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Inhibition of ASK1-p38 pathway prevents neural cell death following optic nerve injury

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Optic nerve injury (ONI) induces retinal ganglion cell (RGC) death and optic nerve atrophy that lead to visual loss. Apoptosis signal-regulating kinase 1 (ASK1) is an evolutionarily conserved mitogen-activated protein kinase (MAPK) kinase kinase and has an important role in stress-induced RGC apoptosis. In this study, we found that ONI-induced p38 activation and RGC loss were suppressed in ASK1-deficient mice. Sequential *in vivo* retinal imaging revealed that post-ONI treatment with a p38 inhibitor into the eyeball was effective for RGC protection. ONI-induced monocyte chemotactic protein-1 production in RGCs and microglial accumulation around RGCs were suppressed in ASK1-deficient mice. In addition, the productions of tumor necrosis factor and inducible nitric oxide synthase in microglia were decreased when the ASK1-p38 pathway was blocked. These results suggest that ASK1 activation in both neural and glial cells is involved in neural cell death, and that pharmacological interruption of ASK1-p38 pathways could be beneficial in the treatment of ONI.
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The eye has a highly specialized structure that converts visual information into neuronal signals. Among various retinal cell types, retinal ganglion cells (RGCs) are the only neurons connected to the brain through the optic nerve. During development, nearly half of RGCs experience programmed cell death.[1] Many transcription factors, guidance molecules, extracellular matrix proteins, neurotrophic factors, cell death-regulating factors and caspases have been reported to be involved in the processes of formation of a precise retino-collicular map and in the regulation of developmental RGC death.[2] On the other hand, during adulthood, RGCs and their projections have to be maintained because they cannot be replaced after injury. Traumatic optic neuropathy is a common clinical problem that occurs in 0.5–5% of patients with closed head injury.[3] A damage to the optic nerve causes shear stress and induces secondary swelling within the optic canal, accompanied by subsequent RGC loss and optic nerve atrophy.[4] Although no large natural history or randomized controlled trial has been published, neither corticosteroid therapy nor optic canal decompression surgery is considered as standard treatments for patients with traumatic optic neuropathy,[5] and there is a lack of effective treatment at present. Other possible therapies to enhance visual recovery following trauma are neuroprotection and axonal regeneration. For example, neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and basic fibroblast growth factor, may rescue RGCs and induce axonal regeneration after optic nerve injury (ONI).[6–9] In addition, inhibitors for glutamate receptors, tumor necrosis factor (TNF) receptors and nitric oxide synthase (NOS) may be effective for RGC protection.[10,11]

Apoptosis signal-regulating kinase 1 (ASK1) has key roles in human diseases closely related to the dysfunction of cellular responses to oxidative stress and endoplasmic reticulum stressors, including neurodegenerative diseases.[12,13] ASK1 relays its apoptotic signals to the stress-activated mitogen-activated protein kinase (MAPK) family members, p38 and c-Jun N-terminal kinase (JNK).[14,15] p38 is activated and phosphorylated by environmental stress such as H_2O_2 and UV-B radiation, as well as by proinflammatory cytokines like TNF and interleukin-1. In addition, axotomy of the optic nerve or intraocular injection of *N*-methyl-D-aspartate (NMDA) activates p38, which leads to neural cell apoptosis.[10] These results suggest the possibility that the ASK1 signaling pathway is involved in RGC death and optic nerve degeneration in various pathological conditions. In this study, we attempted to elucidate a role of ASK1 after ONI and to provide evidence for a critical role of the ASK1-p38 pathway in both neural and glial cells during RGC degeneration.

Results

ASK1 deficiency protects retinal neurons following ONI. The retina of adult ASK1 knockout (KO) mice showed normal organization, and the cell number in the ganglion cell

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Abbreviations: ASK1, apoptosis signal-regulating kinase 1; BDNF, brain-derived neurotrophic factor; GLAST, glutamate/aspartate transporter; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; MIP-1α, macrophage inflammatory protein 1α; NO, nitric oxide; OCT, optical coherence tomography; ONI, optic nerve injury; RANTES, regulated on activation, normal T-cell expressed and secreted; RGC, retinal ganglion cell; TLR4, Toll-like receptor 4; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling
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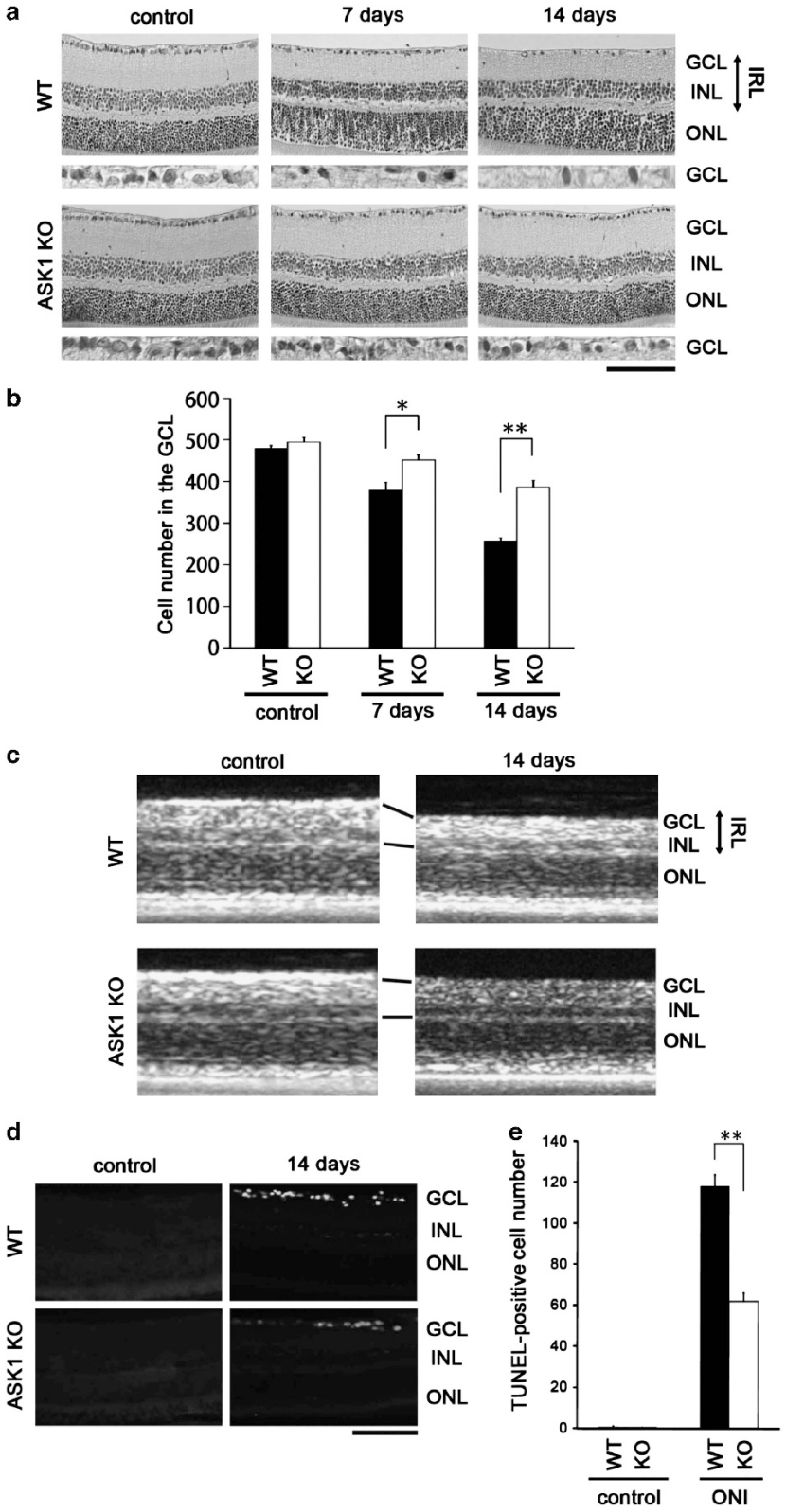


