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Human Neuronal Cells Possess Functional Cytoplasmic and TLR-Mediated Innate Immune Pathways Influenced by Phosphatidylinositol-3 Kinase Signaling

Daniel C. Peltier,* Allison Simms,† Jocelyn R. Farmer,* and David J. Miller*,†

Innate immune pathways are early defense responses important for the immediate control and eventual clearance of many pathogens, where signaling is initiated via pattern recognition receptor (PRR)-mediated events that occur in a ligand- and cell-type specific manner. Within CNS neurons, innate immune pathways are likely crucial to control pathogens that target these essential yet virtually irreplaceable cells. However, relatively little is known about the induction and regulation of neuronal PRR signaling. In this report, we used human neuronal cell lines and primary rat neuronal cultures to examine PRR expression and function. We found that several innate immune receptor ligands, including Sendai virus, the dsRNA mimetic polyinosinic-polycytidylic acid, and LPS all activated differentiation-dependent neuronal innate immune pathways. Functional genetic analyses revealed that IFN regulatory factor 3-mediated pathways that resulted in IFN-β transcriptional upregulation were activated in cultured human neuronal cells by the PRRs TLR3, MDA5, or RIG-I in a ligand-specific manner. Furthermore, genome-wide transcriptional array and targeted genetic and pharmacologic analyses identified PI3K signaling as crucial for the induction of innate immune pathways in neurons. These results indicate that human neuronal cells possess specific and functional PRR pathways essential for the effective induction of innate immune responses, and suggest that neurons can play an active role in defense against neurotropic pathogens. ***The Journal of Immunology*****, 2010, 184: 7010–7021.**

Innate immune pathways are early responses important for pathogen control, and are activated by pattern recognition receptors (PRRs) that bind ligands containing pathogen- or danger-associated molecular patterns, such as modified carbohydrate or nucleic acid structures (1). For antiviral innate immune responses, ligation of these receptors induces a signal transduction cascade that results in the production of type I IFNs, other proinflammatory cytokines, and cell-intrinsic factors important for the generation of an antiviral cellular microenvironment (2). In addition, antiviral PRR signaling is important for activating an appropriate adaptive immune response, which is required for the eventual clearance of many viral infections. Thus, PRR-mediated innate immune pathway signaling serves a pivotal role in stimulating rapid yet nonspecific antiviral activity while also providing activation signals for the production of more specialized adaptive immune responses.

There are three general steps in innate antiviral immune responses: activation, amplification, and effector production. Antiviral PRR signaling is initiated by a variety of receptors, including the transmembrane TLR proteins 2, 3, 4, 7/8, and 9, and the RIG-I–like receptors (RLRs) RIG-I and MDA5 (2). TLR3, TLR7/8, and TLR9 recognize the nonself-nucleic acid moieties dsRNA, ssRNA, and hypomethylated CpG DNA, respectively, whereas TLR4 recognizes viral glycoproteins and the viral ligand for TLR2 remains to be identified. In the cytoplasm, RIG-I binds double-stranded 5′ triphosphorylated RNAs, homopolymeric RNA motifs, and short dsRNAs <2 kb in length (3, 4), whereas MDA5 recognizes complex dsRNAs >2 kb in length (5) that can be mimicked by the synthetic dsRNA molecule polyinosinic-polycytidylic acid [poly(I-C)] (6). Due in part to this ligand specificity, PRRs differentially recognize and respond to distinct viral infections (1). After PRR ligation, signal transduction is mediated by several distinct adaptor proteins, including MyD88, TIR domain-containing adapter-inducing IFN-β (TRIF), and IFN-β promoter stimulator protein 1 (also referred to as Cardif, MAVS, and VISA) (1, 2). These adaptor protein complexes activate the transcription factors NF-κB and IFN regulatory factor 3 (IRF3) via multiple downstream kinases. Activated NF-κB and IRF3 subsequently upregulate the expression of many genes important for mounting a robust antiviral response, including type I IFNs (7), which function in either a paracrine or autocrine manner to induce IFN-stimulated genes that contain IFN-stimulated response elements (ISREs) within their promoters. There are several IFN-stimulated genes that act directly as antiviral effectors, but many are also components of antiviral PRR pathways, which provides a mechanism for positive feedback regulation and amplification (1, 2).

