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Innate immune receptors represent an evolutionarily ancient system that allows organisms to detect and rapidly respond to pathogen- and host-derived factors. TLRs are predominantly expressed in immune cells and mediate such a response. Although this class of pattern recognition receptors is involved in CNS disorders, the knowledge of ligands leading to activation of TLRs and to subsequent CNS damage is limited. We report in this study that ssRNA causes neurodegeneration and neuroinflammation dependent on TLR7 in the CNS. TLR7 is not only expressed in microglia, the major immune cells of the brain, but also in neurons of the CNS. Extracellularly delivered ssRNA40, an oligoribonucleotide derived from HIV and an established ligand of TLR7, induces neuronal cell death dependent on TLR7 and the central adapter molecule MyD88 in vitro. Activation of caspase-3 is involved in neuronal damage mediated by TLR7. This cell-autonomous neuronal cell death induced by ssRNA40 is amplified in the presence of microglia that mount an inflammatory response to ssRNA40 through TLR7. Intrathecal administration of ssRNA40 causes widespread neurodegeneration in wild-type but not in TLR7−/− mice, confirming that neuronal cell death induced by ssRNA40 through TLR7 occurs in vivo. Our results point to a possible mechanism through which extracellularly delivered ssRNA contributes to CNS damage and determine an obligatory role for TLR7 in this pathway. *The Journal of Immunology*, 2012, 189: 1448–1458.

Neuroinflammation, neuronal injury, and neuronal cell death represent major hallmarks of infectious and noninfectious CNS diseases. Emerging evidence suggests a role for innate immune receptors such as TLRs in various forms of CNS damage. TLRs were originally discovered to control host immune responses against invading pathogens through recognition of highly specific molecular patterns derived from microorganisms (1). Eleven members of the TLR family (TLR1–11) have been identified in humans to date. Certain members of the TLR family including TLR1, TLR2, TLR4, and TLR6 are expressed on the plasma membrane where they sense the presence of protein and lipid components derived from bacteria and fungi. Other TLRs such as TLR3, TLR7, TLR8, and TLR9 are localized in intracellular compartments (1). TLR7 localizes to the endolysosomal compartment and is activated by antiviral compounds including imiquimod, guanosine analogues including loxoribine, virus-derived single-stranded oligoribonucleotides such as ssRNA40 from HIV, and whole viruses (1–6). TLRs initiate signaling

through their cytoplasmic Toll/IL-1R (TIR) domains. These interact with other TIR domains such that upon activation, each TLR binds to a specific set of adapter proteins that also contain TIR domains. MyD88 is the universal adapter recruited by all known TLRs except TLR3 (1).

Microglia, the major immune cells of the CNS, express all known TLRs (7, 8) and are involved in various forms of CNS damage. These cells mediate the innate immune response in the context of infections (9) such as viral encephalitis (10–12) and bacterial meningitis (13, 14). Activation of TLRs and the subsequent immune response can be harmful to the CNS and may aggravate the clinical outcome. For example, TLR4 in microglia mediates LPS-induced injury of oligodendrocytes and neurons (15, 16). In addition, infectious processes triggered by TLRs may exacerbate CNS autoimmune disorders, as was shown for pneumococcus-induced infection, which aggravates the outcome of experimental autoimmune encephalitis, an animal model for multiple sclerosis, via TLR2 (17). In contrast to these observations, protective effects mediated by TLRs in CNS injury are clearly present, too. For example, mutations in TLR3 may render individuals more susceptible to encephalitis induced by HSV (18).

Several studies suggest a role for TLRs not only in infectious but also in noninfectious CNS injury, where pathogen-associated molecules are not detectable. In particular, involvement of TLRs and associated signaling pathways were reported in mouse models of Alzheimer's disease (19), Parkinson's disease (20), stroke (21), amyotrophic lateral sclerosis (22), and multiple sclerosis (23). These observations may be explained, at least in part, by the fact that dying CNS cells release endogenous factors such as heat shock protein 60 (HSP60) that are recognized by TLR4 and initiate a neurotoxic microglial response (24). Likewise, a role for TLR2 signaling in the glial response to brain injury was suggested (25).

Whereas widespread expression of TLRs is observed in microglia, only few TLRs are expressed in neurons. TLR3 expression was detected postmortem in neurons of brains from patients with

herpes encephalitis, amyotrophic lateral sclerosis, and Alzheimer's disease (26). TLR8 was detected in CNS neurons, where it serves as a negative regulator of axonal growth (27), and TLR7 expressed in peripheral neurons was reported to mediate pruritus (28). However, the endogenous ligands involved in these contexts are unknown, and the involved downstream signaling pathways are unclear. We report here that activation of TLR7, a receptor sensing ssRNA, in neurons by ssRNA40, a synthetic oligoribonucleotide derived from HIV, causes neuronal cell death through the adapter molecule MyD88 and caspase-3 in a cell-autonomous fashion. In addition, microglia release inflammatory molecules in response to ssRNA40 through TLR7 and enhance this neurotoxic effect. These results demonstrate that extracellularly delivered ssRNA can in principle cause neuronal injury and point to TLR7 as an essential element in this form of CNS damage.

Materials and Methods

Animals and cell lines

C57BL/6J mice were purchased from Charles River (Sulzbach, Germany). TLR7 knockout (TLR7KO) and MyD88 knockout (MyD88KO) mice were generously provided by Dr. S. Akira (Department of Host Defense, Osaka University, Osaka, Japan). All animals were maintained according to the guidelines of the committee for animal care. Animal studies have been reviewed and approved by the Landesamt für Gesundheit und Soziales Berlin. N1E-115 neuroblastoma cells and HEK293 cells (both obtained from American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% heat-inactivated FCS and penicillin–streptomycin. Cells were grown at 37°C in humidified air with 5% CO_2.

Plasmids

The plasmid pCAG-mTLR7-HA was constructed by subcloning the corresponding coding frame from pUNO-TLR7-HA (Invitrogen, Carlsbad, CA) into pCAG-IRES-EGFP (29). pCAG-IRES-EGFP served as control vector.

Intrathecal injection into mice

Intrathecal injection into mice and analysis of the cerebrospinal fluid was performed as described previously (30). Ten micrograms of RNA was used for injections. After transcardial perfusion with 4% paraformaldehyde, brains were removed and cryoprotected in 30% sucrose. Neuronal survival in the brain's cortex was analyzed by quantifying NeuN-positive cells in six fields (×60) from five representative sections of each brain.

Primary culture of cortical neurons, microglia, and astrocytes

Primary cultures of purified cortical neurons, microglia, and astrocytes were generated as described previously (16).

Toxicity assays in vitro

For toxicity studies, indicated amounts of ssRNA40 complexed with LyoVec, imiquimod, loxoribine (all from InvivoGen, San Diego, CA), LPS (List Biological Laboratories, Campbell, CA), and other reagents were added to cell cultures for indicated durations. The control oligoribonucleotide was synthesized by Purimex (Grebenstein, Germany). Control cultures were incubated with PBS or LyoVec (Cayla, Toulouse, France). For each condition, experiments were performed in duplicate. Cells were immunostained with NeuN Ab (Chemicon, Temecula, CA). NeuN-positive cells were quantified by analyzing six high-power fields per coverslip. The viability of control cells was set to 100%, and results were expressed as relative neuronal viability. TUNEL staining of CNS cultures was conducted using the In Situ Cell Death Detection kit, TMR red, following the instruction manual (Roche, Basel, Switzerland). For caspase inhibition assays, 100 μM Z-VAD-FMK or Z-DEVD-FMK (Calbiochem, Gibbstown, NJ) was added 12 h before RNA treatment. For TNF-α inhibition assays, 10 μg/ml mouse TNF-α Ab (cat. no. AF-410-NA) (31, 32) or normal goat IgG control (both from R&D Systems, Wiesbaden-Nordernstadt, Germany) was added to cell cultures in the presence or absence of TLR7 agonists, as indicated.