Figure 1 Effect of ASK1 deficiency on RGC loss and IRL thickness following ONI. (**a**) H&E staining of retinal sections at 7 and 14 days after ONI in wild-type (WT) and ASK1 KO mice. Scale bar: 100 and 400 μm in the upper and immediately lower panels, respectively. ONL, outer nuclear layer. (**b**) Quantification of RGC number in WT and ASK1 KO mice. The number of neurons in the GCL was counted in retinal sections from one ora serrata through the optic nerve to the other ora serrata. (**c**) OCT cross-sectional images of retinas before (control) and 14 days after ONI. (**d**) Representative images of TUNEL staining in WT and ASK1 KO mouse retinas at 5 days after ONI. Scale bar: 100 μm. (**e**) Quantification of TUNEL-positive cells in WT and ASK1 KO mice. The data are presented as means ± S.E.M. of six samples for each experiment. $^{**}P<0.01$, $^{*}P<0.05$

layer (GCL) was comparable to WT mice (Figures 1a and b).[13,16] We first analyzed the histopathology of the retina following ONI. ONI induced RGC death in both WT and ASK1 KO mice, but the number of surviving neurons in ASK1 KO mice was significantly higher than that in WT mice (Figures 1a and b). In addition, the thickness of the inner retinal layer (IRL) was significantly greater in ASK1 KO mice (112 ± 4% at 7 days and 126 ± 6% at 14 days; $n=6$) compared with WT mice (100 ± 3% at 7 days and 100 ± 5% at 14 days; $n=6$) ($P<0.05$). We also visualized retinal layers using optical coherence tomography (OCT), a noninvasive imaging technique that can be used to acquire cross-sectional tomographic images of the retina *in vivo*.[17,18] The IRL thickness at 14 days following ONI was significantly greater in ASK1 KO mice (124 ± 2%; $n=6$) compared with WT mice (100 ± 4%; $n=6$) (Figure 1c, $P<0.05$). The fact that these OCT results are consistent with the *in vitro* histological analysis suggest that *in vivo* mouse retinal imaging using OCT is highly reliable. We next analyzed apoptotic cells in the retina by terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) staining 5 days after ONI. Control animals showed practically no signals in both WT and ASK1 KO mice (Figure 1d). ONI resulted in the detection of TUNEL-positive cells in the GCL in both strains, but the number of such cells in ASK1 KO mice were significantly lower than that in WT mice (Figures 1d and e). These results suggest that ASK1 deficiency prevents ONI-induced RGC loss and secondary retinal degeneration *in vivo*.

ONI activates ASK1-p38 MAPK signaling. ASK1 relays its apoptotic signals to the stress-activated MAPK family members, p38 and JNK.[14,15] To determine whether these pathways are involved in ONI-induced RGC death, we examined total and phosphorylated (i.e. activated) p38 and JNK immunoreactivities in WT and ASK1 KO mice after ONI. Immunoblot analysis revealed that the levels of phosphorylated p38 in control retinas were very low in both strains (Figure 2a). ONI strongly induced p38 phosphorylation in WT, but not in ASK1 KO mice (Figures 2a and b). Phosphorylated p38 immunoreactivity was particularly high at 3 h and disappeared by 24 h after ONI. On the other hand, phosphorylated JNK immunoreactivity was very low throughout our observation period, demonstrating that ONI had little effect on its activation (Figure 2a). Total p38 and JNK immunoreactivities (i.e. including both phosphorylated and nonphosphorylated forms) did not change after ONI in both strains. We also examined the localization of phosphorylated p38 immunoreactivity in the retina (Figure 2c). In control retinas, phosphorylated p38-positive cells were absent, as predicted. In contrast, 3 h after ONI, phosphorylated p38-like immunoreactivity was observed in the GCL in WT, but this was almost absent in ASK1 KO mice.

ASK1-p38 MAPK signaling induces neural cell death following ONI. Our results so far suggest that activation of the ASK1-p38 pathway induces RGC death after ONI, and that pharmacological inhibition of p38 may be beneficial in the treatment of traumatic ONI. To determine this possibility,