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Abbreviations used in this paper: AKT, adenosine kinase protein kinase B; BTK, Bruton's tyrosine kinase; CDK, cyclin-dependent kinase; CK, creatine kinase; C_t, threshold cycle; EGFRK, epidermal growth factor receptor kinase; GSK3, glycogen synthase kinase 3; hpi, hours postinfection; IRF3/7, IFN regulatory factor 3/7; ISRE, IFN-stimulated response element; MOI, multiplicity of infection; mTOR, mammalian target of rapamycin; pIC, poly(I-C); PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; poly(I-C), polyinosinic-polycytidylic acid; PRR, pattern recognition receptor; RLR, RIG-I-like receptor; SEAP, secreted alkaline phosphatase; SEV, Sendai virus; shRNA, short-hairpin RNA; T-pIC, transfected poly(I-C); TRIF, TIR-domain-containing adapter-inducing IFN-β; WNV, West Nile virus.

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The molecular mechanisms of antiviral PRR signaling have been defined primarily using a limited number of cell lines and primary cell types, many of which are derived from small rodent models and are professional immune cells, such as dendritic cells or macrophages. These studies have revealed important cell type-specific differences in antiviral PRR pathways. For example, dendritic cells express relatively high basal levels of TLR7 and TLR9, and as a result vigorously respond to ligands for these receptors (8). In contrast, "nonprofessional" immune cells, such as fibroblasts, use primarily cytoplasmic RLRs for innate antiviral pathway activation (9), although some cell types, such as keratinocytes (10) and respiratory epithelial cells (11), can also mount vigorous TLR-mediated antiviral responses. Plasmacytoid dendritic cells also constitutively express the transcription factor IRF7, which is thought to contribute to their ability to produce IFN-α rapidly after PRR-mediated stimulation (8, 12, 13), whereas IFN-α production in other cell types occurs later if at all, and is linked to IFN-β–mediated induction of IRF7 (14–16). Additional examples of cell type-specific differences in innate antiviral immunity include a lower basal activity of PRR pathways in cardiac fibroblasts compared cardiac myocytes (17), differential responses of specific human hepatocyte cell lines to poly(I-C) and Sendai virus (SEV) stimulation (18), and cell type-specific roles for IRF3 and IRF7 in response to West Nile virus (WNV) infection (19, 20). Furthermore, species-specific differences also exist with respect to TLR expression, regulation, and function (21–24). These observations suggest that caution should be exercised in extrapolating results on innate antiviral pathway activity between species and cell types.

Viruses from several families preferentially infect CNS neurons, and the extent of neurotropic virus-mediated cell death can be an important determinant in the severity and clinical outcome of infection (25). Thus, an effective neuronal innate antiviral response that controls virus replication until an adaptive immune response can be generated may be crucial to prevent the essentially irreversible loss of these critical cells. However, we have limited knowledge regarding the PRR antiviral pathways that are active in CNS neurons. TLR3 expression has been reported in human neurons (26–30), WNV replication is enhanced in cortical neurons isolated from TLR3$^{-/-}$ mice (31), and neural progenitor cells respond to poly(I-C) stimulation by reducing proliferation and neurosphere formation in a TLR3-dependent manner (32). Furthermore, studies have demonstrated virus-mediated induction of type I IFNs in CNS neurons both in vitro (19, 20, 27, 31) and in vivo (33). In addition to putative antiviral functions, PRR pathways have been implicated in neuronal development (34), neuronal regeneration (28, 35), and neuroinflammatory diseases (36, 37). Altogether, these reports suggest that CNS neurons possess active PRRs that may have multiple physiologic functions, but the full extent of their activity and the downstream components that mediate their activation remain to be determined.