Flow cytometry

Purified microglia and cortical neurons were prepared as described above. Abs against CD11b and β3-tubulin (BD Biosciences, Bedford, MA) were used for immunostaining of microglia and neurons, respectively. In addition, a monoclonal anti-mouse Ab against TLR7 (Alexa Fluor 488; Dendritics, Lyon, France) or an IgG1 isotype control (eBioscience, San Diego, CA) was used. Data were collected on a FACSCalibur II and analyzed by Flow Jo Version 8.8.6 (Tree Star, Ashland, OR).

Detection of cytokines and chemokines

Purified microglia were incubated with 10 μg/ml ssRNA40 for 12 h. The concentrations of IL-6, IL-10, RANTES, and MCP-1 in the supernatants were determined by bead-based multiple analyte detection (FlowCytomix; Bender MedSystems) according to the manufacturer's instructions.

TNF-α ELISA

Microglia were incubated for indicated durations with increasing concentrations of ssRNA40 or mutant oligoribonucleotide complexed to the transfection agent LyoVec (Cayla, Toulouse, France). Conditioned medium was collected and analyzed by ELISA with hamster Ab against mouse TNF-α (BD Biosciences, Franklin Lakes, NJ) as a capture Ab and biotin-labeled rabbit anti-mouse as a secondary Ab (BD Biosciences).

RT-PCR

Relative levels of TLR7 mRNA in neurons, astrocytes, and microglia were determined by RT-PCR as described (15). Primers against mouse TLR7 and the SYBR Green dye as the fluorescent reporter were obtained from Qiagen (Hilden, Germany).

Immunocytochemistry and immunohistochemistry

Immunostaining was performed as described previously (16). The following primary Abs were used: anti-neuronal-specific nuclear protein NeuN, anti-neurofilament, and anti-glial fibrillary acidic protein, all purchased from Chemicon (Temecula, CA); anti-TLR7 was purchased from Imgenex (San Diego, CA); anti–TNF-α was obtained from R&D Systems (Wiesbaden-Nordernstadt, Germany); and anti-Iba1 and anti–caspase-3 were from Wako (Neuss, Germany) and Chemicon (Billerica, MA), respectively. IB4 was obtained from Invitrogen (Carlsbad, CA), and DAPI was from Roche (Basel, Switzerland). Fluorescence microscopy was performed with an Olympus BX51 microscope and with a confocal laser scanning Leica TCS SL microscope with sequential analysis.

In situ hybridization

In situ hybridization of P0 mouse brain cryostat sections using a hybridization probe directed against TLR7 (5′-TAATCACATCCACTTTTT-CATC-3′) and a scrambled control motif (5′-GTGACACGTCTATAC-GCCCA-3′; both from Exiqon, Vedbaek, Denmark) was visualized by Fast Red or NBT/BCIP staining and was performed as described previously (33).

Statistical analysis

Data are expressed as mean ± SD or SEM, as indicated. Statistical differences between groups were determined using two-tailed Student t test or one-way ANOVA with subsequent Bonferroni post hoc analysis. Differences were considered statistically significant when the p value was <0.05.

Results

Synthetic TLR7 ligands induce neuronal cell death in vitro

TLR7 is expressed in microglia and astrocytes (8, 34) and was recently detected in peripheral neurons (28). To investigate the expression of TLR7 in neurons of the CNS, RNA from purified mouse cortical neurons, microglia, and astrocytes was reverse-transcribed and analyzed by PCR using primers specific for mouse TLR7. The purity of the neuronal cultures used was confirmed by immunocytochemistry with NeuN Ab to be at least 95%. Spleen tissue served as positive control (Fig. 1A). Similar amounts of transcripts of TLR7 were detected in cortical neurons, astrocytes, and microglia. In situ hybridization of cortical neurons and microglia with a TLR7-specific probe confirmed the presence of TLR7 mRNA in neurons and microglia in vitro (Fig. 1B). Employment of a mismatched hybridization probe and cell preparations derived from TLR7KO mice in this experimental setup confirmed the specificity of the employed TLR7 hybridization probe. Whereas in situ hybridization of brain sections from
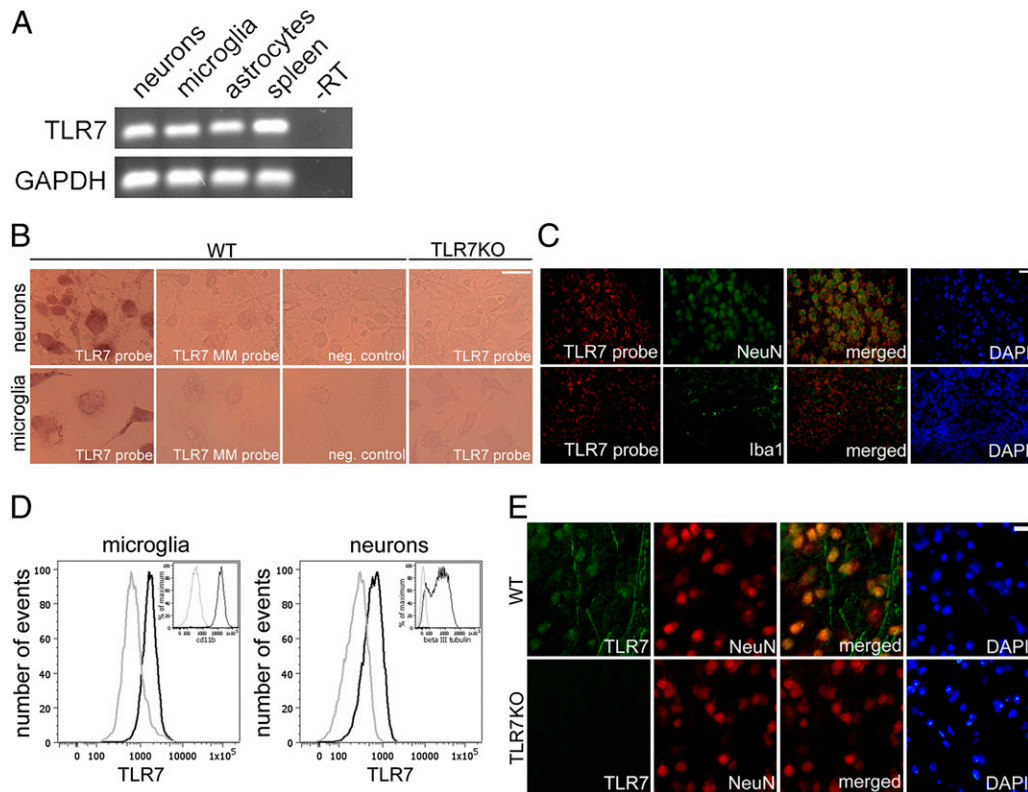
FIGURE 1. Neurons of the CNS express TLR7. (**A**) RNA was isolated from neuronal, microglial, and astrocyte cultures and amplified by RT-PCR with specific primers for TLR7 or GAPDH, which served as a housekeeping gene control. Spleen tissue served as positive control for TLR7 expression. (**B**) In situ hybridization (NBT–BCIP) of primary neurons and microglia isolated from C57BL/6J (wild type; WT) and TLR7KO mice using a DIG-labeled probe specific for *tlr7* or a mismatched probe (MM) with a sequence containing a scrambled *tlr7* sequence. Scale bar, 10 μm. (**C**) In situ hybridization of the P0 mouse brain cortex costained with Iba1 to label microglia, an Ab against NeuN to mark neurons, and DAPI to visualize all nuclei. Subsequently, brain sections were stained with Fast Red to pick up the in situ hybridization signal. Scale bar, 10 μm. (**D**) Flow cytometric analysis of neurons and microglia stained with an mAb specific for TLR7. Abs against β3-tubulin and CD11b served as markers for neurons and microglia, respectively. Data in the large plots are gated on cells positive for β3-tubulin and CD11b as depicted in the small plots (*insets*). Black line, anti-TLR7; gray line, isotype control. (**E**) Brain sections of C57BL/6J (WT) and TLR7KO mice were stained with DAPI and immunostained with Abs against TLR7 and NeuN. Scale bar, 10 μm.