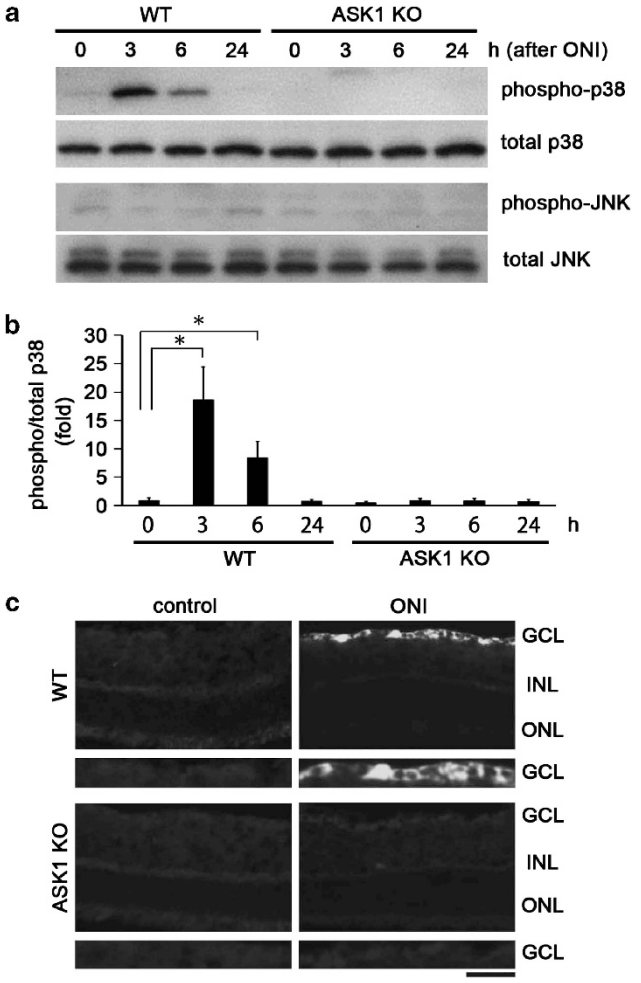


Figure 2 Effect of ASK1 on p38 activation following ONI. (**a**) Immunoblot analysis of phosphorylated p38, total p38, phosphorylated JNK and total JNK in whole retinas of wild-type (WT) and ASK1 KO mice at the indicated times after ONI. (**b**) Quantitative analysis of phosphorylated p38 in whole retinas of WT and ASK1 KO mice at the indicated times after ONI. The data are presented as means ± S.E.M. of six samples for each experiment. *$P<0.01$. (**c**) Immunohistochemical analysis of phosphorylated p38 in retinal sections of WT and ASK1 KO mice 3 h after ONI. Scale bar: 100 and 200 μm in the upper and lower panels, respectively

we evaluated the effect of pre-treatment of the eye with a p38 inhibitor, SB203580, *in vivo* (Figure 3a). Intravitreous injection of SB203580 suppressed ONI-induced p38 phosphorylation, but had no effects on the activation of JNK and ASK1 in the retinas of WT mice (Figures 3b–d). Consistently, SB203580 suppressed ONI-induced p38 phosphorylation in the GCL (Figure 3e). As SB203580 showed a strong effect and had a very good exposure in the eye, we further examined the effect of pre- and post-treatment with SB203580 on ONI-induced RGC death (Figure 4a). Pre-treatment with SB203580 (1 h before ONI) significantly increased the number of surviving retinal neurons in the GCL and the IRL thickness compared with control animals treated with PBS, and ONI-induced retinal degeneration was mild, similar to ASK1 KO mice (Figures 4b–d). Interestingly, post-treatment with SB203580 was also effective when

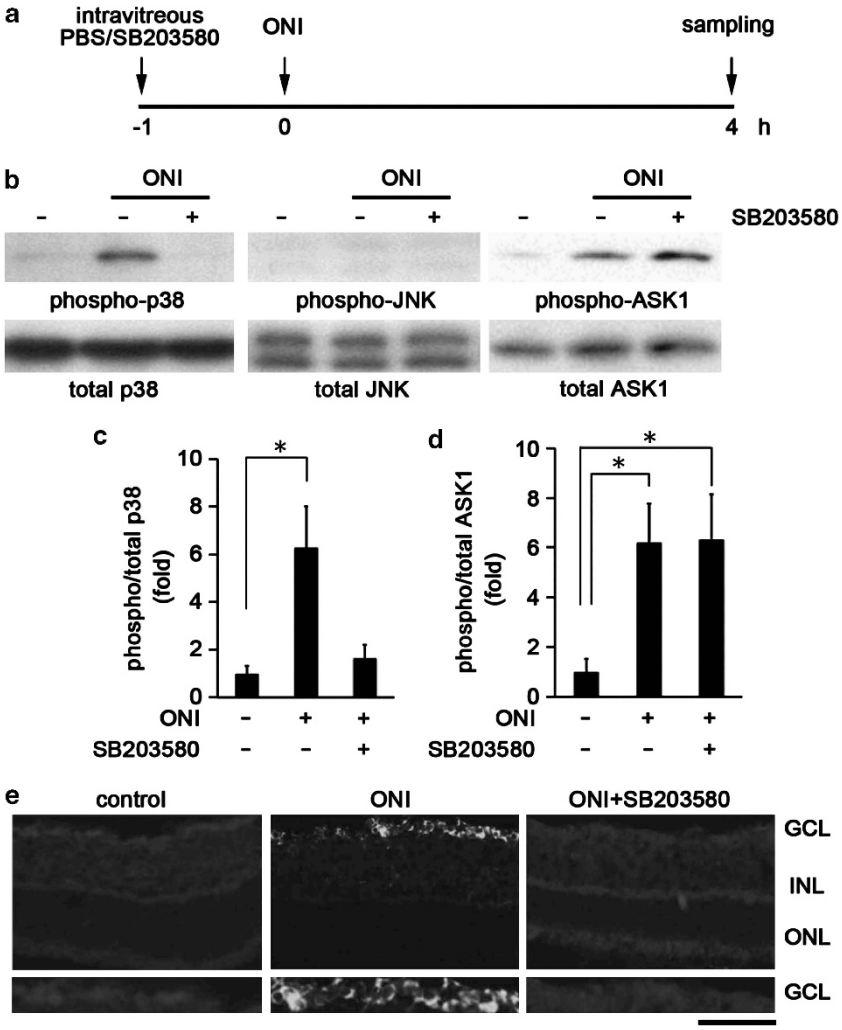


Figure 3 Effect of p38 inhibitor on ONI-induced p38 activation in the retina. (**a**) Animal protocols. Phosphate-buffered saline (PBS) or SB203580 (20 μM), a p38 inhibitor, was intraocularly injected 1 h before ONI, and the animals were killed 4 h after ONI. (**b**) Effect of SB203580 on ONI-induced activation of the ASK1 signaling pathway assessed by immunoblot analyses of phosphorylated p38, total p38, phosphorylated JNK, total JNK, phosphorylated ASK1 and total ASK1 in whole retinas of wild-type (WT) mice. (**c**, **d**) Quantitative analyses of ONI-induced activation of p38 (**c**) and ASK1 (**d**) in whole retinas of WT mice. The data are presented as means ± S.E.M. of six samples for each experiment. $^*P<0.01$. (**e**) Immunohistochemical analysis of ONI-induced p38 phosphorylation in retinal sections. Scale bar: 100 and 200 μm in the upper and lower panels, respectively

treated at 5 min. However, consistent with the time scale of the rise in phosphorylated p38 (Figure 2b), post-treatment with SB203580 could not prevent retinal degeneration when treated at 12 h after ONI (Figures 4b–d). We carried out sequential retinal imaging using OCT, and found that post-treatment (5 min) with SB203580 is effective at 14 days after ONI (Figures 4e and f). These results suggest that ONI induces neural cell apoptosis through the activation of p38 under the regulation of ASK1.