In this report, we use both global and targeted approaches to examine PRR expression and pathway activity in response to RLR and TLR ligands. We found that human neuronal cells show differentiation-dependent selective responses to TLR3-, TLR4-, MDA5-, and RIG-I–mediated stimulation. Furthermore, detailed genetic and pharmacologic studies revealed that select neuronal innate immune pathways were dependent on PI3K activity. These results demonstrate that human neuronal cells are immunologically active and possess specific and nonredundant functional PRR pathways that may play a protective role in neurotropic virus pathogenesis.

Materials and Methods

Plasmids

We purchased the reporter plasmids pISRE-secreted alkaline phosphatase (SEAP) and pNF-κB-SEAP, the wild-type expression plasmid pTLR3, the dominant-negative expression plasmids pDN-TLR3(ΔTIR), pDN-TRIF (TIR), and pDN-RIG-I(ΔN), and the short-hairpin RNA (shRNA) expression plasmid pshRNA-MDA5 from InvivoGen (San Diego, CA). The dominant-negative expression plasmid pDN-IRF3(ΔN) was generously provided by Rongtuan Lin (McGill University, Montreal). We purchased the lentivirus shRNA expression plasmids pGIPZ-shCD14 and pGIPZ-shPI3K110α from Open Biosystems (Huntsville, AL). The lentivirus helper plasmids pCMV-vesicular stomatitis virus (VSV)/G and pCMV-Gag/Pol were generously provided by David Markovitz (University of Michigan, Ann Arbor, MI).

Virus

Recombinant SEV that contains a GFP tag between the viral P and M genes was generously provided by Valery Grdzelishvilli (University of North Carolina at Charlotte, Charlotte, NC) and was expanded twice through Vero cells at a low multiplicity of infection (MOI) to generate viral stocks. SEV growth curves were analyzed by monitoring GFP accumulation in infected cells using a FLUOstar Ω plate reader (BMG Labtech, Durham, NC) and black-walled, translucent-bottomed 96-well tissue culture plates.

Lentivirus production for shRNA knockdown was performed as previously described (38). Briefly, HEK293FT cells at 90% confluence were incubated with 25 μg/ml chloroquine and transfected either pGIPZ-shCD14 or pGIPZ-shPI3K110α and the packaging plasmids pCMV-VSV/G and pCMV-Gag/Pol using calcium chloride. Virus was harvested from clarified supernatants at 24 and 48 h after transfection and stored at 4°C in the dark prior to cell infections.

Abs, cytokines, PRR ligands, and kinase inhibitors

Abs against GAPDH and IRF3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Abs against synaptophysin, neurofilament 200, and glial fibrillary acidic protein were purchased from Sigma-Aldrich (St. Louis, MO), Abs against TLR3 were purchased from either Santa Cruz Biotechnology (clone TLR3.7, catalog no. sc-32232) or Imigenex (San Diego, CA; clone 40C1285.6, catalog no. IMG-315A), Abs against PI3K p110α were purchased from Cell Signaling Technology (Danvers, MA), and Abs against RIG-I were purchased from Alexis Biochemical (San Diego, CA). Abs against MDA5 were generously provided by Paul Fisher (Columbia University, New York, NY). Neutralizing antisera against type I IFNs and the corresponding control sera were obtained from the Biodefense and Emerging Infections (BEI) Research Resources Repository (Manassas, VA). All secondary Abs for immunoblotting and immunofluorescence staining were purchased from Jackson ImmunoResearch (West Grove, PA).

Recombinant human IFN-α–A/D and rat IFN-α were purchased from PBL Biomedical Laboratories (Piscataway, NJ), recombinant human TNF-α was purchased from R&D Systems (Minneapolis, MN), and human leukocyte IFN-α and fibroblast IFN-β were obtained from the BEI Research Resources Repository. All cytokines were stored in single use aliquots at −80°C. Ultrapure *Escherichia coli* K12 LPS, the imidazoquinoline derivative CLO97, and the CpG-containing synthetic oligonucleotide ODN2006 were purchased from InvivoGen. Poly(I-C) was purchased from either Sigma-Aldrich or InvivoGen and stored as a 5–10 mg/ml solution in sterile water at −20°C. We transfected poly(I-C) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at a ratio of 200 μg poly(I-C) per 60 μl Lipofectamine 2000 in a total volume of 150 μl Opti-MEM I (Life Technologies, Grand Island, NY) media.