C57BL/6J mice revealed abundant expression of TLR7 mRNA in cortical neurons, no hybridization signal for this mRNA was detected in microglia (Fig. 1C). These results indicate that, contrary to our findings in vitro, TLR7 mRNA is constitutively expressed in neurons but not in microglia in vivo. To analyze the expression of TLR7 protein in CNS cells, purified microglia and neurons were analyzed by cytofluorometry with an mAb directed against TLR7 (Fig. 1D). Expression of TLR7 protein was detected in both isolated microglia and neurons. Immunohistochemical analysis of sections derived from mouse brains using both NeuN and TLR7 Abs confirmed the expression of TLR7 protein in cortical neurons of wild-type but not TLR7KO mice (Fig. 1E). In the wild-type cortex, incubation with the TLR7 Ab resulted not only in the labeling of the neuronal soma but also of axons (Fig. 1E). However, parallel incubation with various Abs against established axonal markers including neurofilament, tau, or myelin did not result in a colocalization with TLR7, and the identity of these axons remains unclear (data not shown).

Taken together, isolated microglia and neurons express TLR7, whereas constitutive expression of this receptor is detected in neurons but not microglia in vivo, suggesting upregulation of TLR7 in microglia upon manipulation.

TLR7 was initially identified as a receptor recognizing synthetic antiviral compounds such as the guanosine analogues imiquimod and loxoribine (2, 35, 36). We have previously described that activation of TLR2 and TLR4 in the CNS by their respective ligands Pam3CSK4 and LPS causes neuronal injury (14, 16). To investigate the role of TLR7 in CNS damage, purified cortical neurons, microglia, and astrocytes were generated from C57BL/6J mice and incubated with 10 μg/ml imiquimod or PBS (control) for 4 d. Staining of neurons with NeuN Ab revealed cell damage and loss in neuronal cell cultures treated with imiquimod. In contrast, no harmful effects were detected in cell cultures of microglia or astrocytes (Fig. 2A). Quantification of surviving NeuN-positive cells confirmed that imiquimod causes a reduction of neuronal viability, whereas microglia and astrocyte numbers were unaffected by the treatment (Fig. 2B). Likewise, incubation with loxoribine caused cell death in neuronal cultures but not in microglial or astrocyte cultures (data not shown). As expected, incubation with LPS led to a reduction of microglial numbers but not to neuronal cell loss (16), indicating that the observed neurotoxic effects in neuronal cultures incubated with imiquimod and loxoribine are specific for activation of TLR7 (Fig. 2B).

ssRNA40 induces neuronal cell death in vitro through TLR7

ssRNA40 is an HIV-derived 20-mer single-stranded oligoribonucleotide with a GU-rich sequence. For use as a TLR7 ligand in vitro and in vivo, the phosphate backbone is protected by incorporation of phosphothioates (3). ssRNA40 was established as a natural ligand of TLR7. It is generally applied complexed to the cationic lipid LyoVec further to protect it from degradation and to facilitate its uptake. When complexed to LyoVec, ssRNA40 can
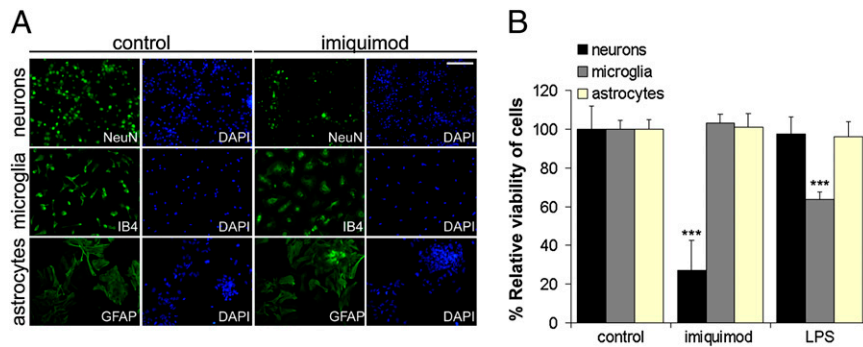
FIGURE 2. Imiquimod induces neuronal cell death. Cortical neurons, microglia, and astrocytes derived from C57BL/6J mice were incubated with 10 μg/ml imiquimod or PBS (control) for 4 d. (**A**) Surviving cells were immunostained with NeuN Ab, IB4, or glial fibrillary acidic protein (GFAP) Ab. All nuclei were stained with DAPI. Scale bar, 50 μm. (**B**) Quantitation of NeuN-, GFAP-, and IB4-positive cells incubated with 10 μg/ml imiquimod, 100 ng/ml LPS, or PBS (control) for 4 d. Six high-power fields per coverslip were analyzed. For each condition, experiments were performed in duplicate. Data shown are representative of five individual experiments. Results are presented as mean ± SD. ***$p < 0.001$ (Student t test).

substitute for viral RNAs in inducing the release of TNF-α and IFN-α from PBMCs (3, 5). To test whether ssRNA40 is capable of inducing toxic effects in neurons similar to imiquimod and loxoribine, primary neurons prepared from cortices of C57BL/6J mice were incubated with increasing amounts of ssRNA40 for various time intervals. A 20-mer oligoribonucleotide with a sequence containing A's and T's instead of G's and U's served as negative control ("mut. oligo"). Cells were immunostained with Abs against neurofilament and neuronal nuclei to label axons and neurons, respectively (Fig. 3A). Incubation with ssRNA40 led to injury of axons and reduction of neurons, whereas neuronal numbers and axonal integrity were not affected in cultures incubated with the control oligoribonucleotide. Quantification of NeuN-positive cells revealed a dose-dependent (Fig. 3B) and time-dependent (Fig. 3C) reduction of the relative neuronal viability induced by ssRNA40. In contrast, incubation of neurons with the control oligoribonucleotide did not affect relative neuronal viability compared with control conditions during the whole round of observation, even when used at the highest concentration of 20 μg/ml. Similarly, both LyoVec alone and LPS did not affect relative neuronal viability. Activity of ssRNA40 closely resembled that of loxoribine, whereas imiquimod reduced neuronal numbers to a greater extent than ssRNA40 and loxoribine (Fig. 3B). The effect of ssRNA40 was not likely to be mediated by soluble secreted factors because conditioned medium from neuronal cultures stimulated with ssRNA40 failed to affect morphology or numbers of freshly plated neurons (Fig. 3D).

To confirm the role of TLR7 in neuronal cell death induced by ssRNA40, TLR7-deficient mice were included in the experimental setup (Fig. 3B, 3C). In contrast to wild-type neurons, neurons prepared from TLR7KO mice were completely protected against neuronal cell death induced by ssRNA40, as assessed by quantification of NeuN-positive cells. Whereas neurotoxic effects caused by loxoribine were also abolished in cultures containing TLR7KO neurons, imiquimod induced cell death in these cultures, although to a lesser extent than in wild-type cultures (Fig. 3B). The fact that neurons lacking TLR7 are not completely protected against neuronal cell death induced by imiquimod may be explained by its interference with an adenosine receptor signaling pathway (37) or by binding of imiquimod to receptors other than TLR7. Increased numbers of TUNEL-positive cells in wild-type but not in TLR7-deficient neuronal cultures treated with ssRNA40, loxoribine, or imiquimod confirmed neuronal cell death induced by ssRNA40 through TLR7 in vitro (Fig. 3E). The fact that not all wild-type

neurons display TUNEL positivity after treatment with TLR7 ligands may be due to inherent differences of TLR7 expression within the neuronal cell culture. Also, because neuronal death was dose- and time-dependent, the maximum effect may not have been reached in our experiment.

