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The neurotoxic effect of astrocytes activated with toll-like receptor ligands

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ABSTRACT

Toll-like receptors (TLRs) are key molecules in the innate immune system in the central nervous system. Although astrocytes are believed to play physiological roles in regulating neuronal activity and synaptic transmission, activated astrocytes may also be toxic to neurons. Here, we show that the ligands for TLRs 2, 4, 5 and 6 induce neuronal cell death in neuron–astrocytes co-cultures through the production of reactive oxygen species (ROS). Inhibition of ROS production by NADPH oxidase inhibitor apocynin significantly suppresses neuronal cell death. ROS induced in astrocytes via TLRs may be involved in neuroinflammation and a therapeutic target for neurotoxicity by activated astrocytes.

1. Introduction

Astrocytes, the major glial cells in the central nervous system (CNS), have a variety of physiological roles in brain functions, including nutritional support for neurons and brain endothelial cells, maintenance of extracellular ion balance, glutamate uptake via glutamate transporters and tissue repair. Astrocytes attenuate microglial activation by reducing microglial production of inflammatory molecules such as interleukin-12 (IL-12), reactive oxygen species (ROS), and inducible nitric oxide synthase (iNOS) (Welser and Milner, 2012).

In contrast, activated astrocytes also play pro-inflammatory roles as innate immune cells. They produce various inflammatory mediators such as tumor necrosis factor α (TNF-α), IL-1β and IL-6, and amplify the local inflammatory reaction (Farina et al., 2007). In response to tissue damage, astrocytes become reactive and proliferate to form the glial scar, gliosis. Axonal regeneration is inhibited by chondroitin sulfate proteoglycans (CSPGs) derived from these reactive astrocytes (Yu et al., 2012). Thus, astrocytes are dual edged sword as microglia.

Toll-like receptors (TLRs) play pivotal roles in the recognition of pathogen-specific patterns and the subsequent initiation of innate and adaptive immune responses (Medzhitov and Janeway, 2000). TLRs are expressed in microglia and astrocytes (Farina et al., 2007). TLRs 2, 3, 4 and 8 are expressed in neurons (van Noort and Bsibsi, 2009). TLR stimulation promotes microglial activation, especially TLR4 stimulation induces neurotoxic molecules such as inflammatory cytokines, glutamate and ROS in microglia, which damage the neurons (Takeuchi et al., 2005; Polazzi and Contestabile, 2006; Yang et al., 2008). While TLR4 signal also induces neurotoxic molecules in astrocytes (Mizuno et al., 2005), the neurotoxicity by activated astrocytes with TLR stimulation remains largely unknown. In this study, we examined the expression of TLRs in astrocytes, neurotoxic functions by astrocytes activated with ligand for TLRs 1–9 in neuron–astrocytes co-cultures, and the major neurotoxic molecules derived from astrocytes.

2. Materials and methods

2.1. Cell cultures

2.1.1. Astrocytes cultures

The protocols for animal experiments were approved by the Animal Experiment Committee of Nagoya University. Astrocyte cultures were prepared from the primary mixed glial cell cultures of newborn C57BL/6 mice (SLC, Shizuoka, Japan), as described previously (Suzumura et al., 1987). Astrocytes were purified from the primary mixed glial cell cultures by three to four repetitions of trypsinization and replating. The purity of astrocytes was greater than 95% astrocytes when examined by indirect immunofluorescence staining with an anti-GFAP antibody. Cultures were maintained with Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 5 μg/ml bovine insulin, and 0.2% glucose. Astrocytes were grown to confluency and the medium was exchanged every 3 days.

2.1.2. Neuron-astrocytes co-cultures

Primary neuronal cultures were prepared from the cortices of embryonic day 17 (E17) C57BL/6 mice embryos as described previously (Doi et al., 2009). Briefly, cortical fragments were dissociated into single

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cells in dissociation solution (Sumitomo Bakelite, Akita, Japan) and re-suspended in Nerve Culture Medium (Sumitomo Bakelite). Neurons were plated onto 12 mm polyethyleneimine (PEI)-coated glass coverslips (Asahi Techno Glass, Chiba, Japan) at a density of 5×10^4 cells/well in 24-well multidishes and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The purity of the cultures was >95% as determined by NeuN-specific immunostaining. For neuron–astrocytes co-cultures, confluent monolayer astrocytes were grown on 12 mm PEI-coated cover glass in 24-well multidishes. Neuronal cells (5×10^4 cells/well) in 10 μl neuron medium were added to astrocytes cultures. After 12 days, cultures were stimulated with TLR 1–9 ligands for 48 h.

2.2. Measurement of IL-1β, TNF-α, NO, and glutamate

To measure molecules produced by astrocytes activated with TLR 1–9 ligands, astrocytes were plated at a density of 3×10^4 cells/well (200 μl) in 96-well multidishes and then treated with ligands for TLRs 1–9 (InvivoGen, San Diego, CA, USA).