Effect of ASK1 on chemokine productions following ONI. We examined whether ONI induces the expression of key chemokines implicated in the pathogenesis of neuro-inflammation,[15] including the monocyte chemoattractant protein-1 (MCP-1), regulated on activation, normal T-cell expressed and secreted proteins (RANTES) and macrophage inflammatory protein-1α (MIP-1α). Real-time PCR (RT-PCR) analysis of messenger RNA (mRNA) extracted from whole retinas at 5 days after ONI revealed that the expression levels of all three chemokines were increased significantly in WT mice (Figures 5a–c). The expression levels of RANTES and MIP-1α were also increased in ASK1 KO mice, but the degree of increase was significantly lower compared with WT mice. There was no change in the expression level of MCP-1 in ASK1 KO mice. We next examined the distribution of MCP-1 in the retina because ONI-induced induction of this chemokine was completely suppressed in ASK1 KO mice (Figure 5a). Immunohistochemical analysis revealed that ONI-induced upregulation of MCP-1 was detected at 1 h after ONI in WT mice, but not in ASK1 KO mice (Figure 5d). To identify the specific MCP-1 immunopositive (IP) cell type(s), we carried out co-staining with retinal cell markers. At 1 h after ONI, MCP-1-IP cells were double-labeled with calretinin (a marker for RGCs and

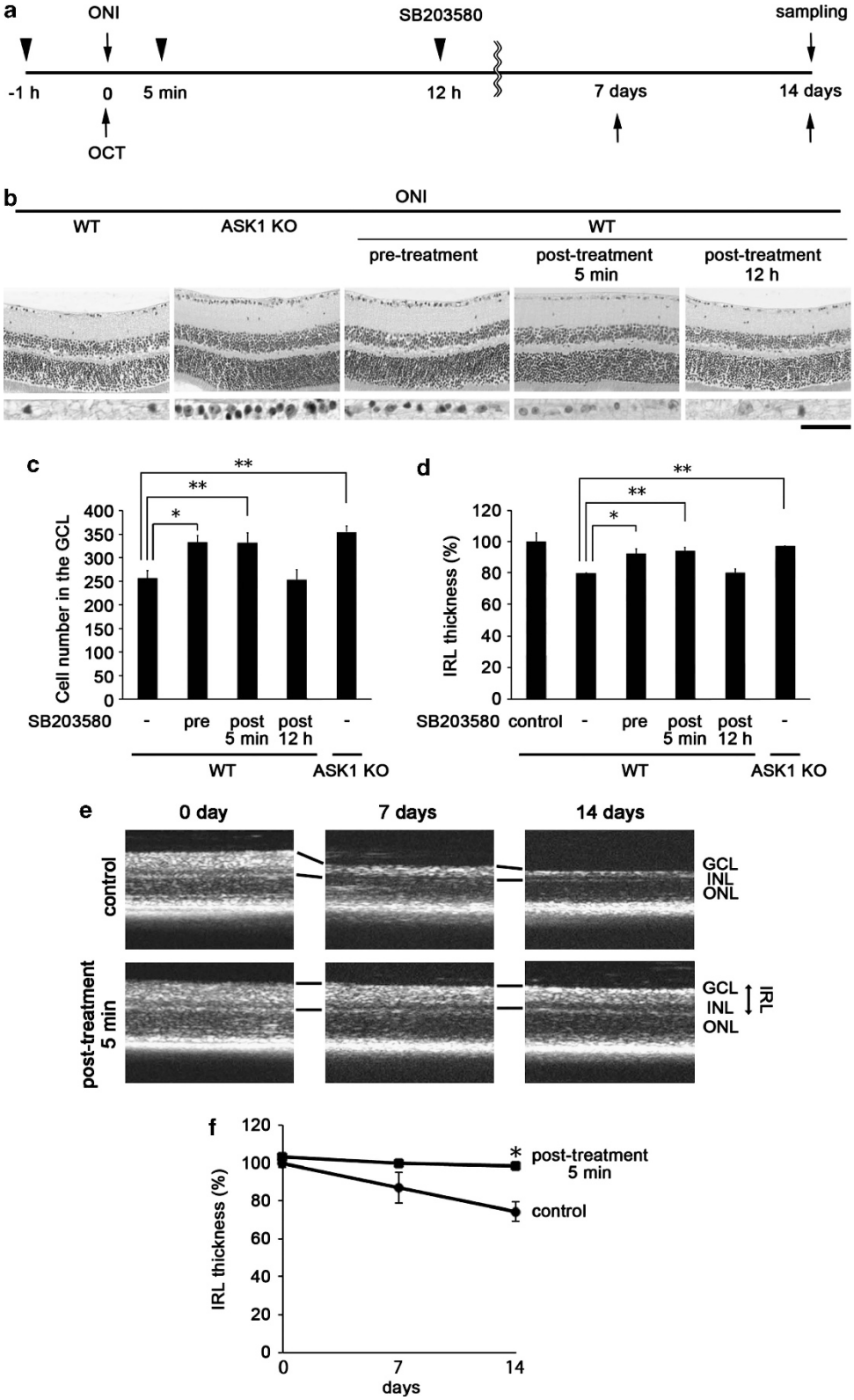


Figure 4 Effect of p38 inhibitor on ONI-induced RGC loss. (**a**) Animal protocols. Phosphate-buffered saline (PBS) or SB203580 (20 μM) was intraocularly injected before or after ONI, and the animals were killed at 14 days after ONI. (**b**) H&E staining of retinal sections in WT and ASK1 KO mice. Pre- and post-treatment with SB203580 prevented ONI-induced RGC loss in WT mice. Scale bar: 100 and 400 μm in the upper and lower panels, respectively. (**c**, **d**) Quantification of RGC number and IRL thickness. (**e**) OCT cross-sectional images of retinas before (0 day) and after (7 and 14 days) ONI. (**f**) Longitudinal evaluation of IRL thickness. The data are presented as means ± S.E.M. of six samples for each experiment. $^{**}P<0.01$, $^{*}P<0.05$

amacrine cells) and TUJ1 (another RGC marker), but not with glial fibrillary acidic protein (GFAP; a marker for astrocytes) and glutamate/aspartate transporter (GLAST; a marker for Müller glial cells) (Figure 5e). Similar findings were observed at 2 days after ONI (data not shown). These results showed that ASK1 KO mice lacked upregulation of neuronal MCP-1 expression after ONI.

