent evidence suggests that cleaved caspase-1 may require the presence of cleaved caspase-11 for the maturation of IL-1β and IL-18 precursors.[44] Cleaved caspase-11 can activate caspase-3 and cause apoptosis in neurons and glial cells under ischemic conditions.[45] Another role of cleaved caspase-1 is to induce apoptotic cell death by cleaving Bid (BH3 Interacting Domain Death Agonist) into its truncated form and activating both executioner caspases-3 and -7, respectively.[46,47] Our data further support the role of the NLRP1 and NLRP3 inflammasome in mediating apoptotic cell death in neurons under ischemic conditions.

It was previously reported that intracerebroventricular administration of the caspase-1 inhibitor Ac-YVAD.CMK reduced brain damage in a rat permanent MCAO stroke model.[47] We found that IVIg and Ac-YVAD.CMK inhibited activation of caspase-3. This link between the inflammasome and apoptotic cascades supports the idea that increased expression levels of cleaved caspase-1 may mediate a number of pleiotropic effects.[13,46,47,48] Besides apoptosis, pyroptosis is another form of cell death directly linked to the inflammasome. Studies suggest that pyroptosis is exclusively regulated by cleaved caspase-1, which induces pore formation in the plasma membrane of cells, thereby allowing an

osmotic influx of water molecules to cause cell lysis and release of pro-inflammatory contents.[13,49] However, whether neurons undergo pyroptosis during cerebral ischemia remains to be determined.

We previously reported that treatment of cultured neurons with IVIg reduced ischemic neuronal cell death, in part, by inhibiting the complement cascade.[21] The present results demonstrate neuroprotective effects of IVIg on ischemia-induced NLRP1 and NLRP3 inflammasome activity in primary cortical neurons. We found evidence that the neuroprotective effects of IVIg are associated with a significant reduction in the levels of NLRP1 and NLRP3 inflammasome proteins as well as precursors of both IL-1β and IL-18 during simulated ischemia *in vitro* and in a mouse model of focal ischemic stroke. IVIg was previously shown to reduce activation of caspase-3 and to protect neurons from undergoing apoptosis under ischemic conditions.[21,24]

Although the molecular basis of the neuroprotective action of IVIg in ischemic stroke-induced NLRP1 and NLRP3 inflammasome activity remains to be established, the present data fit a model in which IVIg inhibits inflammasome priming by decreasing the activity of both intracellular NF-κB and apoptotic MAPK signaling pathways, a possibility consistent with the recent report that IVIg protects neurons from cell death under ischemic conditions by inhibiting the phosphorylation levels of NF-κB, p38, and JNK.[24] This effect would be expected to attenuate the production of NLRP1 and NLRP3 inflammasome proteins and IL-1β and IL-18 precursors, thereby decreasing the production of both cleaved caspase-1 and caspase-11 and hence mature IL-1β and IL-18. Indeed, we found that both IVIg and the caspase-1 inhibitor blocked maturation of IL-1β and IL-18 precursors. We therefore speculate that cleaved caspase-1-dependent pyroptosis and apoptosis would be reduced by IVIg treatment. Consistent with the latter possibility, IVIg can increase the expression of the anti-apoptotic protein Bcl-2 in cultured cortical neurons and in an animal model of stroke[24] (Supplementary Figure 10). Studies have demonstrated that Bcl-2 and Bcl-xL, both anti-apoptotic proteins, directly bind and inhibit the NLRP1 receptor in macrophages by specifically preventing ATP from binding onto the nucleotide-binding domain (NBD) in the NLRP1 receptor.[50,51] Therefore, inhibiting the oligomerization of the NLRP1 receptor is likely to reduce caspase-1 activation and maturation of both IL-1β and IL-18.[50,51] Accordingly, it appears that both Bcl-2 and Bcl-xL are tight regulators of NLRP1 receptor activation; however, whether Bcl-2 and Bcl-xL regulate NLRP3 receptor activation, and how IVIg increases Bcl-2 levels, remain to be determined.

Previous reports have suggested IVIg to be a promising therapeutic modality for targeting a number of injury mechanisms in multiple cell types under ischemic conditions.[19,21,24] The present study has identified a novel mechanism by which IVIg can protect neurons and brain tissue from ischemic damage. These findings suggest that therapeutic interventions targeting inflammasome assembly and activity during ischemia may offer substantial promise. Hence, continued investigation into the mechanisms underlying NLRP1 and NLRP3 inflammasome activity in settings of brain tissue injury and neurodegeneration is warranted.