The kinase inhibitor library was provided by the University of Michigan Center for Chemical Genomics and was originally purchased from TimTec (Newark, DE). The kinase inhibitors LY294002 and TGX-221 were purchased from Calbiochem (San Diego, CA) and the kinase inhibitors AS-252424 and p110α inhibitor 2 were purchased from Cayman Chemical (Ann Arbor, MI).

Cell culture

BE(2)-C, SHSY-5Y, HCN-1A, U937, and Vero cells were all obtained from the American Type Culture Collection (Manassas, VA). We differentiated BE(2)-C cells with all-*trans* retinoic acid as previously described (39), the nonmalignant human cortical neuronal cell line HCN-1A with nerve growth factor, 1-isobutyl-3-methylxanthine, and dibutyryl cAMP as previously described (40), and human monocytic U937 cells with 15 nM PMA for 48 h. To avoid potential confounding effects of cell differentiation on

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transfection or transduction efficiency, we generated stable cell lines prior to differentiation. BE(2)-C and SH-SY5Y cells were transfected with reporter gene-, dominant-negative–, or shRNA-expressing plasmids using Lipofectamine 2000 according to the manufacturer's instruction (Invitrogen), whereas U937 cells were transfected by electroporation using a GenePulser Xcell according to the manufacturer's instructions (Bio-Rad, Hercules, CA). For lentiviral transduction, cells were infected with recombinant lentiviruses in the presence of 8 μg/ml polybrene. Cell lines were passed at least three times in the presence of selection antibiotic prior to use in experiments, and selection agents were removed for retinoic acid- or PMA-induced differentiation.

Primary rat neuronal cultures were prepared from embryonic day 18 Sprague-Dawley rat cortices according to the supplier's recommendations (BrainBits, Springfield, IL). Briefly, cortices were digested with 2 mg/ml papain (Worthington, Lakewood, NJ) for 30 min at 30°C in HibernateE solution (BrainBits) without calcium, followed by gentle trituration. Cell suspensions were allowed to settle by gravity for 1 min to remove large debris, supernatants were collected and centrifuged at 500×*g* for 5 min, cell pellets were gently resuspended in Neurobasal E media supplemented with 2% B27 (Life Technologies), 500 μM L-glutamine, 10 U/ml penicillin, and 10 μg/ml streptomycin, dispensed into poly-D-lysine-coated plates at 1×10^5 cells/cm^2, and incubated at 37°C with 5% CO_2. Eighteen to 24 h after plating, the media were completely replaced and on subsequent days, half of the culture volume was replaced. Cells were routinely used at 12–14 d after plating, at which time immunofluorescence staining showed that ~95% of cells expressed the transmembrane synaptic vesicle glycoprotein synaptophysin and neurofilament 200, which are both markers of mature neurons, but not the astrocyte marker glial fibrillary acid protein. Furthermore, primary rat neuronal cultures were also highly sensitive to glutamate-mediated excitotoxicity (data not shown), which is a well-described phenotype of mature cortical neurons in vitro (41).

Cell viability and SEAP assays

Cell viability was determined with either Alamar Blue according to the manufacturer's instructions (AbD Serotec, Oxford, U.K.) or an MTT assay as previously described (39). SEAP assays were conducted using QuantiBlue substrate according to manufacturer's instructions (InvivoGen). Fluorescence and absorbance end point values for viability and SEAP assays were obtained with a FLUOstar Ω plate reader.

Immunoblotting, immunofluorescence, and RT-PCR analyses

Immunoblotting, immunofluorescence staining of cultured cells, and RT-PCR were performed as previously described (39, 42) with the following modifications. For TLR3 immunoblotting, membranes were blocked with PBS containing 1% BSA and 1% polyvinylpyrrolidone, and TLR3-specific bands were detected with the Imigenex mAb, a biotinylated secondary Ab, and streptavidin-conjugated HRP. For TLR3 immunofluorescence staining, cells were permeabilized with 0.1% Triton X-100 after paraformaldehyde fixation, immunostained with the Santa Cruz mAb, a biotinylated secondary Ab, and streptavidin-conjugated PE. Primer sequences for PCR are available on request.