TLR1 ligand: Pam3CSK4 (0.1, 1, and 10 μg/mL)
TLR2 ligand: HKLM (1×10^7, 1×10^8, and 1×10^9 cells/ mL)
TLR3 ligand: Poly (I:C) (0.1, 1, 10, and 100 μg/mL)
TLR4 ligand: LPS (0.1, 1, and 10 μg/mL)
TLR5 ligand: ST-FLA (0.1, 1, and 10 μg/mL)
TLR6 ligand: FSL1 (0.1, 1, and 10 μg/mL)
TLR7/8 ligand: ssRNA40/LyoVec (0.1, 1, and 10 μg/mL)
TLR9 ligand: CpG (0.1, 1, and 10 μg/mL)

After 48 h of treatment with TLR ligands, supernatants from astrocytes were assessed by ELISA kits for TNF-α and IL-1β (BD Pharmingen, Franklin Lakes, NJ, USA). Measurement of NO was determined using the Griess reaction. To measure glutamate, Glutamate Assay Kit colorimetric assay (Yamasa, Tokyo, Japan) was used as described previously (Doi et al., 2009).

2.3. Measurement of ROS

To measure ROS, we used a novel cell-permeant dye CellROX™ Deep Red reagent (Invitrogen). Astrocytes were treated with or without 100 μM apocynin (Calbiochem, San Diefo, CA, USA) for 1 h, and then treated with TLR 1–9 ligands for 1 h. 5 μM CellROX™ Deep Red reagent was added to the cultures for 30 min. After washing with PBS, the fluorescence with 630/665 nm was measured using a Wallac 1420 ARVOMX (PerkinElmer Japan, Yokohama, Japan). Live cell staining for ROS was also performed in astrocytes activated with TLR ligands using ROS sensor CellROX™ Deep Red reagent (red) and the astrocyte cell surface marker anti-GLAST (Miltenyi Biotec Gmbh, Bergisch Gladbach, Germany) (green) with a deconvolution fluorescent microscopy.

2.4. Immunocytochemistry

Neuron–astrocyte co-cultures were fixed with 4% paraformaldehyde for 30 min at room temperature, then blocked with 5% normal goat serum in PBS and permeabilized with 0.3% Triton X-100. Neurons were stained with mouse polyclonal anti-microtubule-associated protein (MAP)-2 antibody (1:1000; Chemicon, Temecula, CA, USA) and secondary antibodies conjugated to Alexa 488 (1:1000; Invitrogen). Astrocytes were stained with phycoerythrin-conjugated rat anti-mouse GFAP monoclonal antibody (1:1000; BD Pharmingen) before fixation. Images were analyzed with a deconvolution fluorescent microscope system (BZ-8000; Keyence, Osaka, Japan). To assess neuronal death induced by astrocytes stimulated with TLR 1–9 ligands, purified neurons (5×10^4 cells/well) were plated in 24-well multidishes. TLR 1–9 ligands were added to the cultures on day 13 in vitro for 48 h. Surviving neurons were identified by MAP-2 staining as described previously (Doi et al., 2009). Viable neurons were stained strongly with an anti-MAP-2 antibody, whereas damaged and dying neurons were little stained. The number of MAP-2-positive neurons was counted in representative areas per well. More than 200 neurons were examined in each of five independent trials by a scorer blind to the experimental condition. Neuronal survival rate was quantified as follows: the number of MAP-2-positive survival neurons / the number of non-treated healthy neurons.

2.5. Western blotting

For detection of TLR protein, cell lysates of mouse cortical neurons and astrocytes were obtained from primary cultures, respectively. Neuronal cells seeded for 5×10^5 cells/well and astroctyes seeded for 1×10^6 cells/well were harvested in 500 μl of solution containing 50 mM Tris–HCl (pH 7.6), 0.01% NP-40, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, and protease inhibitor cocktail. Soluble, extracellular-enriched proteins were collected from mechanically homogenized lysates following centrifugation for 5 min at 3000 rpm. Collected samples were mixed with sample buffer (200 mmol/L Tris–HCl, 8% SDS, and 1% glycerol). Proteins were separated on a 5 to 20% Tris–glycine SDS–polyacrylamide gel and transferred to Hybond-P polyvinylidene difluoride membrane (GE Healthcare UK, Buckinghamshire, UK). Membranes were blocked with 1% skim milk in Tris-buffered saline (TBS) containing 0.05% Tween20 (TBS-T). Blots were incubated in the first antibody diluted in 1% skim milk overnight at 4 °C. Antibodies for TLRs 1, 4, 5, 7 and 9 were obtained from Imgenex (San Diego, CA, USA), and antibodies for TLRs 2, 3, 6 and 8 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Subsequently, membranes were washed in TBS-T 3×5 min and incubated with a horseradish peroxidase-conjugated second antibody diluted in 1% skim milk for 1 h. After washing in TBS-T for 1×15 min, 2×5 min, and TBS for 1×5 min, signals were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL). The intensity of the bands was calculated by using CS Analyzer 1.0 (Atto, Tokyo, Japan).

2.6. Statistical analysis

Statistical significance of the experiments was assessed with one-way analysis of variance, followed by post hoc Tukey test using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA).