Materials and Methods

Focal cerebral I/R stroke model. Three-month-old C57BL/6J male mice were subjected to transient middle cerebral artery I/R injury, as described previously.[52] Briefly, after making a midline incision in the neck, the left external carotid and pterygopalatine arteries were isolated and ligated with 6-0 silk thread. The internal carotid artery (ICA) was occluded at the peripheral site of the bifurcation with a small clip and the common carotid artery (CCA) was ligated with 5-0 silk thread. The external carotid artery (ECA) was cut, and a 6-0 nylon monofilament with a tip that was blunted (0.20–0.22 mm) with a coagulator was inserted into the ECA. After the clip at the ICA was removed, the nylon thread was advanced to the origin of the middle cerebral artery until light resistance was evident. The nylon thread and the CCA ligature were removed after 1 h to initiate reperfusion. In the sham group, surgery was performed until the arteries were visualized. Mice were administered with either 10 or 20 mg/kg of a caspase-1 inhibitor (20 μl; Ac-YVAD-CMK, Cayman Chemical, Ann Arbor, MI, USA), 1 g/kg of IVIg (250 μl; Privigen, CSL Behring, King of Prussia, PA, USA, CSL) or vehicle by infusion into the femoral vein 3 h after the start of reperfusion. In a separate set of experiments, anesthetized animals from all groups (5–6 mice per group) underwent cerebral blood flow measurements using a laser Doppler perfusion monitor (Moor Lab, Moor Instruments, Axminster, UK).

The functional consequences of I/R injury were evaluated using a five-point neurological deficit score (0, no deficit; 1, failure to extend right paw; 2, circling to the right; 3, falling to the right; and 4, unable to walk spontaneously) and were assessed in a blinded fashion. At 72 h of reperfusion, the mice were euthanized with a lethal dose of isoflurane. Brains were immediately removed and placed into phosphate-buffered saline (PBS; Sigma-Aldrich, Castle Hill, NSW, Australia) at 4 °C for 15 min, and four 2-mm coronal sections were made from the olfactory bulb to the cerebellum. The brain sections were stained with 2% 2,3,5-triphenyltetrazolium chloride in PBS at 37 °C for 15 min. The stained sections were photographed and the digitized images used for analysis. Borders of the infarct in the image of each brain slice were outlined and the area quantified (NIH Image J software). To correct for brain swelling, the infarct area was determined by subtracting the area of undamaged tissue in the left hemisphere from that of the intact contralateral hemisphere. The infarct volume was determined by calculating the percentage of infarcted area in each brain slice, and then integrating the infarct areas for all slices of each brain. All *in vivo* experimental procedures were approved by The University of Queensland Animal Care and Use Committee.

Primary cortical neuronal cultures. Dissociated neuron-enriched cell cultures of cerebral cortex were established from day 16 C57BL/6J mouse embryos, as described.[53] Experiments were performed in 7–9 day-old cultures. Approximately 95% of the cells in such cultures were neurons, and the remaining cells were astrocytes. For glucose-deprivation studies, glucose-free Locke's buffer containing: 154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5 mM HEPES, pH 7.2, supplemented with gentamicin (5 mg/l) was used. The cultured neurons were incubated in glucose-free Locke's buffer for 1–24 h. Controls were incubated in Locke's buffer containing 10 mM glucose. For OGD, neurons were incubated in glucose-free Locke's buffer in an oxygen-free chamber for 3, 6, or 12 h. For simulated I/R experiments, neurons were incubated in glucose-free Locke's medium in an oxygen-free chamber for 3 h and then the mediumreplaced with glucose-containing Neurobasal medium for 3, 6, 12, or 24 h. To observe the effect of a caspase-1 inhibitor (Ac-YVAD-CMK) or IVIg, either drug were added to cultures during and after GD, OGD or simulated I/R. Control conditions included exposure to vehicle or a negative control protein (bovine serum albumin (BSA)) (Sigma-Aldrich).

Cell viability. Neuronal cell viability was determined by trypan blue exclusion assay. The assay is based on the principle that live cells possess intact cell membranes, which will exclude the dye trypan blue, while the membrane of injured or dead cells is permeable to trypan blue. Hence, injured or dead cells are stained blue whereas live cells will show no staining. Following incubation with trypan blue, the plates were emptied and the cells fixed with 4% paraformaldehyde for 20 min at room temperature. The cells were then washed with PBS three times and stored in PBS for latter observation under a light microscope to quantify the percentage of cells that were trypan blue-positive in each culture.

