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*J Immunol* 2006; 176:3804-3812; doi: 10.4049/jimmunol.176.6.3804

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Microglia Recognize Double-Stranded RNA via TLR3

Terrence Town,* David Jeng,* Lena Alexopoulou,‡ Jun Tan,§ and Richard A. Flavell²*†

Microglia are CNS resident innate immune cells of myeloid origin that become activated and produce innate proinflammatory molecules upon encountering bacteria or viruses. TLRs are a phylogenetically conserved diverse family of sensors for pathogen-associated molecular patterns that drive innate immune responses. We have recently shown that mice deficient in TLR3 (TLR3⁻/⁻ mice) are resistant to lethal encephalitis and have reduced microglial activation after infection with West Nile virus, a retrovirus that produces dsRNA. We wished to determine whether microglia recognize dsRNA through the TLR3 pathway. In vitro, murine wild-type primary cultured microglia responded to synthetic dsRNA polyinosinic-polycytidylic acid (poly(I:C)) by increasing IL-6 and IL-12, which play critical roles in the initiation of innate immunity. There are currently 11–12 members of the mammalian TLR family, and each TLR acts to recognize discrete microbial components resulting in induction of specific immune responses (4–7). For example, it is well established that the bacterial components LPS and peptidoglycan (PGN) are recognized by CD14/TLR4 and TLR2/6, respectively (5). Additionally, TLR recognition of viral envelope proteins has also been demonstrated (8–10). We have previously shown that peripheral macrophages and dendritic cells recognize polyinosinic-polycytidylic acid (poly(I:C)), a synthetic dsRNA analog, and dsRNA from Lang reovirus through TLR3 (11). Furthermore, we and others have demonstrated that TLR7 and TLR8 mediate recognition of ssRNA viruses and ssRNA (12–14), and both TLR9 (which recognizes unmethylated CpG-DNA motifs) and TLR3 mediate defense against mouse CMV infection (7).

We have recently shown that mice deficient in TLR3 (TLR3⁻/⁻ mice) are resistant to lethal encephalitis resulting from infection with West Nile virus (15), a mosquito-borne positive ssRNA virus that produces dsRNA during its replicative cycle. In humans, West Nile virus infection is typically asymptomatic, but a subset of elderly and immunocompromised individuals manifest severe neurological disease, including encephalitis or meningitis, following penetration of the virus into the brain through the blood-brain barrier (BBB) (16). In mice, West Nile virus crosses the BBB and diminishes activation of brain microglia (15). Based on these results, we hypothesized two mechanisms underlying resistance of TLR3⁻/⁻ mice to West Nile encephalitis: 1) TLR3 directly or indirectly mediated viral entry into the CNS across the BBB; and/or 2) microglial recognition of dsRNA was
impaired in TLR3−/− mice, resulting in reduced brain inflammation in these animals.

In this report, we wished to explore the second hypothesis by directly examining the potential of microglial cells to recognize dsRNA through the TLR3 signaling pathway. To accomplish this examination, we cultured N9 microglia and established primary cultures of murine microglia from wild-type and TLR3−/− mice, and challenged these three types of microglial cells with various PAMP including poly(I:C) and LPS. Furthermore, we set up an in vivo model of viral or bacterial encephalitis by intracerebroventricular (i.c.v.) injection of wild-type or TLR3−/− mice with poly(I:C) or LPS, respectively, and we evaluated microglial activation in this system. Our data indicate that microglia directly recognize dsRNA through the TLR3 pathway and suggest that these cells are key sensors of CNS-invading viral pathogens.

Materials and Methods

Mice

Generation of TLR3−/− mice has been described previously (11). These mice were backcrossed for 10 generations onto a C57BL/6 background and genotyped using a PCR-based method (11). As such, control wild-type C57BL/6 mice were purchased from The Jackson Laboratory. Adult mice between 8 and 12 wk of age were used for experiments. All animals were maintained in the Yale University School of Medicine Animal Facility under specific pathogen-free conditions, and all experiments were approved by the Yale Animal Resource Committee.

Reagents

Poly(I:C) was purchased from Amersham Biosciences and was solubilized at 2.5 mg/ml in filter-sterilized Tris-EDTA buffer (pH 7.4) containing 15 mM NaCl by gently warming the mixture at 55°C. Once solubilized, poly(I:C) was slowly cooled to ambient temperature to allow for renaturation and then aliquoted and stored at −20°C before use. LPS from Escherichia coli and PGN from Staphylococcus aureus were purchased from Sigma-Aldrich and Fluka, respectively, and both were solubilized in sterile PBS at 1 mg/ml stock concentration. Unmethylated CpG-DNA sequence Sigma-Aldrich and Fluka, respectively, and both were solubilized in sterile erichia coli CD45 by FACS analysis as previously described (19), confirming their More than 98% of these glial cells stained positive for CD11b/Mac-1 or purchased from InvivoGen.