3. Results

3.1. The neurotoxic effect of astrocytes activated with ligands for TLRs 1–9

First, we examined TLR expression in astrocytes and neurons by Western blot analysis. TLRs 1, 2, 4, 5, 6, 7 and 9 were expressed in astrocytes, while the expression of TLRs 3 and 8 was weak. In neurons, TLRs 4, 5 and 9 were clearly expressed, however, the expression of TLRs 2, 3, 6 and 8 was weak, and TLRs 1 and 7 were not detected (Fig. 1A). We examined the neurotoxicity of astrocytes activated with TLR 1–9 ligands in neuron–astrocytes co-cultures using immunocytochemistry (Fig. 1B). Neurons stained with anti-MAP-2 antibody (green) and astrocytes stained with anti-GFAP antibody (red) had no detectable morphologic abnormalities in unstimulated co-cultures (NT), while both addition of 100 μM glutamate, which are shown as positive control, induced neuronal cell death (Glu 100). Astrocytes were not damaged by glutamate. When the co-cultures were stimulated with TLR 1–9 ligands, 1×10^9 cells/mL HKLM (TLR2), 10 μg/mL LPS (TLR4), 10 μg/mL ST-FLA (TLR5) and 10 μg/mL FSL-1 (TLR6), neuronal damage was observed. However, 10 μg/mL Pam3CSK4 (ligand for TLR1), 100 μg/mL Poly (I:C) (TLR3), 10 μg/mL ssRNA40 (TLR7/8) and 10 μg/mL CpG (TLR9) did not induce neuronal cell death. The survival rate of neurons was quantified (Fig. 1C). Treatment with the ligands for TLR2, 4, 5 or 6 significantly decreased the number of survival neurons. We next examined the direct toxic effect of TLR 1–9 ligands on neuronal cells. Neuronal cell death was not induced by these ligands (Fig. 2).

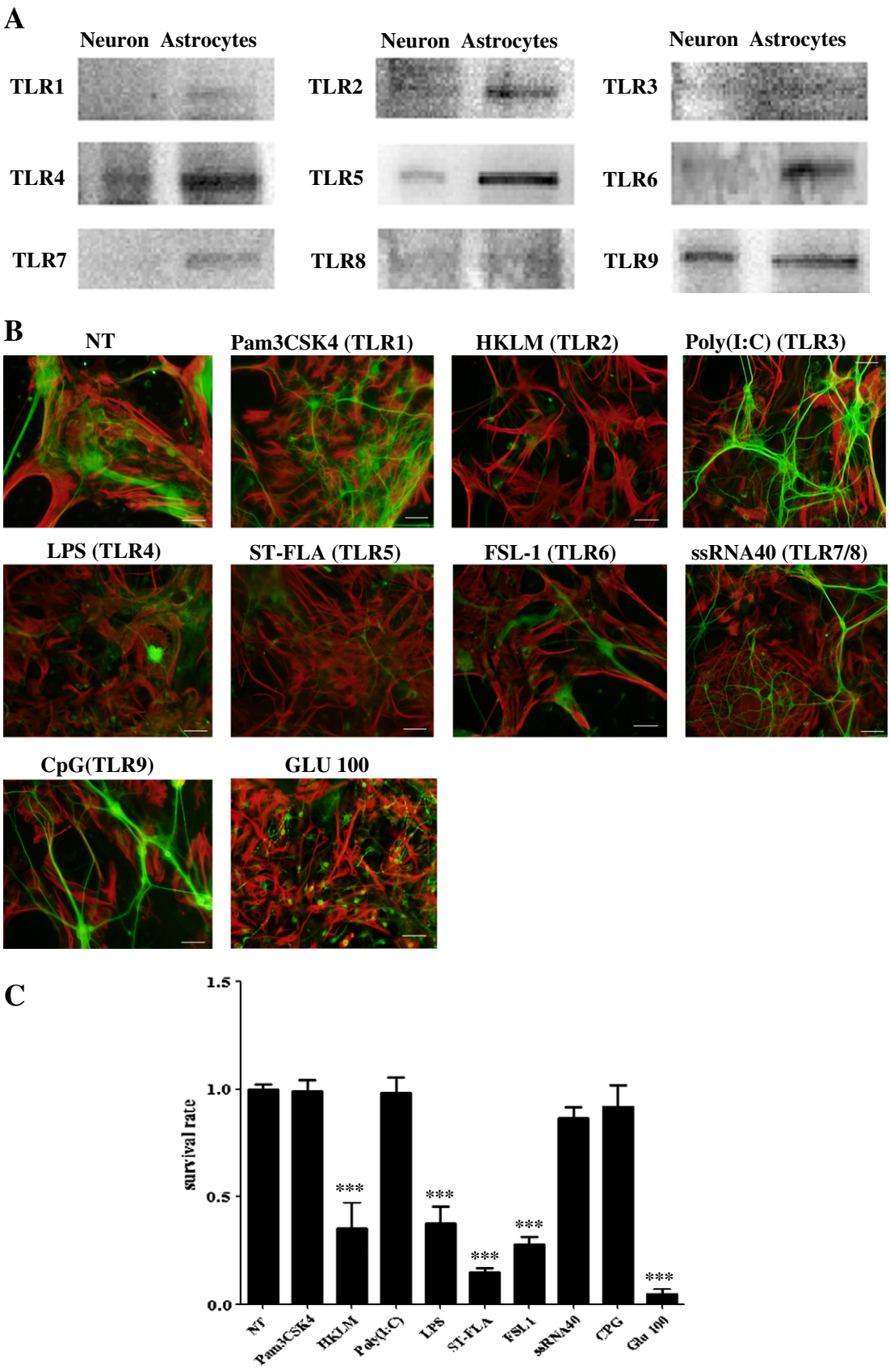

Fig. 1. The neurotoxic effect of astrocytes activated with ligands for TLRs 1–9. (A) The Western blot analysis of TLRs in astrocytes and neurons. (B) Representative deconvolution fluorescent images of neuron–astrocytes co-cultures. Neurons were stained with an anti-MAP-2 antibody (green). Astrocytes were stained with anti-GFAP antibody (red). Co-cultures were treated with TLR 1–9 ligands, or 100 μM glutamate (GLU 100) for 48 h. Scale bar = 50 μm. (C) Neuronal survival rate was quantified. The viability of non-treated neurons (NT) was normalized to 1.0. Each column indicates the mean ± SEM (n = 5). ***, $P<0.001$ as compared to the survival rate of non-treated co-cultures.