Western blot analysis. Protein samples were subjected to sodium dodecyl sulfate–polyacrylamide (10%) gel electrophoresis using a Tris-glycine running buffer. Gels were then electro-blotted using a transfer apparatus (Bio-Rad

Laboratories, Inc., Hercules, CA, USA) in transfer buffer containing 0.025 mol/l Tris base, 0.15 mol/l glycine, and 10% (v/v) methanol for 1.5 h at 15 V onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc.). The membrane was then incubated in blocking buffer (5% non-fat milk in 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.2% Tween-20) for 1 h at 23 °C. The membrane was then incubated overnight at 4 °C with primary antibodies including those that selectively bind NLRP1 (Novus Biologicals, Littleton, CO, USA), NLRP3 (Novus Biologicals), ASC (Abcam, Cambridge, UK), caspase-1 (Abcam), caspase-11 (Abcam), XIAP (Novus Biologicals), IL-1β (Abcam), IL-18 (Abcam), Bcl-2 (Cell Signaling Technology, Danvers, MA, USA), cleaved caspase-3 (Cell Signaling Technology) and β-actin (Sigma-Aldrich). After washing three times (10 min per wash) with Tris-buffered saline-T (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.2% Tween-20), the membrane was incubated with secondary antibodies against the primary antibody and β-actin for 1 h at room temperature. The membrane was washed with Tris-Buffered saline-T and scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Quantification of protein levels was achieved by densitometry analysis using Image J software.

Immunocytochemistry and immunohistochemistry. Coverslips containing cortical neurons subjected to either control Neurobasal medium or GD medium were fixed in 4% buffered paraformaldehyde in PBS. Fixed cells were permeabilized and incubated in blocking solution (1% BSA and 0.1% Triton-X in PBS) at room temperature for 1 h before overnight incubation at 4 °C with microtubule-associated protein 2 antibody (MAP2, mouse monoclonal, Millipore, Temecula, CA, USA) along with primary antibodies that selectively bind NLRP1 (Novus Biologicals), ASC (Abcam), caspase-1 (Abcam), caspase-11 (Abcam), IL-1β (Abcam) or IL-18 (Abcam) diluted in blocking solution. Following incubation with primary antibodies, the cells were incubated with the appropriate Alexa Fluor-conjugated secondary antibodies (Invitrogen) for 1 h at room temperature. The nuclei were counterstained with DAPI (AbD Serotec, Oxford, UK) for 10 min at room temperature. Following secondary antibody incubation, coverslips were sealed with Vectashield Fluorescent Mounting Medium (Vector Laboratories, Burlingame, CA, USA) on glass slides. For immunohistochemistry, frozen cryostat brain sections were obtained from sham and focal ischemic stroke mice following trans-cardiac perfusion with 4% paraformaldehyde and immunostained with primary antibodies against NLRP1 (Novus Biologicals), ASC (Abcam), caspase-1 (Abcam), caspase-11 (Abcam), IL-1β (Abcam) or MAP2 (Abcam). Images were acquired using an Olympus BX61 confocal laser-scanning microscope (Olympus, Tokyo, Japan) with a $\times$ 100 oil immersion objective. Single confocal images were converted to 512 $\times$ 512 pixel 12-bit TIFF images.

Patient brain tissue samples. Human brain tissues were obtained from anonymized autopsy patients from files of the Institute of Neuropathology at the University Medical Center Hamburg-Eppendorf and National Taiwan University Hospital, as approved by the National Taiwan University Hospital ethics committee. Brain specimens had been fixed in 4% buffered formalin for at least 3 weeks before paraffin embedding. Brain sections (3 μm) were stained according to standard immunohistochemistry procedures with primary antibodies against NLRP1 (Novus Biologicals), NLRP3 (Abcam), ASC (Abcam), caspase-1 (Abcam), IL-1β (Abcam) or IL-18 (Novus Biologicals).

Statistical analysis. Numerical values are expressed as mean $\pm$ S.E.M. Statistical analysis of all data except the behavioral score data were performed using one-way ANOVA followed by a Bonferroni *post hoc* analysis to determine between-group differences. Statistical difference was taken as $P<0.05$. Neurological behavior scores were analyzed using a non-parametric Kruskal–Wallis test and Dunn's Multiple Comparison test. Statistical analyses were performed using GraphPad Prism 5.02 software.