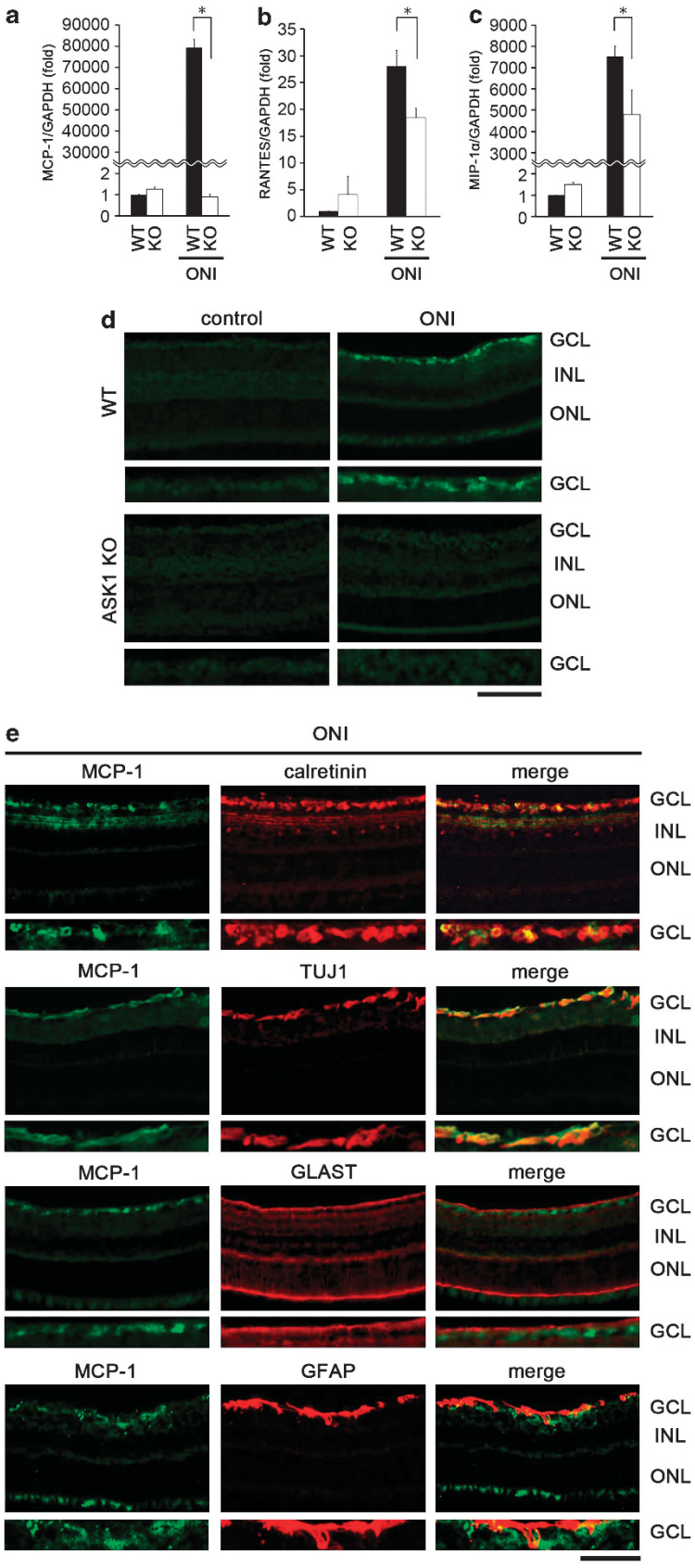
We recently showed that ASK1 is involved in MCP-1 production in microglia in a mouse model of multiple sclerosis.[15] Therefore, we investigated how microglial MCP-1 may be involved in RGC death. Iba1-positive microglial cells were detected in the inner retina[19] and there was no difference in the numbers of these cells between WT ($100 \pm 4\%$; $n = 6$) and ASK1 KO ($82 \pm 7\%$; $n = 6$) mice (Figures 6a and b). The number of iba1-positive cells was increased in both strains at 5 days after ONI, but the degree of increase was significantly lower in ASK1 KO mice ($175 \pm 33\%$; $n = 6$) compared with WT mice ($365 \pm 47\%$; $n = 6$) (Figures 6a and b). Many iba1-IP cells were double-labeled with MCP-1 (Figure 6a) and phosphorylated-p38 (Figure 6c) in WT mice, but this double-labeling was not observed in ASK1 KO mice, similar to control mice. These results suggest that MCP-1 produced in RGCs after ONI allows recruitment of microglia to the injury site, and that ASK1 signaling is involved in this ONI-induced MCP-1 production in RGCs and activated microglia.

Role of ASK1-p38 MAPK signaling in microglial cells following ONI. Axonal damage activates the innate immune response and induces microglial activation. For example, microglia that express Toll-like receptor 4 (TLR4) has a crucial role in lipopolysaccharide (LPS)-induced neuronal damage and cell death both *in vitro* and *in vivo*.[20] RT-PCR analyses of mRNA extracted from cultured microglia revealed that expression of TNFα and inducible NOS (iNOS) were almost absent in untreated cells, but LPS clearly increased expression levels of both TNFα and iNOS (Figures 7a and b). LPS-induced productions of TNFα and iNOS were almost completely suppressed in ASK1-deficient cells. In addition, inhibition of p38 by SB203580 or inhibition of ASK1 by its specific inhibitor MSC2032964A[15] significantly suppressed the expression levels of TNFα and iNOS in WT cells (Figures 7a and b). These results suggest that the TLR4-ASK1-p38 pathway is involved in the productions of TNFα and iNOS in microglial cells. As the ASK1-p38 pathway may regulate TNFα-induced iNOS expression in astrocytes and Müller cells,[13,21] we further examined the direct effect of TNFα on cultured microglia. TNFα-induced iNOS expression from ASK1-deficient cells was significantly lower than that from WT mice (Figure 7c). In addition, SB203580 and MSC2032964A significantly suppressed the expression levels of iNOS in WT cells (Figure 7c). Taken together, loss of ASK1 prevents LPS- and TNFα-induced iNOS expression in microglia. Finally, we examined the effect of ASK1 deficiency on ONI- or LPS-induced productions of TNFα and iNOS in whole retinas. ONI- or LPS-induced upregulation of TNFα and iNOS were observed in WT mice, but were completely suppressed in ASK1 KO mice (Figure 8). These results suggest that the reduced productions of microglial TNFα and iNOS after ONI result in the higher rate of RGC survival in ASK1 KO mice.