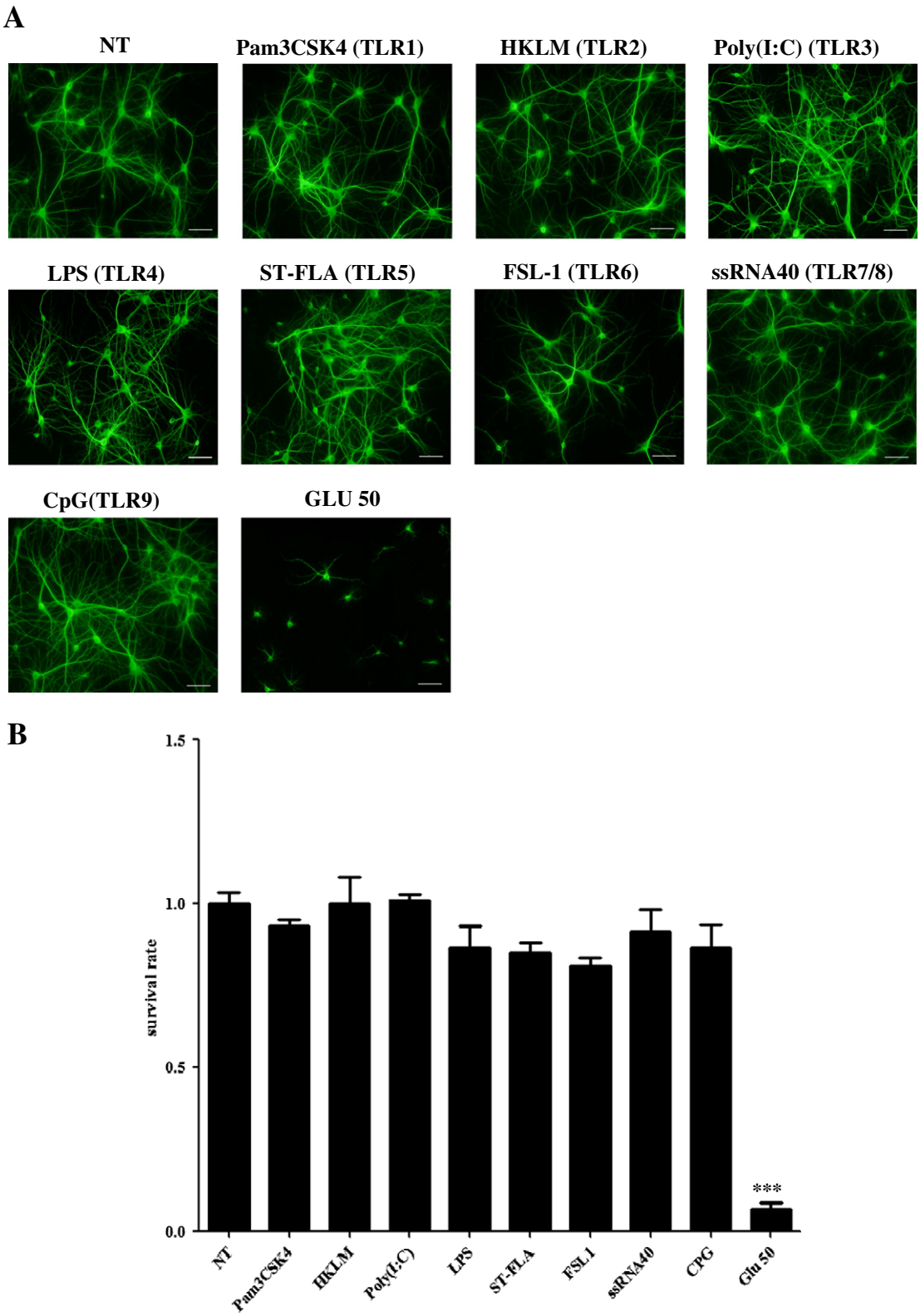

Fig. 2. The effect of TLR 1–9 ligands on neurons. (A) Representative deconvolution fluorescence images of neuron cultures. Neurons were stained with an anti-MAP-2 antibody (green). Neuron cultures were treated with TLR 1–9 ligands, or 50 μM glutamate (GLU 50) for 48 h. Scale bar = 50 μm. (B) Neuronal survival rate was quantified. The viability of non-treated neurons (NT) was normalized to 1.0. Each column shows the mean ± SEM (n = 5). ***, $P<0.001$ compared to the survival rate of non-treated neuron cultures.

3.2. The production of inflammatory molecules by astrocytes activated with various TLR ligands

In order to identify the inflammatory molecules produced by TLR ligand-activated astrocytes, release of nitric oxide (NO), glutamate and pro-inflammatory cytokines including tumor necrosis factor α (TNF-α), and IL-1β were examined. The production of NO, measured as nitrite, was enhanced by Pam3CSK4, HKLM, LPS and ST-FLA. Glutamate was induced by HKLM, Poly (I:C), LPS, ST-FLA, FSL1, ssRNA40 and CpG. IL-1β was mainly enhanced by HKLM, LPS, and ST-FLA. TNF-α was induced by HKLM and LPS (Fig. 3A). We examined that the neurotoxicity induced by astrocytes activated with TLR2, 4, 5, or 6 ligand is suppressed by the inhibitors of these molecules: NO inhibitor L-NMMA, a highly selective non-competitive NMDA receptor antagonist MK801, anti-TNF-α antibody, and anti-IL-1β receptor antibody. For example, 10 μg/ml anti-TNF-α antibody did not suppress the neuronal cell death induced by astrocytes activated with LPS, and 10 μg/ml IL-1β receptor antibody also did not suppress the cell death by ST-FLA (Fig. 3B). Other inhibitors did not show the suppressive effect (data not shown). The contamination of microglia was less than 1% by immunostaining evaluation.