These results demonstrate that extracellularly applied ssRNA40 induces neuronal cell death dependent on TLR7 in vitro.

Neuronal cell death induced by ssRNA40 is cell autonomous and does not require microglia

Neuronal injury and cell death mediated by TLR2 and TLR4 require the presence of microglia (14, 16). So far, highly enriched neuronal preparations were used in this study. We next tested the effect of microglia on ssRNA40-induced neuronal cell death by comparing these purified neuronal cultures with cocultures of neurons and microglia (14). Both cultures were incubated with various concentrations of ssRNA40 for 4 d, as before. Imiquimod and loxoribine were used as additional TLR7-specific ligands. Cells were then immunostained with NeuN Ab or IB4 to label neurons and microglia, respectively (Fig. 4A). Assessment of relative neuronal viability showed that ssRNA40 induces neurotoxic effects in both purified neurons and neurons cocultured with microglia. However, the extent of neuronal cell death was increased in the presence of microglia. Similar results were obtained when neurons and cocultures of neurons and microglia were incubated with imiquimod or loxoribine (Fig. 4A). In contrast, incubation with LPS induced neuronal cell death only in the presence of microglia, as expected (16). The fact that supernatants of purified neurons incubated with LPS did not contain TNF-α or NO, as assessed by ELISA and Griess assay, respectively, confirms the absence of relevant numbers of microglia in these cultures (data not shown). To rule out further the involvement of other cell types in neuronal cell death induced by ssRNA40 and mediated by TLR7, N1E-115 cells derived from a neuroblastoma cell line were incubated with ssRNA40, loxoribine, or imiquimod for 36 h, and relative neuronal viability was assessed after immunostaining with NeuN Ab (Fig. 4B, 4C). Similarly to primary cortical neurons, N1E-115 cells incubated with ssRNA40 underwent cell death, confirming that neuronal cell death induced by ssRNA40 is cell autonomous. Similar results were obtained in experiments using loxoribine or imiquimod (Fig. 4B, 4C).

Taken together, neuronal cell death induced by ssRNA40 is cell autonomous but is enhanced in the presence of microglia.
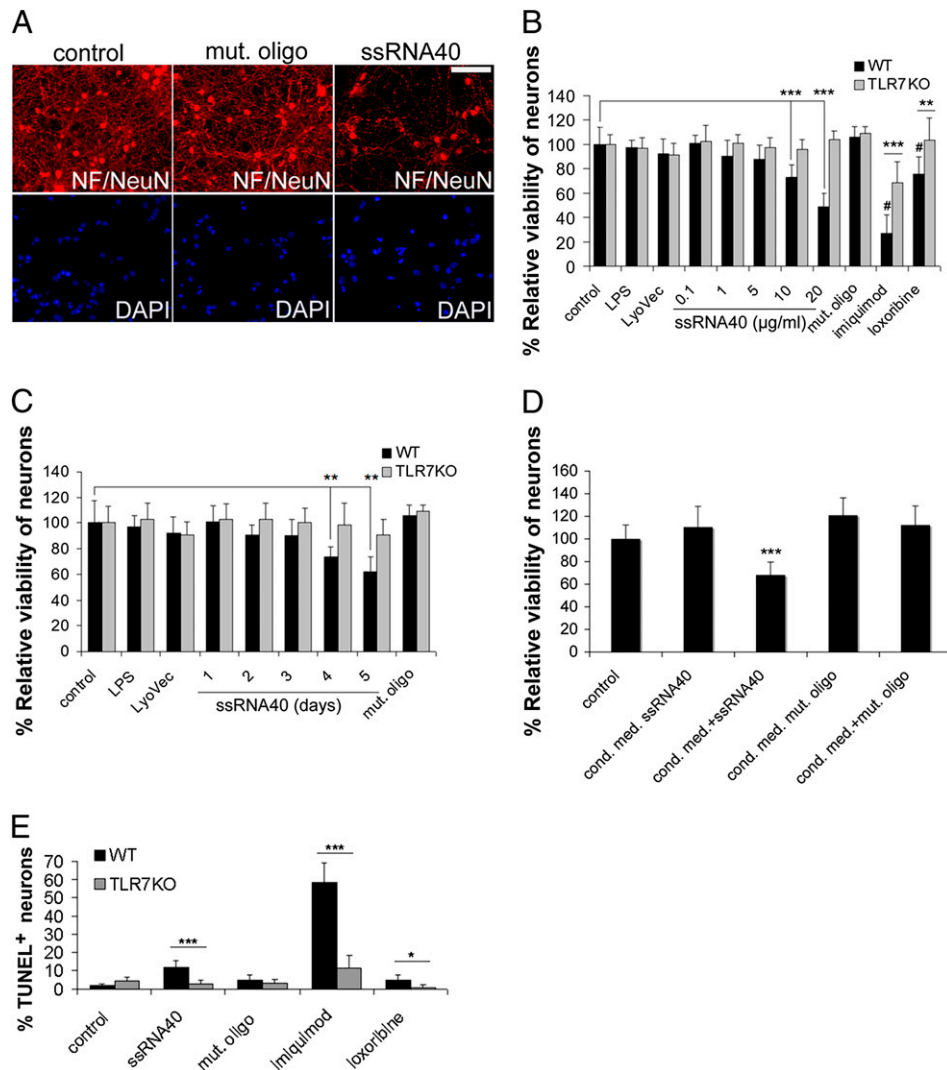
FIGURE 3. ssRNA40 induces cell death in cultures of cortical neurons dependent on TLR7. Cortical neurons were prepared from C57BL/6J (WT) or TLR7KO mouse brains. (**A**) WT neurons were incubated with 10 μg/ml ssRNA40, 10 μg/ml control oligoribonucleotide (mut. oligo), or PBS (control) for 4 d. Subsequently, cells were immunostained with Abs against NeuN and neurofilament. DAPI staining visualized all nuclei. Scale bar, 50 μm. (**B** and **C**) Purified WT and TLR7KO neurons were incubated with increasing concentrations of ssRNA40 for 4 d (B) or with 10 μg/ml ssRNA40 for various incubation periods (C), as indicated. A control RNA oligoribonucleotide and LyoVec alone served as negative controls, whereas LPS was used as a control for TLR specificity. (**D**) In a separate culture, neurons grown in normal medium were first stimulated with 10 μg/ml ssRNA40 or control oligoribonucleotide (mut. oligo) for 24 h, washed thoroughly to eliminate trace amounts of ssRNA40, and further incubated in fresh normal medium for 3 d, after which the supernatant was collected as the conditioned media. Fresh neuronal cultures were incubated with this conditioned media with or without additional 10 μg/ml ssRNA40 or control oligoribonucleotide for 4 d. Cells were then stained with NeuN Ab. NeuN-positive cells were quantified, and results were statistically expressed as relative neuronal viability by setting the viability of control cells to 100%. (**E**) Neurons derived from C57BL/6J (WT) or TLR7KO mice were incubated with 10 μg/ml ssRNA40 or control oligoribonucleotide for 24 h and analyzed by TUNEL assay. TUNEL-positive neurons and DAPI-positive nuclei were quantified. For each condition, experiments were performed in duplicate. Data shown are representative of 10 individual experiments. Results are presented as mean ± SD. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$ (Student t test), $^#p < 0.05$ (for the comparison with control conditions).
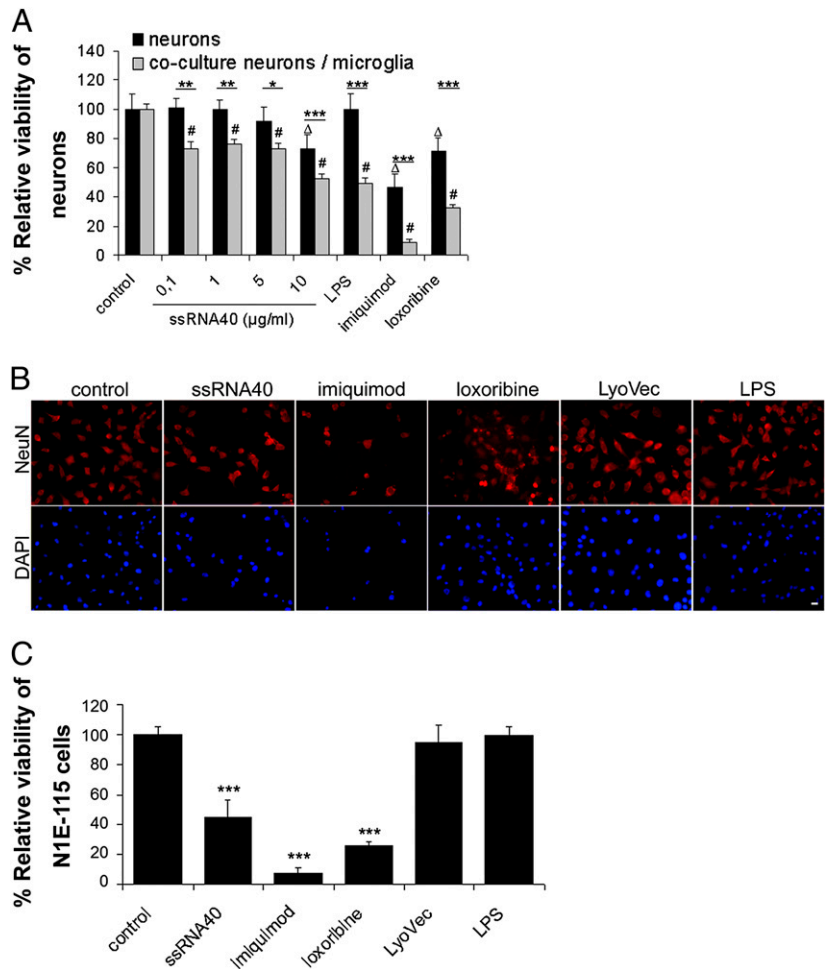
ssRNA40-induced neuronal cell death involves activation of MyD88 and caspase-3