Discussion

Herein, we report for the first time that interruption of the ASK1-p38 pathway prevents RGC death caused by traumatic ONI. In this study, we show that ONI stimulates p38 phosphorylation and productions of MCP-1, RANTES, MIP-1α, TNFα and iNOS in the retina, and these effects are suppressed in ASK1 KO mice. Local administration of a p38 inhibitor, SB203580, before ONI inhibited p38 phosphorylation in the GCL and protected RGCs from death. Strikingly, SB203580 treatment even after ONI prevented RGC death and thinning of the IRL. In the early phase after ONI, MCP-1 expression was upregulated mainly in RGCs, which led to the recruitment of activated microglia into the inner retinal layer (Figures 5 and 6). Consistently, neural MCP-1 in the brain has been reported to mediate microglia recruitment and activation that exacerbates neurodegeneration.[22] Lack of this upregulation in ASK1 KO mice suggested that the microglia were not navigated effectively to the injury site in these mice. Pharmacological inactivation of ASK1 or p38 suppressed LPS-induced TNFα and LPS/TNFα-induced iNOS productions in microglia (Figure 7). Taken together with previous findings that TNF and NO induce RGC death,[11,23] our data suggest that TLR4/TNF-ASK1-p38 pathways in microglia indirectly regulate RGC death. Microglial p38 activation has been reported to contribute to the pathogenesis of Alzheimer's disease,[24] supporting our data that p38 activation is damaging to neural cells. However, microglia secrete several polypeptide neurotrophic factors that protect and regulate the survival of retinal neurons.[19] For example, another microglia-derived factor oncomodulin, a small Ca^{2+}-binding protein, stimulates neurite regeneration of RGCs.[25] Microglia-derived BDNF also stimulates optic nerve regeneration, at least partly through the activation of Dock3, a member of atypical Rho-guanine nucleotide exchange factors.[7] Thus, microglia as well as other immune cells[26] seem to have complex and sometimes paradoxical actions in response to ONI. In addition, we cannot exclude the possibility that microglial invasion to the inner retina may be due to molecules and factors that are released when RGCs die, rather than just the one factor, MCP-1, being released by living RGCs. As glia–glia and glia–neuron networks have important roles in the central nervous system,[6,19,27,28] more detailed functions of ASK1, MCP-1 and MCP-1 receptors should be elucidated using cell type-specific conditional KO mice.

Recent studies have shown that reactive oxygen species are involved in neuronal death from axotomy, and antioxidants such as thioredoxins and spermidine rescue neural cells.[29–31] In resting cells, ASK1 constantly forms an inactive complex with thioredoxin, whereas upon stimulation, ASK1 is dissociated from thioredoxin and activated by conformational changes and covalent modifications.[14] Several reports have suggested a possibility that thioredoxin is a kind of neurotrophic factor and has a crucial role in maintaining retinal cells.[31,32] On the other hand, PB1, a novel phosphineborane complex, promotes RGC protection through the induction of BDNF and activation of the extracellular signal-regulated kinases 1/2, but not ASK1, signaling.[33] The identification of small-molecule compounds that mimic some of the beneficial effects of trophic factors is of clinical interest. Thus,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application of thioredoxin and PB1, in combination with other relevant trophic factors,[6,19,27] may stimulate multiple cellular targets and activate several independent mechanisms to rescue RGCs following ONI. Another important point is that ONI induced activation of p38, but not JNK (Figure 3). Although the reason for the lack of JNK activation is unclear, the ASK1-p38 pathway might be more tightly regulated than pathways involving other MAPK kinase kinases (MAP3Ks) such as TAK1 and MEKK3.[34] A recent study demonstrated that ASK2, a new ASK1 family member, formed a heteromeric complex with ASK1, and ASK2 in this complex exhibited sufficient basal activity toward the p38 and JNK pathways as an MAP3K.[35] Thus, detailed ASK2 gene expression profiles in the central nervous system and functional analysis using ASK2 KO mice will be required in future investigations.

In summary, we demonstrated that ASK1 deficiency-mediated RGC protection after ONI occurs by a number of ways: inhibition of p38 in the GCL, lack of recruitment of microglia to the injury site and suppression of microglial TNF and NO productions. We also show that administration of a p38 inhibitor even after ONI was effective as a treatment, suggesting strongly of a possibility that p38 is a potential therapeutic target for optic neuropathy that may occur independent of, or as a complication of, ONI.[10,13,15,16] In addition, we showed that OCT permits *in vivo*, noninvasive, longitudinal and quantitative assessment of the changes in retinal morphology after ONI and it clearly visualized the effects of the p38 inhibitor. OCT data can provide useful information in experimental animals as well as clinical trials and management.[17,18,36] Although further *in vivo* studies are required, our findings raise intriguing possibilities for the management of ONI by ASK1 and/or p38 inhibitors. Recently, p38 has been represented as an emerging therapeutic target for the treatment of various conditions, including Alzheimer's disease, cardiac ischemia/reperfusion injury and chronic airways disease.[23,37,38] Thus, further efforts to discover new compounds that can specifically inhibit ASK1-p38 signaling for a prolonged period may lead to the development of novel strategies for the management of deteriorating diseases including retinal and optic nerve degeneration.

Materials and Methods

Optic nerve injury. Experiments were carried out using ASK1 KO mice[13,16] in accordance with the Tokyo Metropolitan Institute of Medical Science Guidelines for the Care and Use of Animals. Mice were anesthetized with sodium pentobarbital before ONI. Optic nerves were exposed intraorbitally and crushed at about 0.5–1.0 mm from the posterior pole of the eyeball with fine surgical forceps for 5 s.[7,8,39] In some experiments, SB203580 (20 μM; Cell signaling, Danvers, MA, USA), LPS (5 ng/ml) or PBS was intraocularly injected before or after ONI.

Histological and morphometric studies. Paraffin-embedded retinal sections of 7 μm thickness were cut through the optic nerve and stained with hematoxylin and eosin (H&E). The RGC number and the extent of retinal degeneration were quantified in two ways.[8] First, the thickness of the IRL (between the internal limiting membrane and the interface of the outer plexiform layer and the outer nuclear layer) was analyzed. Second, in the same sections, the number of neurons in the GCL was counted from one ora serrata through the optic nerve to the other ora serrata.