3.3. The production of ROS was correlated with neuronal cell death by astrocytes activated with TLR ligands

Next, we examined ROS production in astrocytes activated with TLR ligands. Live cell staining was performed using ROS sensor CellROX™ Deep Red reagent (red) and the astrocyte cell surface marker GLAST (green) with a deconvolution fluorescent microscopy. ROS was not detected in unstimulated astrocytes. 1×10^9 cells/mL HKLM, 10 μg/mL LPS, 10 μg/mL ST-FLA and 10 μg/mL FSL-1 induced ROS production, while 10 μg/mL Pam3CSK4, 100 μg/mL Poly (I:C), 10 μg/mL ssRNA40 and 10 μg/mL CpG did not (Fig. 4A). ROS production was quantified by the fluorescence microplate reader. HKLM, LPS, ST-FLA and FSL-1 significantly increased ROS production (Fig. 4B). Thus, the production of ROS was correlated with neuronal cell death.

3.4. The NADPH oxidase inhibitor apocynin suppressed neuronal cell death induced by astrocytes activated with TLRs 2, 4, 5 and 6 via ROS inhibition

Then, we examined whether ROS inhibition suppresses neuronal cell death induced by astrocytes activated with TLRs 2, 4, 5 and 6. Treatment of NADPH oxidase inhibitor apocynin (100 μM) inhibited neuronal cell death induced by 1×10^9 cells/mL HKLM, 10 μg/mL LPS, 10 μg/mL ST-FLA and 10 μg/mL FSL-1 in neuron–astrocytes co-cultures (Fig. 5A). The survival rate of neurons was quantified. Treatment of apocynin significantly suppressed neuronal cell death by HKLM, LPS, ST-FLA and FSL-1 (Fig. 5B). Apocynin had no effect on glutamate neurotoxicity. ROS production by these TLR ligands was also significantly inhibited by apocynin as determined by the fluorescence microplate reader (Fig. 5C).

4. Discussion

Astrocytes play important roles to support neurons by uptaking neurotransmitter, supplying glucose for neuronal activities, and regulation of extracellular environment, including free radical scavenging and homeostasis of H^+ (Sidoryk-Wegrzynowicz et al., 2011). On the other hand, astrocytes also play a role in innate immunity by producing a variety of cytokines and chemokines in response to TLR ligands. These activated astrocytes are major component of neuroinflammation, and may also participate in the processes of neuronal degeneration.

In this study, we show that astrocytes become neurotoxic when activated with ligands for TLRs 2, 4, 5 and 6 in neuron–astrocytes co-cultures. Previous studies showed that neuronal TLRs 2 and 4 signaling induce apoptotic neuronal death in ischemia/reperfusion injury (Tang et al., 2007) and TLR3 signaling triggers growth cone collapse and irreversibly inhibits neurite extension (Cameron et al., 2007). In addition, TLR8 signaling reportedly inhibits neurite outgrowth and induces apoptosis (Ma et al., 2006). Thus, we investigated the effect of TLR ligands on neuronal cells. Unexpectedly, all the TLR ligands did not induce neuronal cell death in primary cortical neuronal cultures. Therefore, neurotoxicity in astorocyte–neuron coculture may be induced by factors from activated astrocytes, but not by direct effects on neurons. The reason why all the TLR ligands failed to induce damage of neurons is unclear at this moment. It may come from different culture conditions or different origins of neurons.

TLR expression on astrocytes is reported to be enhanced when inflammation develops (Bsibsi et al., 2002). Therefore, it is possible that neurotoxicity by astrocytes is upregulated in inflammatory conditions in the CNS. In addition, since we have shown that microglia respond to TLR ligands faster than astrocytes and produce more inflammatory cytokines (Sawada et al., 1992), it is possible that microglial activation may synergistically function with astrocytes to damage neurons in neuroinflammation.

The present study shows that ligands for TLRs 2, 4, 5 and 6 induce various neurotoxic molecules such as NO, glutamate, IL-1β, IL-6, TNF-α, and ROS in astrocytes. The excessive glutamate results in neuronal death, which is involved in the pathogenesis of various neurological disorders including inflammation, ischemia, and neurodegenerative diseases (Schwartz et al., 2003; Bruijn et al., 2004). However, the production of NO and glutamate was too little to induce neuronal cell death in this study. NO inhibitor L-NMMA and NMDA receptor antagonist MK801 did not show the neuroprotective effect. IL-1β is reported to inhibit glutamate transport by astrocytes in the spinal cord during viral encephalomyelitis (Prow and Irani, 2008). While TNF-α potentiates glutamate excitotoxicity on motor neurons (Tolosa et al., 2011), we demonstrated previously that TNF-α secreted by astrocytes also functions on astrocytes in an autocrine manner to further upregulate neurotrophic factors such as nerve growth factor and glial cell line-derived neurotrophic factor (Kuno et al., 2006). In this study TNF-α and IL-1β were highly induced by TLR4 and 5 ligands, respectively. However, the neuroprotective effects of inhibitory antibodies were not found.