Microglial cells (N9, wild-type primary, or TLR3−/−) were plated in complete medium at 5 × 105 cells/well in 6-well tissue culture plates (Falcon; BD Biosciences) and stimulated with various PAMP for 16 h. Sandwich ELISA were performed for TNF-α, IL-6, and IL-12 p40 using cytokine-specific purified primary and biotinylated secondary Abs. The Ab set for TNF-α was obtained from eBioscience, and sets for IL-6 and IL-12 p40 were purchased from BD Biosciences. Briefly, medium-binding 96-well microplates were coated overnight at 4°C with primary Abs diluted in PBS (3 μg/ml for anti-TNF-α, 0.5 μg/ml for anti-IL-6, and anti-IL-12). Plates were washed four times in wash buffer (PBS with 0.05% v/v Tween 20) and blocked in blocking buffer (PBS containing 0.05% v/v Tween 20 and 3% w/v BSA) for 2 h at ambient temperature. Samples (50 μl) and standards were then loaded into the plates, and plates were incubated overnight at 4°C. Thereafter, plates were rinsed four times in wash buffer, and biotinylated secondary Abs were added (1 μg/ml for anti-TNF-α, or 0.25 μg/ml for anti-IL-6, and anti-IL-12, diluted in blocking buffer). After an additional 1-h incubation at ambient temperature, plates were rinsed four times in wash buffer, and HRP (Vector Laboratories) (1/2000, diluted in blocking buffer) was added for 30 min at ambient temperature. After a final five times rinsing, the SureBlue TMB substrate (Kirkegaard & Perry Labora- tories) was added followed by stop solution. Plates were read on a micro- plate spectrophotometer (model 550; Bio-Rad) with an emission wave-length of 450 nm.

Microglial cell isolation and culture

Murine primary cultured microglia were isolated according to previously described methods (18). Briefly, cerebral cortices from newborn mice (1- to 2-day-old, wild-type C57BL/6 or TLR3−/−) were isolated under sterile conditions and were kept at 4°C before mechanical dissociation in complete medium (RPMI 1640 containing 5% v/v FCS, 2 mM glutamine, 100 U/ml penicillin, 0.1 μg/ml streptomycin, and 0.05 mM 2-ME). Cells were plated in 75-cm² flasks, and complete medium was added. Primary cultures were kept for 14 days so that only glial cells remained, and microglial cells were isolated by shaking flask at 200 rpm at 37°C in an incubator-shaker. More than 98% of these glial cells stained positive for CD11b/Mac-1 or CD45 by FACs analysis as previously described (19), confirming their identity as microglia. Microglia were cultured in the complete medium described.

RT-PCR analysis

Microglial cells (N9, wild-type primary, or TLR3−/−) were plated in complete medium at 5 × 105 cells/well in 10-cm tissue-culture dishes (BD Falcon) in complete medium. Microglial cells were then restained overnight and went unstimulated (t = 0) or were challenged with LPS (50 ng/ml) or poly(I:C) (50 μg/ml) for a range of time points (t = 5, 10, 15, 30, or 60 min). Microglia were immediately rinsed twice in ice-cold PBS and scraped into cell lysis buffer (containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% v/v Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM PMSF). After lysis for 30 min on ice, cell lysates were centrifuged at 15,000 × g for 30 min, and supernatants were aliquoted for Western blot analysis. Protein concentration was determined using the Bradford method, and an aliquot corresponding to 50 μg of protein was run on 12% polyacryl- amide gels and transferred to nitrocellulose membranes (Millipore). Membranes were blocked in blocking buffer (5% w/v nonfat dry milk in TBS) for 3 h at ambient temperature and incubated overnight at 4°C with rabbit primary Abs directed against phospho- or total p44/42 MAPK, phospho- or total p38 MAPK, phospho- or total JNK, or total IκB-α (diluted at 1/1000 in blocking buffer; Cell Signaling Technology). Membranes were then rinsed three times for 5 min each in distilled H2O, and incubated with anti-rabbit secondary Ab conjugated with HRP (diluted at 1/2000 in blocking buffer) (Amersham Biosciences). After an additional three rinses for 5 min each in distilled H2O, membranes were incubated for 5 min at ambient temperature with the ECL substrate (Pierce), exposed to film, and developed. Band densities were quantified by first digitizing images into a Windows-based computer using an Alpha Innotech FluorChem 8800 Imager and then using Scion Image for Windows software, release beta 4.0.2, to calculate background-subtracted band density ratios of phospho-MAPK to total MAPK.

Stereotactic i.c.v. injection

Mice were anesthetized by i.p. injection with ketamine/xylazine. Animals were placed in a stereotactic device (Stoelting). A cranial window was
opened by drilling the area with a small burr bit, and a 5-mm sterile plastic guide cannula (Plastics One) was implanted and fixed into the left lateral ventricle (coordinates relative to bregma: −0.3 mm anterior/posterior, +1.0 mm medial/lateral, and −3.0 dorsal/ventral) using physiology-grade acrylic dental cement. After surgery, mice were given acetaminophen and rested for 48 h. Thereafter, 1 µg of LPS, 50 µg of poly(I:C), or vehicle (Tris-EDTA buffer) were i.c.v. injected in a volume of 5 µl each over a period of 1 min using a Hamilton microsyringe modified with a solder stop to prevent overinsertion of the needle. Hours later (24–48 h), brains were isolated as detailed below.