Microarray, pathway analysis, and validation

Total RNA was isolated from five independent sets of cultures containing similar numbers of immature BE(2)-C or differentiated BE(2)-C/m cells using TRIzol (Invitrogen), digested with RQ1 DNaseI (Promega, Madison, WI), and repurified using an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA integrity and quantity were assessed using a microfluidics-based Agilent 2100 Bioanalyzer (Foster City, CA). RNA labeling, hybridization, and array scanning were performed by either SeqWright DNA Technology Services (Houston, TX) or the University of Michigan Microarray core facility using biotinylated amplified cRNAs and Affymetrix Human U133 Plus 2.0 microarray chips. Complete original data files for all microarray experiments have been deposited in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/) under the accession number GSE16452.

The Genomatix ChipInspector software package (Genomatix Software, Ann Arbor, MI; www.genomatix.de) was used for primary microarray data analysis. This program uses a single probe method with an enhanced statistics package based on the original SAM algorithm (43) that incorporates a *t* test with a permuted artificial background to reduce false-positive results. The following parameters were chosen to identify sets of differentially regulated transcripts: 1) false-discovery rate of 1%; 2) three probe minimum coverage; and 3) expression level $\log_2$ change ≥0.5 (~1.4-fold) compared with control. Similar results were obtained when microarray data were analyzed with the Affymetrix package of Bioconductor (44). The list of genes preferentially upregulated in differentiated BE(2)-C/m cells were analyzed using Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood, CA; www.ingenuity.com). This analysis used the Ingenuity Pathway Analysis library of 103 signaling and 80 metabolic canonical pathways to identify those that were most significant to the data set. This significance was measured by determining the ratio of the number of genes from the data set that map to a particular canonical pathway to the total number of genes for that pathway, and calculating a subsequent *p* value using a Fischer exact test. The association with a particular canonical pathway was considered significant if the *p* value <0.05.

To validate microarray results, PI3K-adeosine kinase protein kinase B (AKT) pathway real-time RT-PCR arrays (SABiosciences, Frederick, MD) were used to analyze transcript expression level differences between two independent sets of immature BE(2)-C and differentiated BE(2)-C/m cultures. Total RNA was isolated as described previously and RNA integrity and quantity was assessed using nondenaturing agarose gel electrophoresis and spectrophotometry, respectively. cDNA synthesis and real-time PCR were conducted using the manufacturer's recommended reagents and protocols for the BioRad iCycler iQ thermocycler, and fluorescence threshold cycle (C_t) values were calculated using SDS 700 system software (Bio-Rad). Results were normalized to the average C_t for five housekeeping genes contained on the RT-PCR array and $\Delta\Delta C_t$ values were calculated to determine expression changes. Genes that reached the C_t maximum of 35 in either trial were excluded from the analysis.

Statistical analysis

Microarray and pathway statistical analyses are described previously. For comparative analyses we used a two-tailed Student *t* test assuming unequal variances where a *p* value of <0.05 was considered significant. Quantitative EC_{50} and IC_{50} values were calculated using Prism GraphPad 3.0 software. Unless otherwise indicated, presented results are representative of at least three independent experiments, where quantitative data represent the mean ± SEM.

Results

Poly(I-C) induces PRR pathway activation and IFN-β production in differentiated human neuronal cells