A critical test for the hypothesis that TLR7 activation leads to cell death in neurons is the demonstration that key elements of the TLR7 signal transduction pathway are required. The intracellular adapter MyD88 is situated downstream of TLR7, and its involvement in ssRNA40-induced neuronal cell death was investigated next. MyD88 is expressed in cortical neurons (27) and is required for neuronal damage mediated by TLR2 and TLR4 (14, 24). To investigate the role of MyD88 in neuronal cell death induced by ssRNA40, neurons were isolated from wild-type and MyD88KO mice and incubated with ssRNA40 at various con-

centrations for various time periods (Fig. 5A, 5B). Assessment of relative neuronal viability by immunostaining with NeuN Ab revealed that neurons lacking MyD88 are completely protected against ssRNA40-induced neuronal cell death.

The caspase family of cysteine-directed proteases represents one of the key regulators of apoptosis. Caspase-3 is a terminal effector molecule that, once activated, commits cells to death (38). To test if caspase-3 is involved in neuronal cell death induced by ssRNA40, neurons were incubated with ssRNA40, loxoribine, or imiquimod and subsequently immunostained with Abs against activated caspase-3 and NeuN (Fig. 5C). Neuronal cultures incubated with ssRNA40, imiquimod, or loxoribine contained increased numbers

FIGURE 4. Neuronal cell death induced by ssRNA40 is cell autonomous. (**A**) Purified neurons and neurons supplemented with microglia derived from C57BL/6J mouse brains were incubated with ssRNA40 as indicated, 10 μg/ml imiquimod, 1 mM loxoribine, 1 μg/ml LPS, or PBS (control). After 4 d, cell cultures were fixed and stained with NeuN Ab and with IB4 to mark neurons and microglia, respectively. Relative neuronal viability was assessed. (**B**) N1E-115 cells were incubated with 10 μg/ml ssRNA40, 10 μg/ml imiquimod, 1 mM loxoribine, 5 μg/ml LyoVec, 1 μg/ml LPS, or PBS (control) for 36 h and immunostained with NeuN Ab. All nuclei were stained with DAPI. Scale bar, 10 μm. (**C**) Quantitation of NeuN-positive N1E-115 cells incubated as indicated above. Six high-power fields per coverslip were analyzed. For each condition, experiments were performed in duplicate. Data shown are representative of three individual experiments. Results are presented as mean ± SD. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$ (for the comparison of indicated groups; Student t test), $^{\#}p < 0.05$, $^{\Delta}p < 0.05$ (for the comparison with the respective control conditions).

of caspase-3-positive neurons compared with control conditions where PBS, the control oligoribonucleotide, or LPS were used. To analyze further the functional role of caspase-3 in neuronal cell death induced by ssRNA40, neurons were incubated with the caspase-3-specific inhibitor Z-DEVD-FMK or the pan caspase inhibitor Z-VAD-FMK before treatment with ssRNA40. Surviving
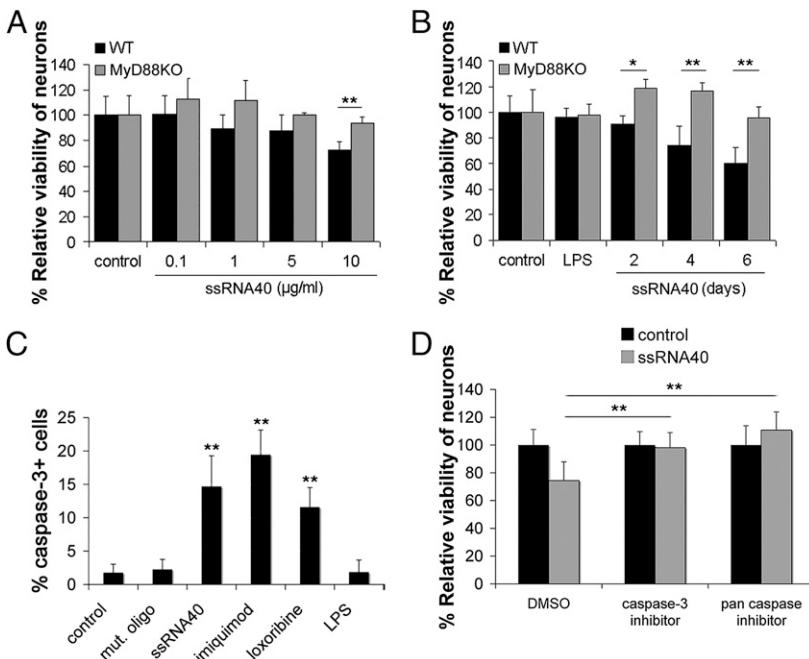