**Immunofluorescence microscopy**

Mice were perfused with ice-cold PBS under isofluorane anesthesia, and brains were rapidly isolated and halved down the midline using a mouse brain slicer (World Precision Instruments). Brain hemispheres were routinely imbedded in OCT compound (Sakura Tissue-Tek) and frozen at −80°C. Cryosections were cut at 8 microns on a Leica model CM1850 freezing microtome, applied to Superfrost Plus Gold slides (Fisher Scientific), air dried, and PAP pen applied (Zymed Laboratories). Sections were blocked for 30 min at ambient temperature with serum-free protein block (DakoCytonation) and reacted with various primary Abs overnight at 4°C. Primary Abs were diluted in serum-free protein block and consisted of rat anti-CD11b (1/150, clone M1/70.15; Serotec), rat anti-F4/80 Ag (1/10, clone CI:A3-1; Serotec), rabbit anti-cow glacial fibrillary acidic protein (GFAP, 1/1000; DakoCytonation), rabbit anti-phospho-p44/42 MAPK, or rabbit anti-phospho-JNK (1/50 each; Cell Signaling Technology). Sections were then rinsed three times for 5 min each in a coplin jar containing PBS, and reacted with Alexa488- and/or Alexa594-conjugated anti-rat IgG or F(ab’2) of anti-rabbit IgG Abs (used at 1/200 each; Molecular Probes) for 1 h at ambient temperature. After three rinses for 5 min each in PBS, excess PBS was removed and sections were mounted in VectaShield HardSet Fluorescence mounting medium containing 4’,6-diamidino-2-phenylindole (Vector Laboratories). Sections were observed under darkfield in independent fluorescence channels using an automated Olympus BX-61 microscope.

**Image analysis**

Four para-median sagittal 8-µm sections, spaced 100-µm apart (to eliminate double-sampling of activated glia), were obtained from each wild-type or TLR3−/− mouse subjected to i.c.v. injection of vehicle, poly(I:C), or LPS, either 24 or 48 h after injection (described earlier). Darkfield images of cortical areas demonstrating the greatest glial activation (the area immediately surrounding the cannula tract was avoided) were captured at ×20 magnification, and digitized into a Windows XP-based computer using an attached MagnaFire charge-coupled device camera system. A single image with the greatest pathology was then taken for each animal and used for image analysis, which was performed essentially as described (15). Briefly, photomicrographs were converted into 8-bit grayscale images and imported into Scion Image for Windows, release beta 4.0.2. The entire area of these images was then selected and lookup tables were adjusted to achieve high signal-to-noise ratios. Mean density of immunolabeled area was then calculated using the software. Image analysis was performed in a blinded fashion.

**Statistical analysis**

Mean with SD was calculated and data were analyzed using t test for independent samples (in instances of single mean comparisons) or one-way ANOVA followed by post hoc testing using Bonferroni’s method (in instances of multiple comparisons of the mean). Values of p < 0.05 were accepted as statistically significant. The Statistical Package for the Social Sciences, release 12.0, was used to analyze the data.

**Results**

**Microglia respond to poly(I:C) challenge by increasing expression of TLR3 and IFN-β and by morphological change**

It has recently been reported that human and murine microglia constitutively express a wide range of TLRs, including TLR3 (20, 21). We first wished to examine TLR3 expression in murine primary cultured microglia and in the N9 microglial cell line (22, 23) to determine whether stimulation of microglia with the dsRNA analog poly(I:C) might result in increased expression of TLR3. As shown in Fig. 1A, RT-PCR results demonstrate constitutive expression of TLR3 by primary microglia and N9 microglia (data not shown), and increased expression after treatment with poly(I:C), whereas LPS, PGN, or CpG-DNA gave only a slight increase compared with unstimulated microglia. In macrophages, stimulation with either poly(I:C) or LPS has been shown to signal through a Toll/IL-1R (TIR) domain-containing adaptor (TIR domain-containing adaptor-inducing IFN-β (TRIF)/TIR-containing adaptor molecule-1 (TICAM-1)), resulting in activation of IFN regulatory factor-3 and production of IFN-β, which positively regulates TLR3 expression (24, 25). We also found inducible IFN-β mRNA in N9 (data not shown) and primary microglia after poly(I:C) or LPS stimulation, and consistent with Yamamoto et al. (24) report in macrophages, we noted that IFN-β treatment induced both IFN-β and TLR3 mRNA (Fig. 1A). IFN-α was not detected in
microglia after PAMP stimulation (although it was clearly present in LPS-stimulated macrophages) (Fig. 1A). Quantitative real-time PCR results revealed an ~8-fold increase in TLR3 expression after poly(I:C) treatment of primary microglia, and this increase was ~4-fold after LPS challenge (Fig. 1B). Quantitative real-time PCR showed a similar pattern of results when considering IFN-β expression, which showed ~15-fold increase after poly(I:C) treatment and ~4-fold up-regulation after LPS treatment (Fig. 1C). These increases in TLR3 and IFN-β expression appear to be specific to LPS and poly(I:C), as they were not observed with PGN or CpG-DNA. The adapter molecules MyD88 and TIR domain-containing adapter protein TIRAP, both of which are known to mediate TLR4 signaling (26, 27), and TRIF, which mediates both TLR3 and TLR4 signaling (24), were all constitutively expressed by primary microglia and N9 cells (data not shown), but were not further induced by PAMP stimulation (Fig. 1A). Finally, poly(I:C) induced in a dose-dependent manner morphological change in primary microglia reminiscent of “amoeboid”-activated microglia with fewer cytoplasmic extensions (Fig. 1D).