Imaging acquisition of spectral-domain-OCT. Spectral-domain-OCT (RS-3000; Nidek, Aichi, Japan) examinations were performed at 0, 7 and 14 days after ONI with modifications.[17,18] For fundus imaging, polymethyl methacrylate contact lenses optimal for mice (UNICON, Osaka, Japan) were placed on the corneas. Use of the lenses prevents anesthesia-induced cataract progression. A 60-D adapter lens was placed on the objective lens of the Multiline OCT to focus on the mouse retina. All the images were location matched, scanning vertically through the center of the optic nerve head at 3 disc diameter length above the optic nerve head. In this study, the maximum number of B-scans set by the manufacturer (50 for line scans) were used for averaging.

TUNEL staining. Frozen retinal sections were treated with 10 μg/ml Proteinase K and then incubated in 0.26 U/μl terminal deoxynucleotidyl transferase in the supplied 1 $\times$ buffer (Invitrogen, Carlsbad, CA, USA) and 20 μmol/l biotinylated-16-dUTP (Roche, Basel, Switzerland) for 1 h at 37°C. The sections were blocked for 15 min with 2 $\times$ SSC (Nippon Gene, Tokyo, Japan) and washed three times in PBS. The sections were then incubated with streptavidin-fluorescein isothiocyanate (Chemicon, Temecula, CA, USA) for 30 min for detection.

Immunohistochemistry. Retinas and optic nerves were examined by immunostaining as reported previously.[6–8] Immunohistochemistry was performed using the following primary antibodies: phosphorylated p38 (1 : 1000; BD Biosciences Pharmingen, San Diego, CA, USA), MCP-1 (1 : 100; R&D, Minneapolis, MN, USA), calretinin (1 : 500; Chemicon), TUJ1 (1 : 100; R&D), GFAP (1 : 50; Abcam, Cambridge, UK), GLAST (1.0 μg/ml)[40] and iba1 (1.0 μg/ml).[19] Quantitative analysis of the IP cell number or stained region was carried out using BZ-H1C (Keyence Software, Osaka, Japan).

Immunoblot analysis. Immunoblotting was performed as reported previously.[41] Membranes were incubated with an antibody against ASK1 (1 : 200; Cell Signaling), phospho-ASK1 (1 : 200; Cell Signaling), p38 (1 : 1000; BD Biosciences), phospho-p38 (1 : 1000; BD Biosciences), JNK (1 : 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or phospho-JNK (1 : 1000; Santa Cruz Biotechnology).

Cell culture. Primary microglial cells derived from WT and ASK1 KO mice were stimulated with LPS (5 ng/ml) or TNFα (50 ng/ml) for 6 h, and processed for quantitative PCR analysis.[15] In some experiments, microglial cells were pre-treated with SB203580 (20 μM; Cell Signaling) or MSC2032964A[15] (10 μM) for 1 h before stimulation.

Quantitative real-time PCR. Quantitative RT-PCR was performed using the ABI 7500 fast RT-PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR Master Mix (Applied Biosystems) as reported previously.[42] Complementary DNA reverse-transcribed from total RNA was amplified by using primers specific for MCP-1 (sense: 5′-AACTGCATCTGCCCTAAGGT-3′; antisense: 5′-ACGGGTCAACTTCACATTCA-3′), RANTES (sense: 5′-GCCCACGTCA AGGAGTATTT-3′; antisense: 5′-TGACAAACACGACTGCAAGA-3′), MIP-1α

Figure 5 Effect of ASK1 deficiency on ONI-induced activation of RGCs. (**a**–**c**) Impaired chemokine productions in whole retinas of wild-type (WT) and ASK1 KO mice at 5 days after ONI. mRNA expression levels of MCP-1 (**a**), RANTES (**b**) and MIP-1α (**c**) were determined using quantitative real-time PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The data are presented as means ± S.E.M. of six samples for each experiment. $^*P<0.01$. (**d**) Immunohistochemical analysis of MCP-1 expression in the retinas of WT and ASK1 KO mice at 1 h after ONI. (**e**) Immunostaining of MCP-1-positive cells (green). Calretinin, TUJ1, GFAP and GLAST were used as cell type-specific markers (red). Overlapping immunoreactivities (yellow) of MCP-1 and calretinin or TUJ1 indicate the expression of MCP-1 in RGCs. Scale bar: 100 and 200 μm in the upper and lower panels, respectively