Conflict of Interest

The authors declare no conflict of interest.

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1. Hou ST, MacManus JP. Molecular mechanisms of cerebral ischemia-induced neuronal death. *Int Rev Cytol* 2002; **221**: 93–148.
2. Broughton BR, Reutens DC, Sobey CG. Apoptotic mechanisms after cerebral ischemia. *Stroke* 2009; **40**: e331–e339.
3. Sims NR, Muyderman H. Mitochondria, oxidative metabolism and cell death in stroke. *Biochim Biophys Acta* 2010; **1802**: 80–91.
4. Gelderblom M, Leypoldt F, Steinbach K, Behrens D, Choe CU, Siler DA *et al.* Temporal and spatial dynamics of cerebral immune cell accumulation in stroke. *Stroke* 2009; **40**: 1849–1857.
5. Iadecola C, Anrather J. Stroke research at a crossroad: asking the brain for directions. *Nat Neurosci* 2011; **14**: 1363–1368.
6. Lamkanfi M, Dixit VM. Inflammasomes and their roles in health and disease. *Annu Rev Cell Dev Biol* 2012; **28**: 137–161.
7. Abulafia DP, de Rivero Vaccari JP, Lozano JD, Lotocki G, Keane RW, Dietrich WD *et al.* Inhibition of the inflammasome complex reduces the inflammatory response after thromboembolic stroke in mice. *J Cereb Blood Flow Metab* 2009; **29**: 534–544.
8. Boyden ED, Dietrich WF. Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nat Genet* 2006; **38**: 240–244.
9. De Rivero Vaccari JP, Lotocki G, Alonso OF, Bramlett HM, Dietrich WD, Keane RW *et al.* Therapeutic neutralization of the NLRP1 inflammasome reduces the innate immune response and improves histopathology after traumatic brain injury. *J Cereb Blood Flow Metab* 2009; **29**: 1251–1261.
10. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 2002; **10**: 417–426.
11. Andrei C, Margiocco P, Poggi A, Lotti LV, Torrisi MR, Rubartelli A *et al.* Phospholipases C and A2 control lysosome-mediated IL-1 beta secretion: Implications for inflammatory processes. *Proc Natl Acad Sci USA* 2004; **101**: 9745–9750.
12. Brough D, Rothwell NJ. Caspase-1-dependent processing of pro-interleukin-1beta is cytosolic and precedes cell death. *J Cell Sci* 2007; **120**: 772–781.
13. Fink SL, Bergsbaken T, Cookson BT. Anthrax lethal toxin and Salmonella elicit the common cell death pathway of caspase-1-dependent pyroptosis via distinct mechanisms. *Proc Natl Acad Sci USA* 2008; **105**: 4312–4317.
14. Sagulenko V, Thygesen SJ, Sester DP, Idris A, Cridland JA, Vajjhala PR *et al.* AIM2 and NLRP3 inflammasomes activate both apoptotic and pyroptotic death pathways via ASC. *Cell Death Differ* 2013; **20**: 1149–1160.
15. Mastronardi C, Whelan F, Yildiz OA, Hannestad J, Elashoff D, McCann SM *et al.* Caspase 1 deficiency reduces inflammation-induced brain transcription. *Proc Natl Acad Sci USA* 2007; **104**: 7205–7210.