Our data indicated that ROS is the most possible candidate for neuronal cell death caused by TLR ligand-activated astrocytes, because activation of astrocytes with TLR ligands 2, 4, 5 and 6 commonly induce the production of ROS. Suppression of neurotoxicity by NADPH oxidase inhibitor apocynin in neuron–astrocytes co-cultures also support the notion. Because ROS is produced through the NF-κB regulated phagocyte NADPH oxidase (Barakat et al., 2012), the brain is vulnerable to oxidative stress. ROS-induced damage is a common feature of neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD). ROS is also reported to alter microglial activity from beneficial to chronic detrimental neuroinflammation (Rojo et al., 2010). TLR4 signal induces amyloid β (Aβ) production by astrocytes (Zhao et al., 2011). Astrocytes activated with Aβ produce IL-1β, TNF-α, and ROS (Johnstone et al., 1999). Oligomeric Aβ also induces neuronal ROS through a mechanism requiring NMDA receptor activation (De Felice et al., 2007). Oxidative stress is obviously involved in the pathogenesis of AD (Ansari and Scheff, 2010) and PD (Tretter et al., 2004). These findings suggest the possibility that ROS produced by astrocytes may be involved in pathogenesis of neurodegeneration either directly or indirectly. TLRs 3, 7, 8 and 9 are localized in endosomes where they detect nucleic acids. The ligands for these receptors did not induce neurotoxic effect via ROS in astrocytes in the present study. The pathway for ROS production may be different between endosomal TLRs and cell surface TLRs. Further studies are needed to elucidate such differences. Recently, ROS production through TLR4 is reported to be involved in spleen tyrosine kinase (SYK) signaling. SYK binds to the cytoplasmic domain of TLR4. Phosphorylated SYK induces PKC activation, which contributes to Nox2-dependent ROS generation (Miller et al., 2012). SYK signaling in TLRs 2, 5 and 6 may also be involved in ROS production.