**TLR3 mediates microglial secretion of innate proinflammatory cytokines after poly(I:C) challenge**

It has recently been reported that wild-type primary microglia secrete proinflammatory cytokines after poly(I:C) challenge (21). To determine whether microglial secretion of innate cytokines in response to poly(I:C) was dependent on TLR3, we challenged wild-type C57BL/6 or TLR3−/− microglia with a dose range of poly(I:C), and measured cytokine secretion in culture supernatants by ELISA at 8 or 24 h after stimulation. As shown in Fig. 2, A and C, poly(I:C) dose-dependently induced TNF-α or IL-6 production after 8 h in wild-type primary microglia, effects that were clearly diminished at each dose point in TLR3−/− microglia. Although not as marked, probably owing to secondary autocrine/paracrine effects of proinflammatory cytokines, we also observed reduced production of TNF-α or IL-6 after 24 h of poly(I:C) stimulation, particularly at the 25 or 125 μg doses (Fig. 2, B and D, respectively). We also observed poly(I:C) dose-dependent induction of IL-12p40 in wild-type microglia, but only observed a modest (albeit reproducible and statistically significant) reduction in

**FIGURE 2.** Wild-type microglia secrete innate cytokines after poly(I:C) challenge that are diminished in TLR3−/− microglia. ELISA was performed on cultured supernatants from wild-type or TLR3−/− primary microglia after 8 h (A, C, E, G, and H) or 24 h (B, D, and F–H) stimulation with the indicated dose range (A–F) of poly(I:C), or 50 μg/ml PGN or 10 ng/ml LPS (G and H). ELISA (n = 3 for each condition presented) for TNF-α (A and B), IL-6 (C and D), or IL-12p40 (E and F) show dose-dependent proinflammatory cytokine production after poly(I:C) challenge of wild-type microglia. These levels are generally comparatively reduced in TLR3−/− microglia. Challenge with the TLR3-independent PAMPs PGN or LPS shows that TLR3−/− microglia are not deficient in TNF-α production (G) or IL-6 secretion (H) vs wild-type microglia. nd, not detected. Results similar to wild-type microglia were observed in N9 microglia. *, p < 0.05, **, p < 0.01 vs TLR3−/− microglia. Similar results were observed in two to three independent experiments.
TLR3−/− microglia at the 125 μg/ml dose point after 8 h of stimulation (Fig. 2E) and at the 25 or 125 μg/ml dose points following 24 h of challenge. We also measured IL-12p70 levels via the Bio-Plex innate cytokine bead assay and did not find induction of IL-12p70 after either LPS or poly(I:C) stimulation (levels in unstimulated or stimulated wild-type or TLR3−/− microglia were <100 pg/ml). To ensure that the observed diminished cytokine production in TLR3−/− microglia was specific to poly(I:C) stimulation as opposed to a general phenomenon in response to other PAMPs, we stimulated both groups of microglial cells with PGN (50 μg/ml) or LPS (10 ng/ml). As shown in Fig. 2, G and H, TLR3−/− microglia did not demonstrate any deficit in response to 8 or 24 h challenge with these other PAMPs when measuring TNF-α secretion or IL-6 secretion, respectively. In fact, TLR3−/− microglia even responded slightly better to PGN at the 8-h time point on TNF-α release and to LPS at the 24-h time point on IL-6 production. As additional controls, we treated microglia with the TLR7/8 stimulators loxoribine (100 μM) or imiquimod (R-837, 10 μg/ml) for 24 h. We did not detect a significant difference between wild-type and TLR3−/− microglia on TNF-α secretion measured as the mean TNF-α release in picograms per milliliter ± SD in wild-type vs TLR3−/− microglia (n = 3 for each condition): media, 0 ± 0 vs 0 ± 0; loxoribine, 14.0 ± 4.4 vs 9.9 ± 3.9; imiquimod, 159.1 ± 22.5 vs 118.8 ± 11.8; p > 0.05 for each comparison. We also observed TNF-α, IL-6, and IL-12p40 production in a dose-dependent manner after poly(I:C) challenge in the N9 microglial cell line (data not shown).

Microglial TLR3 mediates activation of MAPK and reduces cytoplasmic IκB-α levels following poly(I:C) stimulation

We have previously shown in macrophages and dendritic cells that poly(I:C) stimulates phosphorylation of MAPK, including p44/42 MAPK, p38 MAPK, and JNK (11). We challenged wild-type primary microglia, TLR3−/− microglia, or N9 microglia (data not shown). Background-subtracted band density ratios of phospho-IκB-α were evident in a time-dependent fashion following LPS or poly(I:C) challenge of wild-type primary microglia. TLR3−/− microglia respond as wild-type cells after LPS stimulation, but show delayed kinetics of phospho-MAPKs and less reduction in cytoplasmic IκB-α levels after poly(I:C) stimulation. N9 microglia were subjected to the same kinase assay and behaved similar to wild-type primary microglia (data not shown). Similar results were observed in two to three independent experiments.