16. Jedrzejowska-Szypułka H, Larysz-Brysz M, Kukla M, Snietura M, Lewin-Kowalik J. Neutralization of interleukin-1beta reduces vasospasm and alters cerebral blood vessel density following experimental subarachnoid hemorrhage in rats. *Curr Neurovasc Res* 2009; **6**: 95–103.
17. Gelfand EW. Intravenous immune globulin in autoimmune and inflammatory diseases. *N Engl J Med* 2012; **367**: 2015–2025.
18. Schwab I, Nimmerjahn F. Intravenous immunoglobulin therapy: how does IgG modulate the immune system? *Nat Rev Immunol* 2013; **13**: 176–189.
19. Arumugam TV, Selvaraj PK, Woodruff TM, Mattson MP. Targeting ischemic brain injury with intravenous immunoglobulin. *Expert Opin Ther Targets* 2008; **12**: 19–29.
20. Arumugam TV, Woodruff TM, Lathia JD, Selvaraj PK, Mattson MP, Taylor SM *et al.* Neuroprotection in stroke by complement inhibition and immunoglobulin therapy. *Neuroscience* 2009; **158**: 1074–1089.
21. Arumugam TV, Tang SC, Lathia JD, Cheng A, Mughal MR, Chigurupati S *et al.* Intravenous immunoglobulin (IVIG) protects the brain against experimental stroke by preventing complement-mediated neuronal cell death. *Proc Natl Acad Sci USA* 2007; **104**: 14104–14109.
22. Lux A, Aschermann S, Biburger M, Nimmerjahn F. The pro and anti-inflammatory activities of immunoglobulin G. *Ann Rheum Dis* 2010; **69**: i92–i96.
23. Walberer M, Nedelmann M, Ritschel N, Mueller C, Tschernatsch M, Stolz E *et al.* Intravenous immunoglobulin reduces infarct volume but not edema formation in acute stroke. *Neuroimmunomodulation* 2010; **17**: 97–102.
24. Widiapradja A, Vegh V, Lok KZ, Manzanero S, Thundyil J, Gelderblom M *et al.* Intravenous immunoglobulin protects neurons against amyloid beta-peptide toxicity and ischemic stroke by attenuating multiple cell death pathways. *J Neurochem* 2012; **122**: 321–332.
25. Bauernfeind F, Ablasser A, Bartok E, Kim S, Schmid-Burgk J, Cavlar T *et al.* Inflammasomes: current understanding and open questions. *Cell Mol Life Sci* 2011; **68**: 765–783.
26. Fogal B, Li J, Lobner D, McCullough LD, Hewett SJ. System x(c)- activity and astrocytes are necessary for interleukin-1 beta-mediated hypoxic neuronal injury. *J Neurosci* 2007; **27**: 10094–10105.
27. Yuen CM, Chiu CA, Chang LT, Liou CW, Lu CH, Youssef AA *et al.* Level and value of interleukin-18 after acute ischemic stroke. *Circ J* 2007; **71**: 1691–1696.
28. Caso JR, Pradillo JM, Hurtado O, Lorenzo P, Moro MA, Lizasoain I *et al.* Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke. *Circulation* 2007; **115**: 1599–1608.
29. Tang SC, Wang YC, Li YI, Lin HC, Manzanero S, Hsieh YH *et al.* Functional role of soluble receptor for advanced glycation end products in stroke. *Arterioscler Thromb Vasc Biol* 2013; **33**: 585–594.