FIGURE 5. ssRNA40-induced neuronal cell death via TLR7 involves MyD88 and activation of caspase-3. (**A** and **B**) Cortical neurons were prepared from C57BL/6J (WT) or MyD88KO mouse brains and were incubated with increasing concentrations of ssRNA40 for 4 d (A) or with 10 μg/ml ssRNA40 for various incubation periods (B), as indicated. Cells were then stained with NeuN Ab, and relative neuronal viability was assessed. Data shown are representative of three individual experiments. (**C**) Neurons from C57BL/6J mice were incubated with 10 μg/ml ssRNA40, 10 μg/ml control oligoribonucleotide, 10 μg/ml imiquimod, 1 mM loxoribine, 1 μg/ml LPS, or PBS (control) for 3 d. Cells were immunostained with an Ab directed against activated caspase-3 and with DAPI and were quantified. (**D**) Neurons from C57BL/6J mice were incubated with either Z-DEVD-FMK (caspase-3 inhibitor) or Z-VAD-FMK (general caspase inhibitor) for 12 h before the incubation with 10 μg/ml ssRNA40 or PBS (control) for 4 d. Relative neuronal viability was assessed. Experiments are presented as mean ± SD. One representative experiment of three to four independent experiments is shown. $*p < 0.05$, $**p < 0.005$ (for the comparison of indicated groups with control conditions; Student t test).

cells were immunostained with NeuN Ab and quantified (Fig. 5D). Both global inhibition of caspases and specific inhibition of caspase-3 prevented ssRNA40-induced cell death.

In summary, the intracellular pathway induced by ssRNA40 in neurons requires MyD88 and involves activation of caspase-3.

Activation of TLR7 in microglia by ssRNA40 leads to an inflammatory response

All known TLR ligands induce a release of inflammatory cytokines from immune cells (1). Although microglia are not required for neuronal cell death induced by ssRNA40, an amoeboid shape of microglia was observed in response to incubation with ssRNA40, loxoribine, and imiquimod, indicating an activated state. In contrast, treatment of TLR7KO microglia with the respective TLR7 ligands did not cause such morphological changes (Fig. 6A). To analyze further the activation state of microglia in response to ssRNA40, wild-type and TLR7KO microglia were incubated with

increasing amounts of ssRNA40 for various time periods and were subsequently analyzed for the release of TNF-α by ELISA (Fig. 6B, 6C). Wild-type microglia released TNF-α in response to incubation with ssRNA40 in a dose- and time-dependent manner, whereas no TNF-α was detected in supernatants of microglial cultures incubated with the control mutant oligoribonucleotide. The amount of TNF-α detected in supernatants of microglial cultures incubated with ssRNA40 was similar to the amount detected in microglial cultures incubated with loxoribine, imiquimod, or LPS. TLR7 was required for the ssRNA40-induced TNF-α response, as microglia prepared from TLR7KO mice failed to release relevant amounts of TNF-α (Fig. 6B, 6C). To determine the contribution of TNF-α to TLR7-mediated neurotoxicity through microglia, cortical neurons cocultured with microglia were treated with either ssRNA40 or imiquimod alone or in combination with a neutralizing TNF-α Ab (Fig. 6D). Whereas both TLR7 ligands induced a reduction in the number of
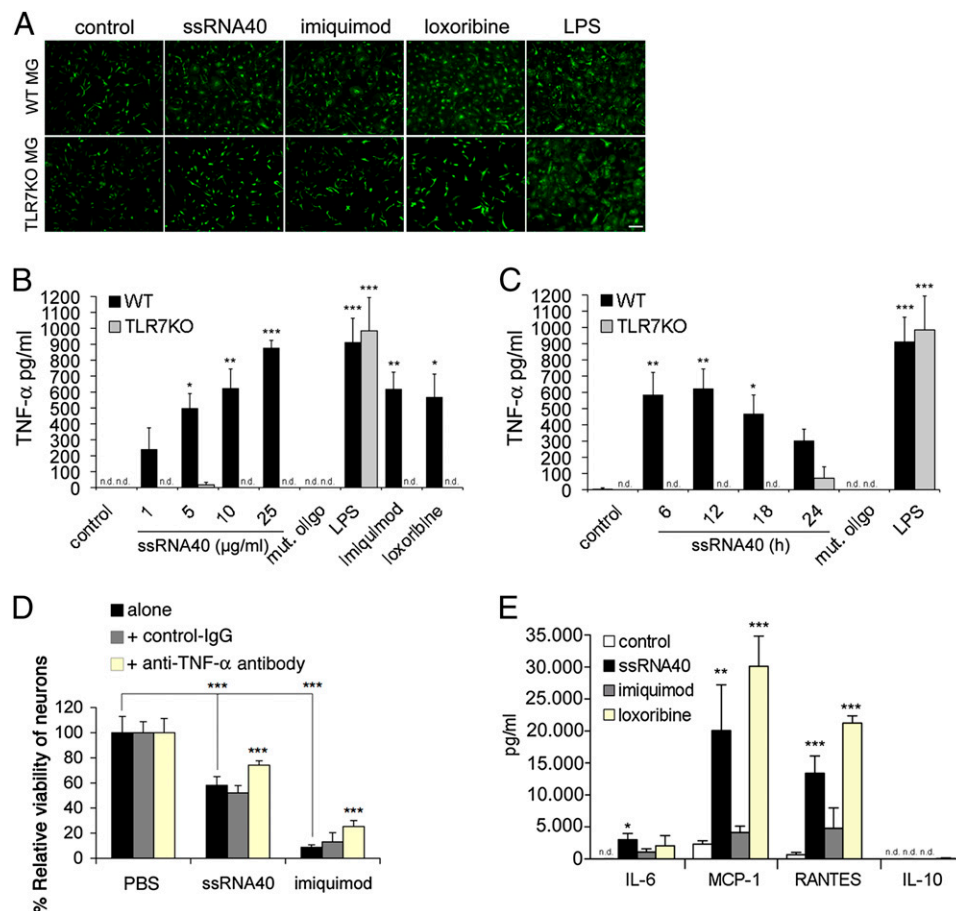

FIGURE 6. ssRNA40 induces the release of TNF-α and other inflammatory molecules from microglia dependent on TLR7. (**A**) Microglia isolated from C57BL/6J (WT) and TLR7KO mice were incubated with 10 μg/ml ssRNA40, PBS (control), 5 μg/ml imiquimod, 1 mM loxoribine, or 1 μg/ml LPS for 12 h. Subsequently, cells were stained with IB4. Scale bar, 10 μm. (**B** and **C**) Microglia from C57BL/6J (WT) and TLR7KO mice were incubated with various doses of ssRNA40 or 10 μg/ml control oligoribonucleotide for 12 h (B) or with 10 μg/ml ssRNA40 or 10 μg/ml control oligoribonucleotide for various durations (C), as indicated. Imiquimod and loxoribine were used as further TLR7-specific ligands, whereas LPS served as a specific activator of TLR4. Subsequently, the amount of TNF-α in the culture supernatants was determined by ELISA. Data shown are pooled from three experiments with three preparations of cells. Results are presented as mean ± SEM. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ (ANOVA test). (**D**) Neurons supplemented with microglia derived from C57BL/6J mouse brains were incubated with 10 μg/ml ssRNA40 or imiquimod alone or in combination with 10 μg/ml TNF-α blocking Ab, as indicated. PBS and 10 μg/ml normal goat IgG served as control. After 4 d, cell cultures were fixed and stained with NeuN Ab to mark neurons. Relative neuronal viability was assessed. Experiments are presented as mean ± SD. One representative experiment out of three independent experiments is shown. ***$p < 0.001$ [for the comparison of indicated groups with PBS alone or after addition of control IgG (+ control IgG), as indicated; Student t test]. (**E**) Microglial cultures derived from C57BL/6J mice were incubated with 10 μg/ml ssRNA40, PBS (control), 5 μg/ml imiquimod, or 1 mM loxoribine for 12 h. Resulting supernatants were analyzed for the presence of IL-6, RANTES, MCP-1, and IL-10 using a cytokine multiplex assay. Data shown are pooled from three experiments with three preparations of cells. Results are presented as mean ± SEM. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ (ANOVA test). n.d., Not detectable.