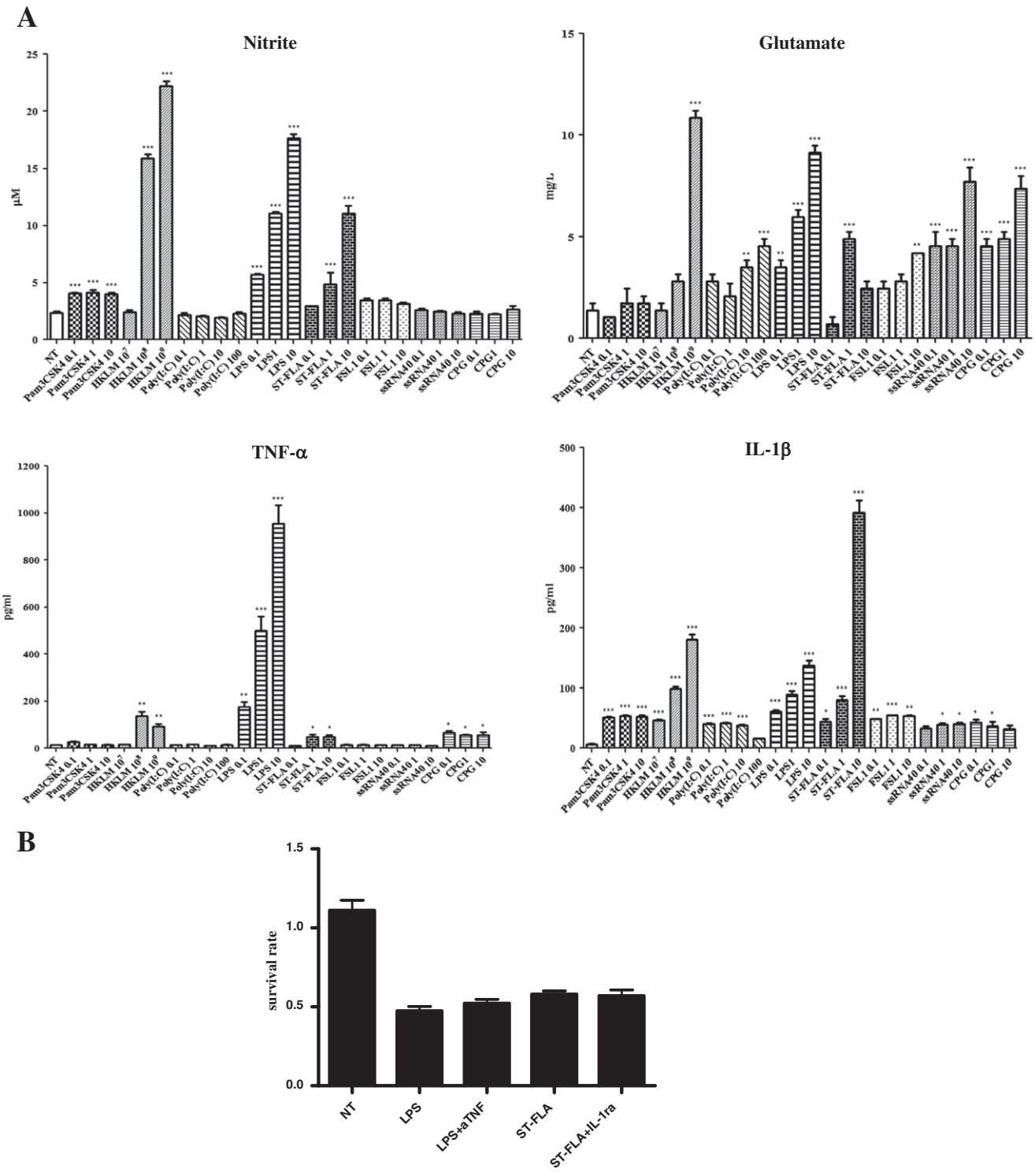

Fig. 3. The production of inflammatory molecules by astrocytes activated with ligands for TLRs 1–9. (A) The measurement of inflammatory molecules including nitrite, glutamate, TNF-α, and IL-1β produced by astrocytes activated with TLR ligands was performed. After 48 h treatment with TLR ligands, the supernatants of astrocytes cultures were analyzed for above molecules. Each column indicates the mean ± SEM (n = 5). *, $P<0.05$ as compared to non-treated astrocytes (NT). **, $P<0.01$;***, $P<0.001$. (B) The neuroprotective effects of anti-TNF-α antibody and IL-1β receptor antibody were evaluated using neuron–astrocytes co-cultures. Neuronal survival rate was quantified. The viability of non-treated neurons (NT) was normalized to 1.0. Each column indicates the mean ± SEM (n = 5).

In conclusion, astrocytes activated with TLR ligands 2, 4, 5 and 6 can be toxic to neurons via ROS production. NADPH oxidase inhibitor, including apocynin, may be useful therapeutics against neurodegeneration associated with activated astrocytes.

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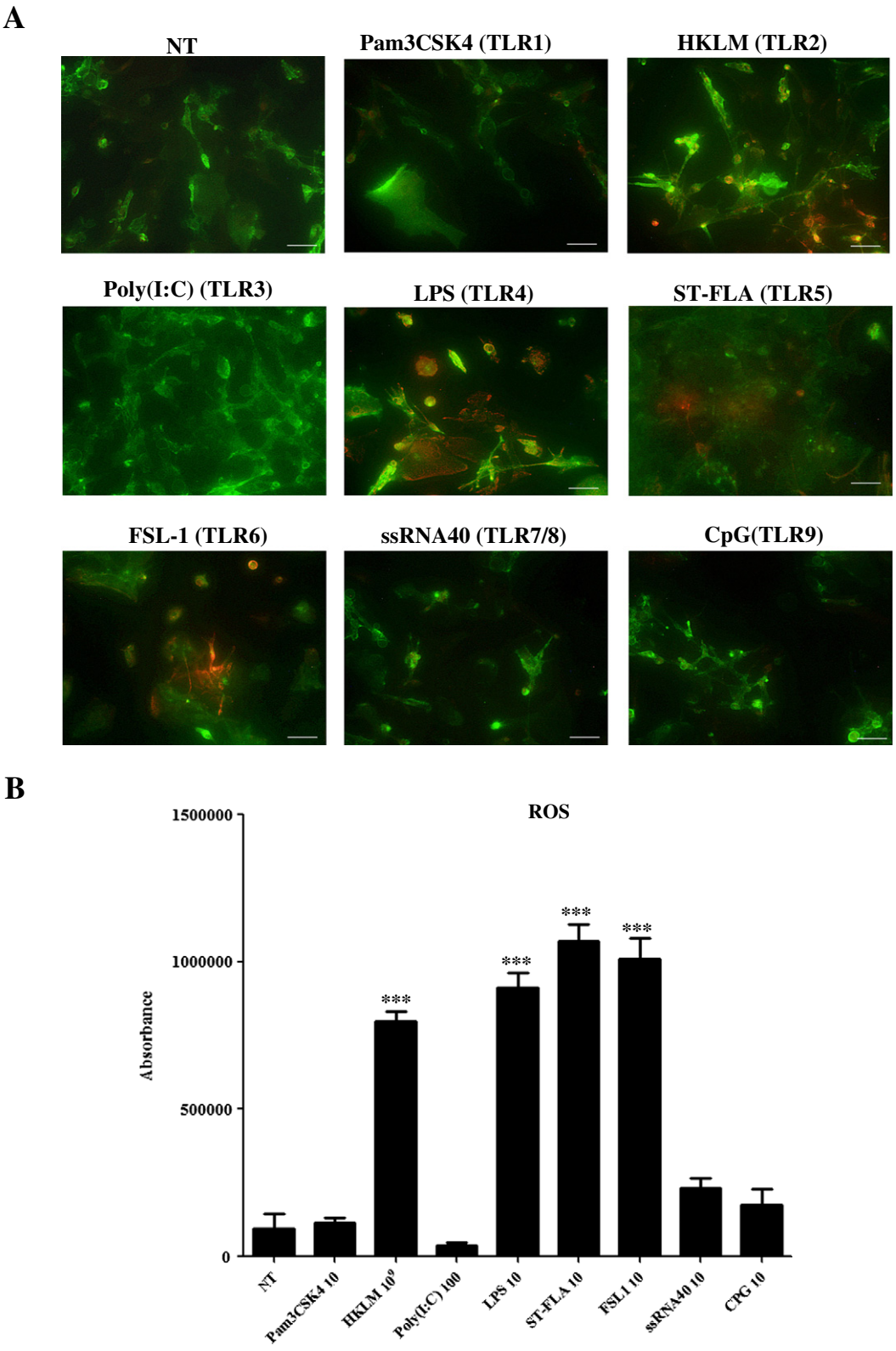

Fig. 4. The production of ROS by astrocytes activated with TLR ligands. (A) Live cell staining was performed in astrocytes activated with TLR ligands using ROS sensor CellROX™ Deep Red reagent (red) and the astrocyte cell surface marker GLAST (green) with a deconvolution fluorescent microscopy. Scale bar = 50 μm. (B) ROS production was quantified by the fluorescence microplate reader. Each column indicates the mean ± SEM (n = 5). ***, $P<0.001$ as compared to non-treated astrocytes (NT).