To initially study neuronal PRR pathway activity, we used the previously characterized human BE(2)-C neuronal culture model (45). This neuroblastoma cell line can be differentiated in the presence of retinoic acid to form cells with morphological, biochemical, and physiological characteristics of mature human neurons, and it has been used to demonstrate differentiation-dependent responses of human neuronal cells to type I IFN stimulation and neurotropic virus infection (39). We generated stable cell lines that expressed either an NF-κB promoter-driven or IRSE promoter-driven SEAP reporter gene, induced differentiation with retinoic acid, and examined reporter gene activity in tissue culture supernatants of cells stimulated with poly(I-C), which is a dsRNA mimetic and potent inducer of PRR pathway activation (6). We used increasing concentrations of poly(I-C) delivered either extracellularly for cell surface or endosomal TLR activation or complexed with lipofectamine and transfected for intracellular RLR stimulation, and examined responses in both undifferentiated and differentiated BE(2)-C cell lines (Fig. 1). Both NF-κB (Fig. 1*A*) and ISRE (Fig. 1*B*) promoter-driven reporters showed dose responsive expression in differentiated BE(2)-C/m cells with both extracellular and transfected poly(I-C), whereas essentially no responses were seen in undifferentiated cells. Calculated poly(I-C) concentrations that produced 50% maximal responses (EC_{50} values) in differentiated BE(2)-C/m cells were between ~10 ng/ml and 10 μg/ml (Table I). The inability of undifferentiated BE(2)-C cells to respond to poly(I-C) was not due to inactive ISRE or NF-κB promoters, as universal type I IFN-α–A/D stimulated ISRE-SEAP activity, albeit with a 4-fold higher IFN-α–A/D EC_{50} compared with differentiated BE(2)-C/m cells (39). Furthermore, TNF-α, a potent NF-κB inducer, stimulated NF-κB–SEAP activity in undifferentiated BE(2)-C cells, although EC_{50} values were ~30-fold higher in undifferentiated compared with differentiated cells (850

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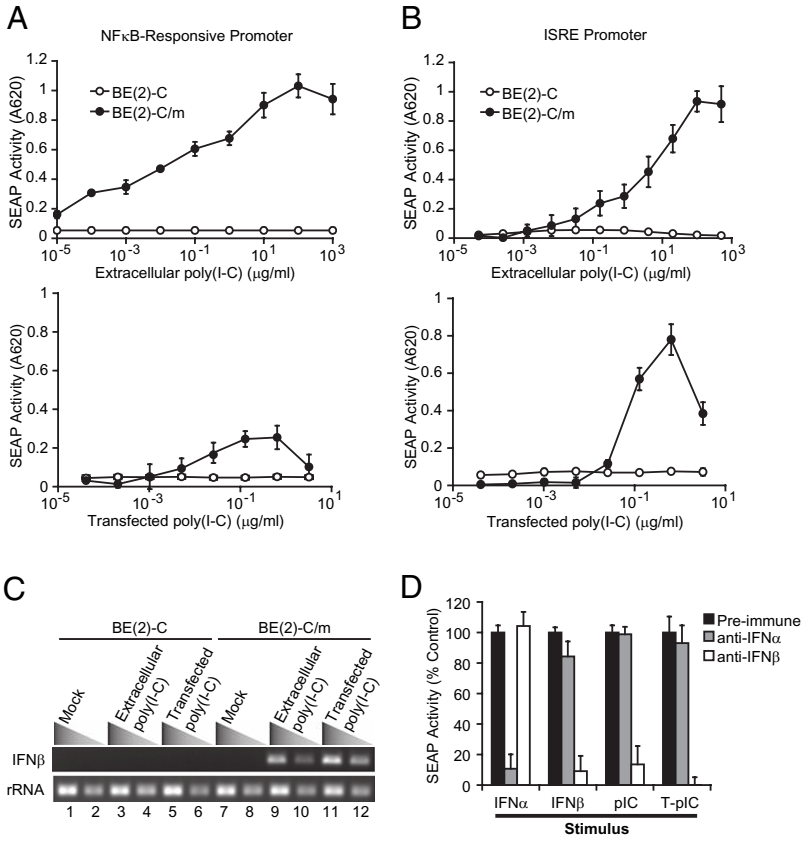