neurons compared with control conditions, TLR7-mediated neurotoxicity was significantly decreased in cultures cotreated with the TNF-α Ab. In wild-type microglia, ssRNA40 induced a specific pattern of an inflammatory response characterized by secretion of IL-6, MCP-1, and RANTES, whereas no IL-10 was detected (Fig. 6E).

ssRNA40 causes neurodegeneration dependent on TLR7 in vivo

We next investigated whether neurotoxic effects induced by ssRNA40 in vitro are relevant in models of neuronal damage in vivo. We have established a model of neuronal damage and cell loss by intrathecal injection of the TLR2 ligand Pam3CSK4. Intrathecal administration of this synthetic lipopeptide in mice induced the pathophysiological hallmarks typically associated with bacterial meningitis induced by pneumococcus including neuronal injury (30). To analyze the effect of ssRNA40 on the mouse brain in vivo, ssRNA40 or the control oligoribonucleotide were injected intrathecally into C57BL/6J mice. Brain sections were analyzed by immunostaining using NeuN and neurofilament Ab (Fig. 7A–C). Of the total of nine animals from the C57BL/6J strain treated with ssRNA40, eight showed axonal loss in the corpus callosum and underlying structures (Fig. 7A) and in the hippocampus (Fig. 7B). In addition, neuronal loss in the cerebral cortex was detected in seven of these animals (Fig. 7C). In contrast, no signs of axonal or neuronal damage were observed in the brain's cortex of mice injected with the control oligoribonucleotide (eight animals) or carrier (control, six animals) (Fig. 7A–C).

To confirm the role of TLR7 in neurodegeneration induced by ssRNA40 in vivo, TLR7KO mice were included in the experimental setup described earlier (Fig. 7A–C). No mortality was observed in wild-type or TLR7KO mice over 3 d. None of the TLR7KO mice that received ssRNA40 (nine animals) or control oligoribonucleotide (seven animals) showed axonal or neuronal damage. Quantitative analysis of the surviving cortical neurons in wild-type and TLR7KO mice confirmed these results (Fig. 7D). Total numbers of NeuN-positive cells in the cortex of wild-type mice were reduced by ~20% after injection of ssRNA40 compared with animals treated with the control oligoribonucleotide. In contrast, neuronal numbers of TLR7KO mice injected with ssRNA40 did not differ from those of animals under control conditions or those of wild-type mice injected with the control oligoribonucleotide.

FIGURE 7. Intrathecal administration of ssRNA40 causes neurodegeneration dependent on TLR7. (A–D) Ten micrograms of ssRNA40, 10 μg control oligoribonucleotide, or water (carrier) were injected intrathecally into 8- to 10-wk-old C57BL/6J [(A–D) WT: ssRNA40 $n = 9$; mut. oligo $n = 8$; water $n = 6$] or TLR7-deficient [(A–D) TLR7KO: ssRNA40 $n = 9$; mut. oligo $n = 7$; water $n = 6$] mice. After a further 3 d, brain sections were analyzed by immunostaining with neurofilament (NF) Ab [(A) corpus callosum; (B) hippocampus] and NeuN Ab [(C) cortex]. DAPI staining marked all nuclei. Scale bar, 50 μm. (D) Quantification of cortical NeuN-positive cells of WT and TLR7KO mice intrathecally injected with RNA, as indicated above. (E) Numbers of leukocytes in the cerebrospinal fluid of WT and TLR7KO mice 12 h after intrathecal application of 10 μg ssRNA40, 10 μg control oligoribonucleotide, or 10 μg Pam3CSK4 that served as positive control for leukocyte influx. Results are presented as mean ± SD. ***$p < 0.001$ (Student t test). (F) Sections of the brain's cortex were immunostained with both Iba1 and TNF-α Ab after intrathecal injection of 10 μg ssRNA40 or 10 μg mutant oligoribonucleotide into WT and TLR7KO mice. Scale bar, 50 μm.
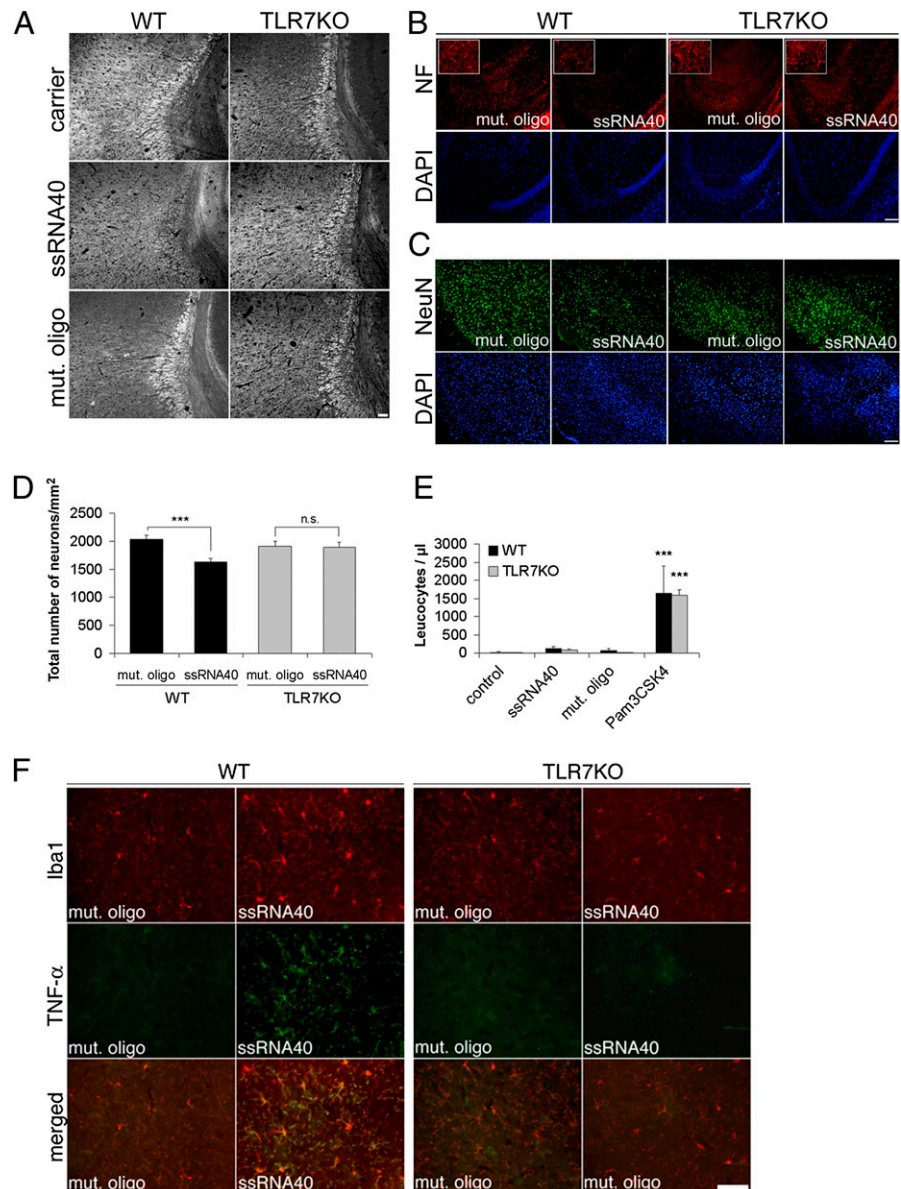
To investigate the inflammatory response induced by intrathecal injection of ssRNA40 into mice, cerebrospinal fluid from wild-type and TLR7KO mice treated with ssRNA40 or control oligoribonucleotide was analyzed (Fig. 7E). Intrathecal injection of the positive control Pam3CSK4 caused an influx of leukocytes in both wild-type and TLR7KO mice, as expected (30). In contrast, no significant increase in the number of leukocytes was observed in the cerebrospinal fluid after intrathecal application of ssRNA40 or control oligoribonucleotide compared with control conditions. Immunostaining of microglia with an Ab directed against TNF-α revealed an activated state of these cells in the cerebral cortex of all wild-type but none of the TLR7KO mice after administration of ssRNA40 (Fig. 7F).