FIGURE 3. Poly(I:C)-induced activation of MAPK and reduced cytoplasmic IκB-α levels in microglia are TLR3 dependent. Wild-type or TLR3−/− primary microglia went unstimulated (t = 0) or were stimulated with 50 ng/ml LPS or 50 μg/ml poly(I:C) for the indicated time range (t = 5, 10, 15, 30, and 60 min). Cytoplasmic cell extracts were Western blotted using the indicated Abs against phospho (p) or total (as loading controls) MAPKs or total IκB-α. Background-subtracted band density ratios of phospho to total MAPKs were calculated using Scion Image and are shown in the boxes below the bands; for phospho-IκB-α, the ratio to total p38 MAPK was calculated. Phosphorylation of p44/42 MAPK, p38 MAPK, and JNK and reduced cytoplasmic levels of IκB-α are evident in a time-dependent fashion following LPS or poly(I:C) challenge of wild-type primary microglia. TLR3−/− microglia respond as wild-type cells after LPS stimulation, but show delayed kinetics of phospho-MAPKs and less reduction in cytoplasmic IκB-α levels after poly(I:C) stimulation. N9 microglia were subjected to the same kinase assay and behaved similar to wild-type primary microglia (data not shown). Similar results were observed in two to three independent experiments.

mean IL-6 release in picograms per milliliters ± SD in wild-type vs TLR3−/− microglia (n = 3 for each condition): media, 0 ± 0 vs 0 ± 0; loxoribine, 14.0 ± 4.4 vs 9.9 ± 3.9; imiquimod, 159.1 ± 22.5 vs 118.8 ± 11.8; p > 0.05 for each comparison. We also observed TNF-α, IL-6, and IL-12p40 production in a dose-dependent manner after poly(I:C) challenge in the N9 microglial cell line (data not shown).

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FIGURE 4. In vivo gliosis induced by i.c.v. administration of poly(I:C) is markedly reduced in TLR3−/− mice. Wild-type or TLR3−/− mice were i.c.v. injected with vehicle, 50 μg/ml poly(I:C), or 1 μg/ml LPS, and 48 h later, brains were isolated and subjected to immunofluorescence microscopy for activated microglial cell surface markers CD11b or F4/80 (A) Ag (red signal) or GFAP (B), a marker of activated astrocytes (green signal). Representative photomicrographs are shown of the cerebral cortex (original magnification, ×20). A, Both poly(I:C) and LPS promote microgliosis in wild-type mice. However, TLR3−/− animals are essentially refractory to poly(I:C)-induced microglial activation but do activate in response to LPS. B, Astrocytosis is clearly evident in wild-type microglia after poly(I:C) or LPS stimulation. TLR3−/− mice respond normally to i.c.v. LPS challenge, but are essentially nonresponsive to astroglial activation induced by poly(I:C). Sections were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI; blue nuclear signal). Data shown are from n = 4 mice for each group and were reproduced in two independent experiments. Similar results were obtained when immunostaining for the activated microglial marker CD45.
Having shown that microglial activation induced by poly(I:C) was dependent on TLR3 in vitro, we next wished to determine the effect of poly(I:C) stimulation on microglia in vivo. We established models of viral or bacterial encephalitis by injecting poly(I:C) or LPS into the brain of wild-type or TLR3−/− mice via an i.c.v. route. We then assessed microglial activation by cell surface marker immunofluorescence staining either 24 or 48 h after i.c.v. injection. As shown in Fig. 4A, i.c.v. injection of poly(I:C) resulted in increased expression of the activated microglial markers F4/80 Ag, CD11b, and showing almost no degradation of cytoplasmic I kB-α. Yet, TLR3−/− microglia generally responded similarly to wild-type cells when challenged with LPS (with the exception of p38 MAPK phosphorylation, in which a modest delay was noted) (Fig. 3), suggesting that these effects are poly(I:C)-specific as opposed to a general defect in TLR3−/− microglial PAMP response.

An i.c.v. injection of poly(I:C) produces activated glial cell surface markers via TLR3

To further validate the observed reduction in CD11b, F4/80 Ag, or GFAP signal in TLR3−/− mice following i.c.v. poly(I:C) stimulation by increasing TLR3 and IFN-β expression in astrocytes, identified as expressing GFAP, are often found in close vicinity of activated microglia in neurodegenerative diseases such as multiple sclerosis, AIDS dementia, Parkinson’s disease, and Alzheimer’s disease (18, 28–34). Furthermore, it was recently shown that astrocytes can express TLR3 in vitro, and may therefore be able to directly respond to dsRNA (20, 35, 36). Thus, we examined GFAP expression in both groups of mice following i.c.v. administration of either poly(I:C) or LPS. As shown in Fig. 4B, activated astrocytes were copiously present after poly(I:C) challenge in wild-type mice, but not in TLR3−/− mice; yet, LPS stimulation produced astrocytosis similarly in both groups of mice.

To further validate the observed reduction in CD11b, F4/80 Ag, or GFAP signal in TLR3−/− mice following i.c.v. poly(I:C) administration, quantitative image analysis was performed. As shown in Fig. 5, each of these markers was clearly induced following i.c.v. injection of poly(I:C) or LPS vs vehicle in wild-type mice at either the 24- or 48-h time point, and these markers were generally significantly augmented at 48 vs 24 h (with the exception of GFAP signal after LPS challenge). However, when considering TLR3−/− mice, a different pattern of results was evident. Although i.c.v. LPS treatment did not produce a difference between wild-type and TLR3−/− mice at either time point, these mice were essentially refractive to poly(I:C) stimulation at 24 or 48 h after i.c.v. administration when quantifying CD11b (Fig. 5A), F4/80 Ag (Fig. 5B), or GFAP signal (Fig. 5C). Thus, expression of activated microglia or astrocyte surface Ags in response to poly(I:C) in vivo is TLR3 dependent.