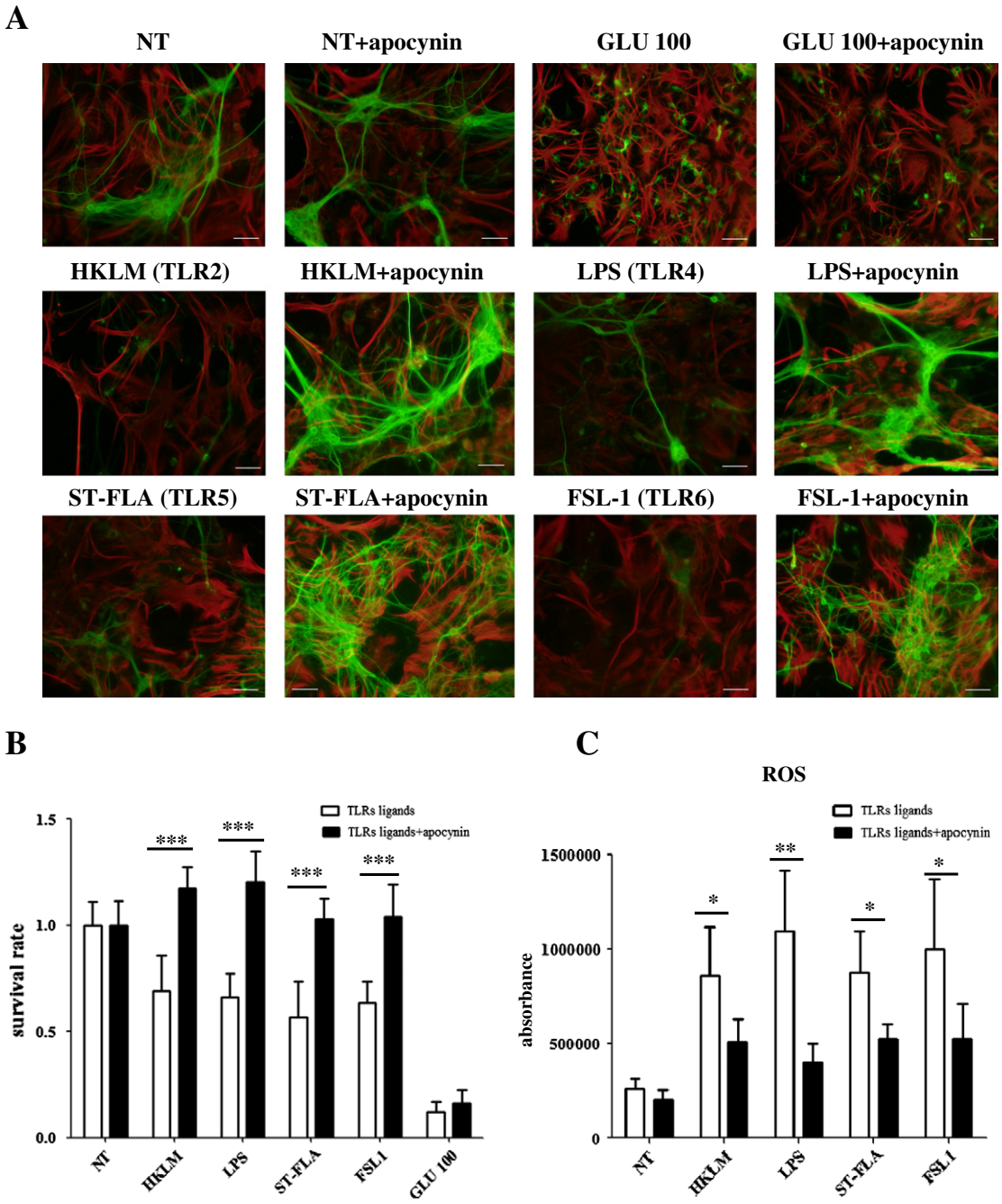

Fig. 5. The effect of NADPH oxidase inhibitor apocynin on neuronal cell death induced by astrocytes activated with TLRs 2, 4, 5 and 6. (A) Representative deconvolution fluorescent images of neuron-astrocytes co-cultures treated with or without apocynin, and then treated with TLR2, 4, 5, 6 or glutamate. Neurons were stained with an anti-MAP-2 antibody (green). Astrocytes were stained with anti-GFAP antibody (red). Scale bar = 50 μm. (B) Neuronal survival rate was quantified. The viability of non-treated neurons (NT) was normalized to 1.0. Each column indicates the mean ± SEM (n = 5). ***, $P<0.001$ as compared to the survival rate of TLR-stimulated co-cultures without treatment of apocynin. (C) ROS production of TLR-stimulated astrocytes with or without treatment of 100 μM apocynin was quantified by the fluorescence microplate reader. Each column indicates the mean ± SEM (n = 5). *, $P<0.05$ as compared to TLR-stimulated astrocytes without treatment of apocynin. **, $P<0.001$.

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