In summary, the results discussed above demonstrate an obligatory role for TLR7 in neurodegeneration induced by ssRNA40 in vivo.

Discussion

Although cause and individual susceptibility factors of infectious and noninfectious CNS disorders vary widely, common mechanisms of neuronal damage seem to exist. TLRs recognize both pathogen- and host-derived factors and hence may be crucial players in such processes of CNS damage independent of the initial cause of the respective disease.

In the current study, we confirm that TLR7 is expressed in neurons of the CNS. Although it was long believed that microglia and other glial cells were the only intrinsic cell types of the brain expressing TLRs and responding to TLR ligands (7, 8), there is increasing evidence that TLRs play a role in neurons in a cell-autonomous manner. Expression of TLRs varies between different types of neurons. All known TLRs have been detected in neural progenitor cells, whereas TLR2 was described as the predominant receptor in hypothalamic neurons (39, 40). Therefore, TLRs in neurons seem to fulfill differential tasks in different parts of the CNS.

In the context of viral infections such as rabies and herpes simplex encephalitis, TLRs have been detected in neurons (26). Furthermore, recent data suggest that neurons are capable of mobilizing an immune response against microbes by expressing inflammatory genes (41). It remains unknown whether neurons use TLR7 to generate such an inflammatory response. We have now shown that ssRNA40, which was originally derived from HIV and therefore may be representative of pathogen-associated ligands for TLR7, induces cell-autonomous neurodegeneration. This cell death response may promote neuronal decay in an effort to limit further viral infection and may represent a primary protective mechanism common to antiviral defense. At the same time, ssRNA40 is a small synthetic oligoribonucleotide that may be exemplary for other, even host-derived, small RNA molecules. Activation of TLR7 by ssRNAs such as ssRNA40 depends on a specific nucleotide sequence (3, 5, 42, 43). It is conceivable that host-derived small nucleotides such as small interfering RNAs or microRNAs exist that activate TLR7 similar to ssRNA40. These molecules may be similar in length to ssRNA40 and share sequence patterns that are responsible for activation of TLR7 by ssRNA40 (3, 44). As outlined later, such small RNA molecules may act as endogenous ligands of TLR7 in the CNS.

A role of host-derived TLR ligands in various forms of CNS damage is discussed in several studies. For example, TLR expression is observed in CNS neurons of patients with amyotrophic lateral sclerosis or Alzheimer's disease in the apparent absence of pathogens (26, 45). In addition, studies in noninfectious animal models of CNS damage including focal cerebral ischemia, experimental autoimmune encephalitis, or lesion of the entorhinal cortex reveal a deleterious role for TLR activation in the brain (21, 23, 25). Moreover, TLRs expressed in neurons were suggested to relate to the inhibition of axonal growth and cell death under noninfectious, nonpathological circumstances, namely development and neurogenesis (27, 40, 41). In glial cells, the host-derived microtubule regulator stathmin serves as an agonist of TLR3 (46). Besides this, the identity of the endogenous TLR ligands involved in pathways of the CNS is largely unknown.

In this report, we demonstrate that exposure of neurons to an extracellular RNA molecule causes neuronal cell death that is dependent on TLR7 in vitro and in vivo. In a state of disease, RNA molecules might be released after neuronal damage. In support of this hypothesis, extracellular RNA is commonly detected in brains of patients with diverse progressive neurodegenerative disorders, such as Alzheimer's disease (47), and injured or dying cells release factors into their local environment that activate innate immune receptors (48). These signals are believed to serve as a first alert to the organism, indicating that normal cellular function has been disrupted. The endogenous activators of innate immune receptors identified to date are evolutionarily conserved proteins or nucleotides with the common feature of being "foreign" to the extracellular environment in normal tissue (49–52). The observed harmful effect of an oligoribonucleotide on neurons in our study may be similar to the self-reinforcing effect described for the ubiquitously expressed HSP60 and TLR4, in which release of cytoplasmic HSP60 from dying cells promotes further neuronal cell death in vitro (24). It was suggested that cytoplasmic RNA species may act as pathological chaperones for the accumulation of CNS lesions and subsequently may be cell toxic in neurodegenerative diseases (53–55). In such lesions, RNAs were predominately derived from neurons (56). It is tempting to speculate that such RNA molecules, at least in part, are recognized by TLR7, thereby contributing to the spread of CNS damage.

Neurotoxic effects induced by HSP60 are not cell autonomous but mediated by TLRs expressed in microglia (24). Considerable data exist demonstrating that microglial activation and the subsequent inflammatory response occurring in the setting of diverse neuronal injury models can be mediated through TLRs (14, 16, 57). Although TLR7 was expressed in both neurons and microglia in our model, experiments including cocultures of neurons and microglia and employment of a neuroblastoma cell line revealed that neuronal damage induced by ssRNA40 is cell autonomous and involves activation of neuronal caspase-3. Nevertheless, we have observed increased neuronal injury induced by ssRNA40 in the presence of microglia. TLR7 was shown to contribute to virus-induced neuroinflammation (58), and inflammatory molecules released from microglia can be responsible for TLR-mediated neuronal injury (59). Thus, inflammatory molecules released from microglia activated by ssRNA40 may unfold harmful effects on neurons in addition to the cell-autonomous neuronal damage directly caused by ssRNA40. As a consequence, a dual injurious effect, namely cell-autonomous neurodegeneration and microglia-mediated neuroinflammation, induced by stimulation of one single TLR may contribute to the outcome of CNS injury.

We demonstrate that MyD88 and caspase-3 are involved in TLR7-mediated neuronal cell death. The partial blocking effect of the caspase-3 inhibitor may be attributed to incomplete inhibition of caspase-3 or, alternatively, to the implication of other, yet unknown, mechanisms of cell death. It remains unclear at this stage how the TLR7–MyD88 pathway and activation of caspase-3 are linked. Although the functional role of neuronal TLRs was investigated in several studies, the intracellular signaling pathways involved remain undefined. However, it was suggested that the canonical pathway linking TLRs and NF-κB is not involved upon

TLR activation in neurons (27). Such differences in the signaling pathways induced by TLR7 activation in microglia and neurons may be important in determining the outcome of TLR7 stimulation and may explain the observed vulnerability of neurons, but not of microglia, toward TLR7 ligands in our studies. Interesting candidate molecules for future investigations include IRAK4, which may play a proapoptotic role (60), and JNK, which is suggested to link TLR4 and activation of caspase-3 in neurons challenged by Abeta protein (61).

In summary, we report that an extracellularly applied ssRNA induces neurodegeneration mediated by an innate immune receptor. Specifically, we demonstrate that ssRNA40 is an activator of TLR7 in both neurons and microglia with the consequence of neuronal damage in vitro and in vivo. We characterize the expression and function of TLR7 in neurons, thereby determining a new role for this receptor as neuronal death receptor. We speculate that both pathogen- and host-derived molecules can stimulate TLRs in the CNS under pathological conditions and thus send danger signals to neurons that accelerate their decay. Further research will be needed to establish the clinical impact of RNA-triggered neuronal injury through TLR7 in specific CNS disorders.

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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