Having shown that wild-type primary microglia respond to IFN-β stimulation by increasing TLR3 and IFN-β mRNA (see Fig. 1A), we wondered whether induction of gliosis by i.c.v. poly(I:C) stimulation might result in increased levels of type I IFN mRNAs clearly demonstrated time-dependent phosphorylation of p44/42 MAPK, p38 MAPK, and JNK after LPS (50 ng/ml) or poly(I:C) (50 μg/ml) stimulation. Furthermore, these cells had time-dependent reduced cytoplasmic levels of I kB-α in response to either LPS or poly(I:C). Most importantly, TLR3−/− microglia responded more poorly to poly(I:C) compared with wild-type primary microglia, demonstrating slower kinetics for all of the MAPK examined and showing almost no degradation of cytoplasmic I kB-α. Yet, TLR3−/− microglia generally responded similarly to wild-type cells when challenged with LPS (with the exception of p38 MAPK phosphorylation, in which a modest delay was noted) (Fig. 3), suggesting that these effects are poly(I:C)-specific as opposed to a general defect in TLR3−/− microglial PAMP response.

A

B

C

FIGURE 5. Quantitation of microglial and astrocytic activation after i.c.v. administration of poly(I:C) or LPS in wild-type vs TLR3−/− mice. Wild-type or TLR3−/− mice were i.c.v. injected with vehicle, 50 μg/ml poly(I:C), or 1 μg/ml LPS, and 24 or 48 h later, brains were isolated and subjected to immunofluorescence microscopy for activated microglial surface markers CD11b (A) or F4/80 Ag (B) or GFAP (C), a marker of activated astrocytes. Quantitative image analysis was performed using Scion Image. Data are presented as mean staining density ± 1 SD (n = 3 mice) for the 24-h time point or (n = 4 mice) for the 48-h time point. *, p < 0.05, **, p < 0.01 vs TLR3−/− or within wild-type mice, 24- vs 48-h time point for either poly(I:C) or LPS, shown above wild-type mice at the 48-h time point.

shown) with poly(I:C) or LPS and evaluated kinetics of MAPK phosphorylation or cytoplasmic I kB-α levels by Western immunoblot and densitometry. As shown in Fig. 3, wild-type microglia
in wild-type mouse brains, and whether this putative effect may be dependent on TLR3. Thus, we isolated mRNA from brain hemispheres of wild-type or TLR3\(^{-/-}\) mice 24 h after i.c.v. administration of vehicle, 50 \(\mu\)g of poly(I:C), or 1 \(\mu\)g of LPS and quantified IFN-\(\alpha\) or IFN-\(\beta\) mRNA by quantitative real-time PCR. Although IFN-\(\alpha\) was not induced after either poly(I:C) or LPS i.c.v. challenge (consistent with lack of IFN-\(\alpha\) mRNA induction after either poly(I:C) or LPS treatment of wild-type microglia in vitro, see Fig. 1A) (data not shown), we did observe significant induction of IFN-\(\beta\) in brains of wild-type mice after poly(I:C) or LPS i.c.v. stimulation. In addition, the poly(I:C) effect was significantly attenuated in TLR3\(^{-/-}\) mice brains, whereas IFN-\(\beta\) mRNA levels after LPS challenge of TLR3\(^{-/-}\) mice brains were not significantly different from levels in wild-type mice brains. Values are mean starting quantity of IFN-\(\beta\) mRNA (unitless ratio normalized to \(\beta\)-actin mRNA) \(\pm\) 1 SD in wild-type vs TLR3\(^{-/-}\) brains (\(n = 3\) for each condition): vehicle, 0.50 \(\pm\) 0.04 vs 0.42 \(\pm\) 0.14, \(p > 0.05\); poly(I:C), 7.86 \(\pm\) 4.16 vs 2.20 \(\pm\) 1.42, \(p < 0.05\); and LPS, 0.90 \(\pm\) 0.32 vs 1.23 \(\pm\) 0.39, \(p > 0.05\). Thus, IFN-\(\beta\) mRNA level positively correlates with cell surface activation markers of microglia (i.e., CD11b or F4/80 Ag) or astroglia (i.e., GFAP).