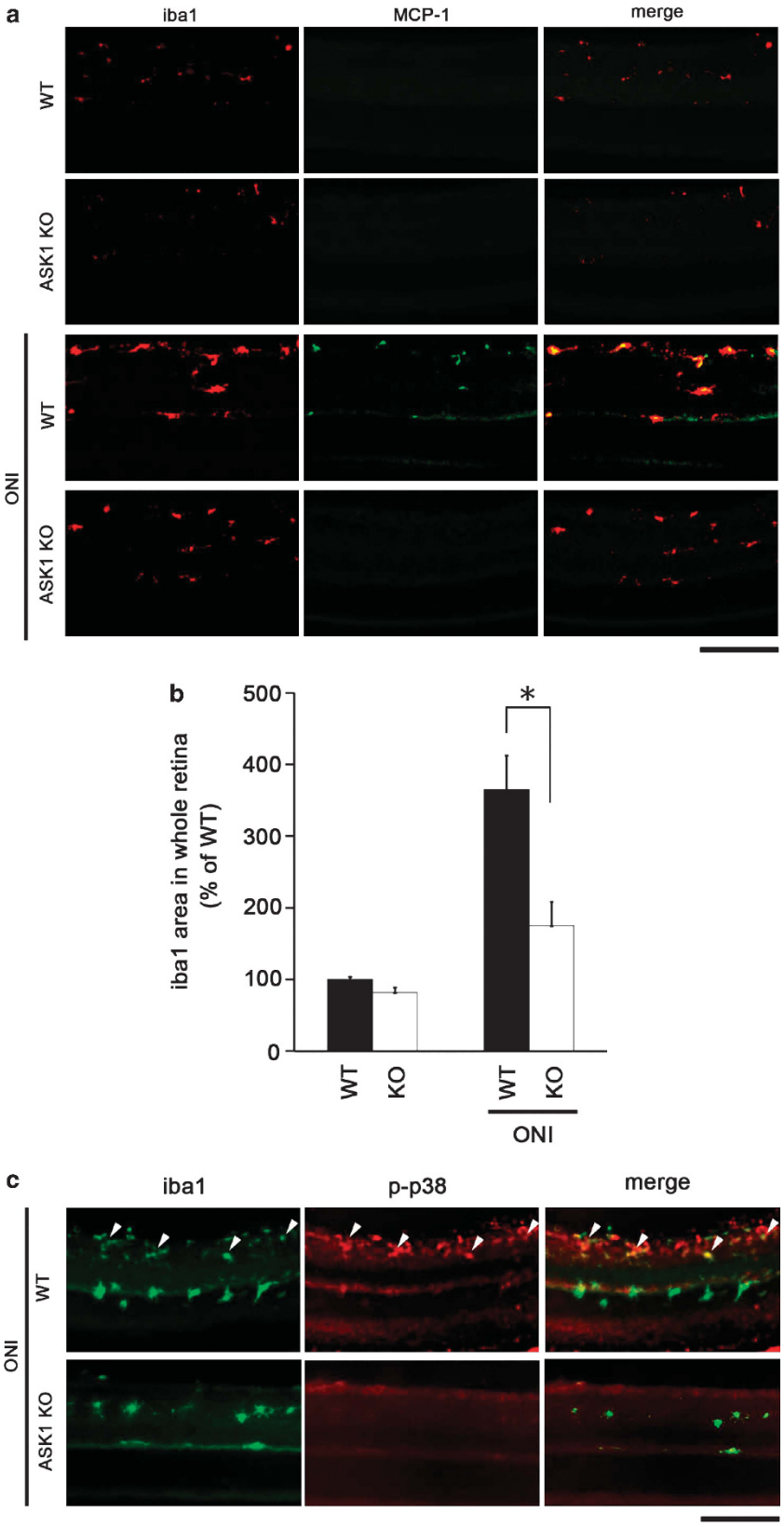

Figure 6 Effect of ASK1 deficiency on ONI-induced migration of microglia. (**a**) Double-labeling immunohistochemistry of iba1 (red) and MCP-1 (green) at 5 days after ONI. Overlapping immunoreactivities (yellow) indicate the expression of MCP-1 in microglial cells. (**b**) Quantification of iba1-stained areas in whole retinas. The data are presented as means ± S.E.M. of six samples for each experiment. $^{*}P<0.01$. (**c**) Double-labeling immunohistochemistry of iba1 (green) and phosphorylated p38 (p-p38; red) at 5 days after ONI. Overlapping immunoreactivities (yellow) indicate the activation of microglial cells. Scale bar: 100 μm

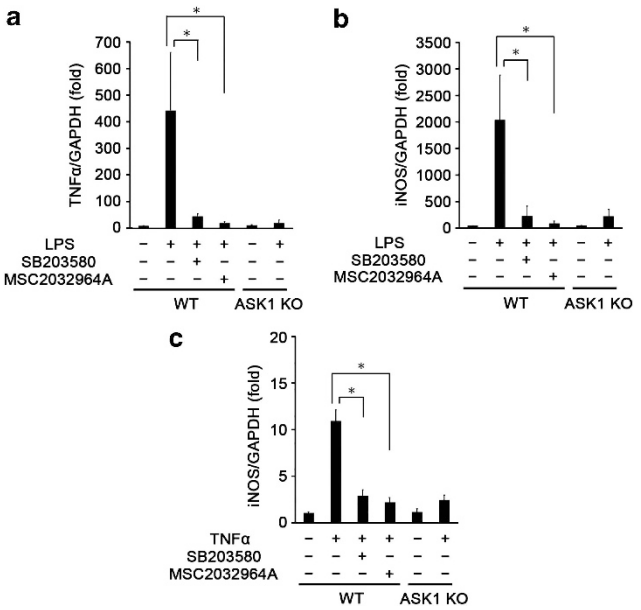

Figure 7 ASK1-p38 pathway is required for TNFα and iNOS productions in microglial cells. Cultured microglial cells were pre-treated with SB203580 (20 μM) or MSC2032964A (10 μM) for 1 h, and then stimulated with LPS (5 ng/ml) or TNFα (50 ng/ml) for 6 h. Cell lysates were subjected to quantitative real-time PCR to measure mRNA expression levels of TNFα (**a**) and iNOS (**b**, **c**). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The data are presented as means ± S.E.M. of six samples for each experiment. *$P<0.01$

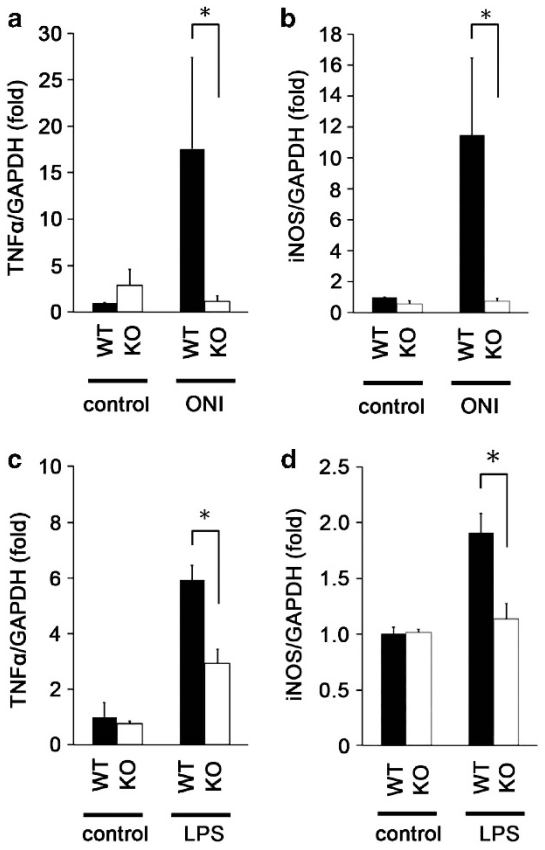

Figure 8 Effect of ASK1 deficiency on productions of TNFα and iNOS in whole retinas. mRNA expression levels of TNFα (**a**, **c**) and iNOS (**b**, **d**) were measured by quantitative real-time PCR after ONI (**a**, **b**) or intraocular injection of LPS (**c**, **d**). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The data are presented as means ± S.E.M. of six samples for each experiment. *$P<0.01$

(sense: 5′-AGATTCCACGCCAATTCATC-3′; antisense: 5′-CAGATCTGCCGGTTTCTCTT-3′), TNFα (sense: 5′-CGTCAGCCGATTTGCTATCT-3′; antisense: 5′-CGGACTCCGCAAAGTCTAAG-3′), iNOS (sense: 5′-ACTGTGTGCCTGGAGGTTCT-3′; antisense: 5′-GGCAGCCTCTTGTCTTTGAC-3′) and GAPDH (sense: 5′-TGCACCACCAACTGCTTAG-3′; antisense: 5′-GGATGCAGGGATGATGTTC-3′).

Statistics. For statistical comparison of two samples, we used a two-tailed Student's *t*-test. Data are presented as means ± S.E.M. $P<0.05$ was regarded as statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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