30. Tang SC, Arumugam TV, Xu X, Cheng A, Mughal MR, Jo DG *et al.* Pivotal role for neuronal Toll-like receptors in ischemic brain injury and functional deficits. *Proc Natl Acad Sci USA* 2007; **104**: 13798–13803.
31. Pradillo JM, Denes A, Greenhalgh AD, Boutin H, Drake C, McColl BW *et al.* Delayed administration of interleukin-1 receptor antagonist reduces ischemic brain damage and inflammation in comorbid rats. *J Cereb Blood Flow Metab* 2012; **32**: 1810–1819.
32. Gross O, Thomas CJ, Guarda G, Tschopp J. The inflammasome: an integrated view. *Immunol Rev* 2011; **243**: 136–151.
33. Taxman DJ, Holley-Guthrie EA, Huang MT, Moore CB, Bergstralh DT, Allen IC *et al.* The NLR adaptor ASC/PYCARD regulates DUSP10, mitogen-activated protein kinase (MAPK), and chemokine induction independent of the inflammasome. *J Biol Chem* 2011; **286**: 19605–19616.
34. Zheng Y, Lilo S, Brodsky IE, Zhang Y, Medzhitov R, Marcu KB *et al.* A Yersinia effector with enhanced inhibitory activity on the NF-κB pathway activates the NLRP3/ASC/caspase-1 inflammasome in macrophages. *PLoS Pathog* 2011; **7**: e1002026.
35. He Q, You H, Li XM, Liu TH, Wang P, Wang BE *et al.* HMGB1 promotes the synthesis of pro-IL-1β and pro-IL-18 by activation of p38 MAPK and NF-κB through receptors for advanced glycation end-products in macrophages. *Asian Pac J Cancer Prev* 2012; **13**: 1365–1370.
36. Savage CD, Lopez-Castejon G, Denes A, Brough D. NLRP3-inflammasome activating DAMPs stimulate an inflammatory response in glia in the absence of priming which contributes to brain inflammation after injury. *Front Immunol* 2012; **3**: 288.
37. De Rivero Vaccari JP, Bastien D, Yurcisin G, Pineau I, Dietrich WD, De Koninck Y *et al.* P2 × 4 receptors influence inflammasome activation after spinal cord injury. *J Neurosci* 2012; **32**: 3058–3066.
38. Ferrari D, Pizzirani C, Adinolfi E, Lemoli RM, Curti A, Idzko M *et al.* The P2 × 7 receptor: a key player in IL-1 processing and release. *J Immunol* 2006; **176**: 3877–3883.
39. Petrilli V, Papin S, Dostert C, Mayor A, Martinon F, Tschopp J *et al.* Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ* 2007; **14**: 1583–1589.
40. Minkiewicz J, de Rivero Vaccari JP, Keane RW. Human astrocytes express a novel NLRP2 inflammasome. *Glia* 2013; **61**: 1113–1121.
41. Silverman WR, de Rivero Vaccari JP, Locovei S, Qiu F, Carlsson SK, Scemes E *et al.* The pannexin 1 channel activates the inflammasome in neurons and astrocytes. *J Biol Chem* 2009; **284**: 18143–18151.
42. Srinivasan D, Yen JH, Joseph DJ, Friedman W. Cell type-specific interleukin-1beta signaling in the CNS. *J Neurosci* 2004; **24**: 6482–6488.
43. De Rivero Vaccari JP, Lotocki G, Marcillo AE, Dietrich WD, Keane RW *et al.* A molecular platform in neurons regulates inflammation after spinal cord injury. *J Neurosci* 2008; **28**: 3404–3414.
44. Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J *et al.* Non-canonical inflammasome activation targets caspase-11. *Nature* 2011; **479**: 117–121.
45. Kang SJ, Wang S, Hara H, Peterson EP, Namura S, Amin-Hanjani S *et al.* Dual role of caspase-11 in mediating activation of caspase-1 and caspase-3 under pathological conditions. *J Cell Biol* 2000; **149**: 613–622.
46. Guegan C, Vila M, Teismann P, Chen C, Onténiente B, Li M *et al.* Instrumental activation of bid by caspase-1 in a transgenic mouse model of ALS. *Mol Cell Neurosci* 2002; **20**: 553–562.
47. Zhang WH, Wang X, Narayanan M, Zhang Y, Huo C, Reed JC *et al.* Fundamental role of the Rip2/caspase-1 pathway in hypoxia and ischemia-induced neuronal cell death. *Proc Natl Acad Sci USA* 2003; **100**: 16012–16017.
48. Rabuffetti M, Sciorati C, Tarozzo G, Clementi E, Manfredi AA, Beltramo M *et al.* Inhibition of caspase-1-like activity by Ac-Tyr-Val-Ala-Asp-chloromethyl ketone induces long-lasting neuroprotection in cerebral ischemia through apoptosis reduction and decrease of proinflammatory cytokines. *J Neurosci* 2000; **20**: 4398–4404.
49. Fink SL, Cookson BT. Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. *Cell Microbiol* 2006; **8**: 1812–1825.
50. Bruey JM, Bruey-Sedano N, Luciano F, Zhai D, Balpai R, Xu C *et al.* Bcl-2 and Bcl-XL regulate proinflammatory caspase-1 activation by interaction with NALP1. *Cell* 2007; **129**: 45–56.
51. Faustin B, Chen Y, Zhai D, Le Negrate G, Lartigue L, Satterthwait A *et al.* Mechanism of Bcl-2 and Bcl-X(L) inhibition of NLRP1 inflammasome: loop domain-dependent suppression of ATP binding and oligomerization. *Proc Natl Acad Sci USA* 2009; **106**: 3935–3940.
52. Arumugam TV, Salter JW, Chidlow JH, Ballantyne CM, Kevil CG, Granger DN *et al.* Contributions of LFA-1 and Mac-1 to brain injury and microvascular dysfunction induced by transient middle cerebral artery occlusion. *Am J Physiol Heart Circ Physiol* 2004; **287**: H2555–H2560.
53. Okun E, Arumugam TV, Tang SC, Gleichmann M, Albeck M, Sredni B *et al.* The organotellurium compound ammonium trichloro(dioxoethylene-0,0') tellurate enhances neuronal survival and improves functional outcome in an ischemic stroke model in mice. *J Neurochem* 2007; **102**: 1232–1241.

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