**Poly(I:C) i.c.v. infection results in phosphorylation of microglial MAPK in wild-type, but not TLR3\(^{-/-}\) mice**

As shown in Fig. 3, in vitro stimulation of microglia with poly(I:C) results in time-dependent phosphorylation of MAPK, which occurs with delayed kinetics in TLR3\(^{-/-}\) microglia. Because we have shown marked reduction of microglial cell surface activation markers in vivo after i.c.v. injection of poly(I:C) in TLR3\(^{-/-}\) vs wild-type mice, we wanted to validate whether activation of MAPK downstream of TLR3 might also be diminished in these knockout animals. Thus, we injected both groups of mice i.c.v. with either poly(I:C) or LPS as described and performed double immunofluorescence staining for either phospho-p44/42 MAPK or phospho-JNK in combination with F4/80 Ag as a maker of activated microglia. As shown in Fig. 6A, phospho-p44/42 MAPK was clearly detected in activated microglia after poly(I:C) or LPS challenge of wild-type mice. Yet, although TLR3\(^{-/-}\) mice also demonstrated cells double-positive for phospho-p44/42 MAPK and F4/80 Ag after LPS stimulation, when these animals were i.c.v. injected with poly(I:C) both signals were nearly absent. We also performed double immunofluorescence staining for phospho-JNK in combination with F4/80 Ag as shown in Fig. 6B. Although activated microglia that were also positive for phospho-JNK were rarely seen in both groups of mice after LPS injection (data not shown), we did consistently observe double-positive cells in wild-type mice after poly(I:C) injection, which were almost absent in TLR3\(^{-/-}\) mice. We obtained similar results when using CD11b as a marker of activated microglia in combination with the phospho-MAPK Abs (data not shown).

**Discussion**

Microglia form the first line of defense against invading pathogens that enter the CNS. Many encephalitic retroviruses (e.g., West Nile virus, Japanese encephalitic virus) produce dsRNA during replication in the CNS (14). We have previously shown that peripheral immune cells respond to dsRNA in a TLR3-dependent fashion (11), and that cultured murine microglia respond to West Nile virus infection by up-regulating TLR3 mRNA (15). Thus, we sought to determine whether murine microglial cells could recognize dsRNA through TLR3 both in vitro and in vivo. We initially found that cultured microglia (both wild-type primary microglia and the N9 microglial cell line) up-regulate mRNA for both TLR3 and IFN-\(\beta\) after poly(I:C) challenge and undergo morphological change indicative of activation (Fig. 1). These results are in concert with a previous report in macrophages that showed poly(I:C) stimulation results in activation of the transcription factor IFN regulatory factor-3 and production of IFN-\(\beta\), resulting in increased expression of TLR3 and macrophage activation (24). Yet, a recent report in murine microglia did not find increased expression of TLR3 in response to poly(I:C) treatment (21). One possible explanation for this discrepancy is the background of the mice used in our study (C57BL/6) vs that used in the Olson and Miller report (SJL/J) (21). We have found large differences in responses of murine innate immune cells to PAMP, including poly(I:C), depending on the mouse strain used (data not shown).

Peripheral immune cells, such as macrophages and dendritic cells, respond to TLR simulation by secreting innate proinflammatory cytokines that provide a “danger signal” alert to the presence of invading pathogens (37). We evaluated secretion of innate proinflammatory cytokines following poly(I:C) stimulation of microglia derived from wild-type or TLR3\(^{-/-}\) mice. Results show dose-dependent induction of TNF-\(\alpha\), IL-6, and IL-12p40 in wild-type microglia, effects that are diminished in TLR3\(^{-/-}\) mice. Results show dose-dependent induction of TNF-\(\alpha\), IL-6, and IL-12p40 in wild-type microglia, effects that are diminished in TLR3\(^{-/-}\) microglia (Fig. 2). This defect in TLR3\(^{-/-}\) microglia was not a general phenomenon in response to any PAMP, as stimulation via LPS or PGN did not produce reduced cytokine secretion in TLR3\(^{-/-}\) vs wild-type microglia. Interestingly, although TLR3\(^{-/-}\) microglia...
secreted significantly less of these cytokines, their levels were still detectable. We previously showed a similar pattern of results in TLR3−/− macrophages and dendritic cells (11). One possibility is that TLR3, TLR7, TLR8, and TLR9 are all able to recognize a wide range of nucleic acids with differing affinities as opposed to an “all-or-none” model in which each of these TLRs only recognizes a particular nucleic acid species. Given that we still observe a response (albeit diminished) to poly(I:C) in TLR3−/− microglia, our data are in concert with the latter model. Another possibility is that other non-TLRs may recognize dsRNA and exhibit some functional redundancy with TLR3. In support of this model, it is becoming increasingly clear that TLR3 is a member of a much larger family of dsRNA binding proteins that mediate diverse cellular functions, including host defense, RNA-mediated gene silencing, development, translation, and RNA editing/stability (38, 39). Recently, a member of this family known as RIG-1 (retinoic acid inducible gene I) has been identified as an RNA helicase that mediates dsRNA-induced antiviral responses, including production of type I IFNs by innate immune cells (40, 41). Another member of this family, protein kinase R, has been shown to recognize and bind to dsRNA and to mediate proinflammatory cytokine production by innate immune cells, possibly by modulating the MAPK pathway (42, 43). Thus, functional redundancy may exist between TLR3 and protein kinase R and/or RIG-I, which could also explain the modest innate cytokine responses in vitro to poly(I:C) (and presence of some phospho-MAPK signal) in TLR3−/− microglia that we observed, and also the low levels of microglial cell surface activation markers CD11b and F4/80 Ag that were present in TLR3−/− mice after i.c.v. application of poly(I:C).

The adapter molecule MyD88 directly binds to a conserved TIR domain of TLRs and, in conjunction with TIRAP, mediates intracellular signaling of most TLRs (26, 27). Once recruited to the TLR, MyD88 activates members of the IL-1R-associated kinase family, which associate with TIRAP-associated factor 6, leading to activation (via IL-1R-associated kinase-2/3) or suppression (by IL-1R-associated kinase-M) of the NF-κB transcription factor and MAPKs (including p44/42 MAPK, p38 MAPK, and JNK) (44–46). However, MyD88-independent signaling has also been described, and TLR3 is a prototypic example of this as it uses TRIF/TICAM-1 as an adapter molecule and activates downstream IFN regulatory factor-3 to promote IFN-β production (24, 25). Our data show time-dependent activation of p44/42 MAPK, p38 MAPK, and JNK following stimulation of N9 or wild-type primary microglia with poly(I:C) or LPS (Fig. 3). Yet, although TLR3−/− microglia responded as wild-type primary cells to LPS, they demonstrated delayed kinetics of MAPK phosphorylation after poly(I:C) challenge. A similar pattern of results was observed when cytosolic IκB-α levels were measured. Specifically, although wild-type primary microglia and N9 microglia (data not shown) showed time-dependent degradation of cytosolic IκB-α in response to poly(I:C) or LPS, TLR3−/− microglia responded poorly to poly(I:C) stimulation but a consistent defect was not observed in response to LPS (Fig. 3).

We have recently shown that TLR3−/− mice are protected from encephalitis resulting from infection with a lethal dose of West Nile virus, a positive ssRNA virus that produces dsRNA during its life cycle (15). These mice had reduced penetration of virus into the brain across the BBB, diminished activation of brain microglia, and reduced brain inflammation as measured by mRNA levels of TNF-α, IL-6, IL-12, and IFN-β. These data raised the possibility that microglia might recognize dsRNA produced by West Nile virus. As we currently have evidence that West Nile virus is recognized by other TLRs in addition to TLR3 (data not shown), we sought to directly address whether microglia recognize dsRNA in vivo. Thus, we established a simplified model of viral or bacterial encephalitis by i.c.v. injection of poly(I:C) or LPS, respectively, into wild-type or TLR3−/− mouse brains. Strikingly, although the microglial cell surface activation markers CD11b and F4/80 Ag were increased in the cerebral cortex after poly(I:C) or LPS challenge of wild-type mice, activated microglia were nearly absent after poly(I:C) (but not LPS) challenge of TLR3−/− mice (Figs. 4A and 5A and B). In concert with our findings in the mouse brain, another group showed that systemic injection of poly(I:C) into the rat spinal cord resulted in activation of microglia in that CNS region (47). We also double-stained for activated microglial cell surface markers in conjunction with either phospho-p44/42 MAPK or phospho-JNK, and observed a similar pattern of results (Fig. 6). Interestingly, reduction of phospho-p44/42 MAPK or phospho-JNK was more marked in TLR3−/− mice in this i.c.v. administration of poly(I:C) paradigm than the more marginal effects that we observed in cultured microglia (Fig. 3). Yet, there are a number of possible explanations for this apparent discrepancy: 1) the time course of poly(I:C) treatment is very different in vitro (acute treatment from 0 to 60 min) compared with in vivo (chronic treatment after 48 h of stimulation); 2) total levels of MAPK are not expected to change in the in vitro assay, whereas after 48 h of stimulation in vivo, they may change thereby influencing levels of phospho-MAPK; and 3) in the in vitro assay was performed with monotypic cultures of microglia while all brain cells were present in the in vivo assay, and the presence of other TLR3-expressing cells, particularly astrocytes (discussed below), may affect MAPK activation in microglia in a paracrine fashion.

It has been shown recently that human and murine astrocytes express TLR3 and respond to poly(I:C) by producing proinflammatory innate cytokine mRNAs (20, 35, 36). Given that astrocytes, along with vascular endothelial cells, smooth muscle cells, and pericytes, are an integral cellular constituent of the BBB, it stands to reason that astrocytes would be important “front-line” responders to dsRNA associated with viruses entering the brain parenchyma from the blood. We examined astrocytic activation in the previously mentioned i.c.v. injection paradigm and found that, although wild-type animals had copious activated cortical astrocytes (i.e., by GFAP immunoreactivity) after poly(I:C) stimulation, TLR3−/− mice did not (Figs. 4B and 5C). Thus, it appears that, in addition to microglia, astrocytes are also able to respond to dsRNA. Given that astrocytes express TLR3, this reactive astrogliosis response may be directly due to dsRNA. Alternatively, this response may be secondary to dsRNA-mediated microglial activation via the TLR3 pathway, in which activated microglia secrete numerous proinflammatory cytokines and acute phase reactants that positively regulate astrogliosis.

Taken together, our results demonstrate that murine microglial cells respond to dsRNA through TLR3. Specifically, these cells are able to mount a robust innate immune response, including activation of MAPKs, production of proinflammatory cytokines, and increased surface activation markers in a TLR3-dependent fashion. These findings have relevance for inflammatory CNS diseases, and in particular viral encephalitides. If brain inflammation, including microgliosis, plays a damaging role in these diseases as has previously been suggested (15, 48), then therapeutic intervention targeting TLR3 may provide a novel approach to suppressing gliosis and bystander injury associated with viral encephalitis.
Acknowledgments
We thank F. Manzo for assistance in the preparation of this manuscript and F. S. Sutterwala for critically reading this manuscript. We also thank P. Ricciardi-Castagnoli for gifting the N9 microglial cell line.

Disclosures
The authors have no financial conflict of interest.

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