FIGURE 1. Poly(I-C) activates NF-κB and ISRE promoters and induces IFN-β production in human neuronal cells. Similar numbers of NF-κB (*A*) or ISRE (*B*) promoter-reporter cells were stimulated with increasing amounts of extracellular poly(I-C) (*upper graphs*) or transfected poly(I-C) (*lower graphs*), and SEAP reporter activity in culture supernatants was measured 20 h after stimulation. *C*, BE(2)-C (*lanes 1–6*) or BE(2)-C/m (*lanes 7–12*) cells were stimulated with 100 μg/ml extracellular poly(I-C) (*lanes 3, 4, 9, 10*) or 700 ng/ml transfected poly(I-C) (*lanes 5, 6, 11, 12*) for 10 h, and IFN-β and rRNA transcript levels were assessed via RT-PCR. Adjacent lanes for individual samples represent results using 10-fold dilutions of cDNA. *D*, BE(2)-C/m ISRE reporter cells were stimulated with either 20 IU/ml human leukocyte IFN-α, 20 IU/ml human fibroblast IFN-β, 100 μg/ml extracellular pIC, or 600 ng/ml T-pIC in the presence of neutralizing IFN-α or IFN-β antisera at concentrations capable of neutralizing 4000 IU/ml of IFN-α or IFN-β, respectively. Results are expressed as the percent SEAP activity compared with control samples incubated with preimmune serum. pIC, poly(I-C); T-pIC, transfected poly(I-C).

versus 25 pg/ml, respectively). We obtained similar results using reporter cell lines generated from SH-SY5Y cells, another human neuronal cell line unrelated to BE(2)-C cells (46) (data not shown).

The poly(I-C) stimulated ISRE promoter-driven SEAP expression seen in differentiated BE(2)-C/m cells could have been due to type I IFN production and autocrine activity or IFN-independent ISRE activation (47). To initially examine endogenous IFN-β transcription in response to poly(I-C) stimulation we used semi-quantitative RT-PCR (Fig. 1*C*). Poly(I-C) delivered both extracellularly and by transfection stimulated IFN-β mRNA upregulation in differentiated BE(2)-C/m cells, whereas undifferentiated cells showed no responses, consistent with the reporter gene expression results (Fig. 1*A*, 1*B*). We also observed poly(I-C)-stimulated IFN-β mRNA induction with differentiated HCN-1A cells (data not shown), a nonmalignant human cortical neuronal cell line (40). Furthermore, we observed ~10- and 100-fold increases in IFN-β mRNA upregulation in differentiated primary rat cortical neurons stimulated with extracellular or transfected poly(I-C), respectively (Fig. 5*B*). These results suggested that transcriptional upregulation of type I IFNs in response to PRR stimulation in differentiated BE(2)-C/m cells was not due to their derivation from neuroblastoma cells.

To further examine the potential for autocrine type I IFN activity in human neuronal cells, we conducted Ab neutralization experiments (Fig. 1*D*). We simultaneously incubated cells with poly(I-C) and control preimmune serum or antisera specific for human IFN-α or IFN-β, and measured SEAP activity in tissue culture supernatants. To determine Ab specificity and neutralization efficiency, we stimulated control wells with either human leukocyte IFN-α or fibroblast IFN-β instead of poly(I-C). The ISRE-SEAP responses of differentiated BE(2)-C/m cells to both extracellular and transfected poly(I-C) were significantly reduced by IFN-β, but not IFN-α–specific antisera (Fig. 1*D*). We obtained similar results with differentiated SH-SY5Y cells (data not shown). These results indicated that differentiated human neuronal cells activated NF-κB and ISRE promoters in response to poly(I-C) stimulation, and that ISRE promoter activation was due to IFN-β production and autocrine activity.

Table I. *Human neuronal responses to PRR ligands*

Stimulus	NF-κB Promoter		ISRE Promoter	
	BE(2)-C	BE(2)-C/m	BE(2)-C	BE(2)-C/m
Extracellular poly(I-C)	>1000 μg/ml	0.093 ± 0.036 μg/ml	>1000 μg/ml	8.0 ± 3.5 μg/ml
Transfected poly(I-C)	>400 μg/ml	0.015 ± 0.007 μg/ml	>400 μg/ml	0.043 ± 0.003 μg/ml
LPS	>100 μg/ml	0.012 ± 0.004 μg/ml	>100 μg/ml	>100 μg/ml
Imidazoquinoline	>25 μg/ml	>25 μg/ml	>25 μg/ml	>25 μg/ml
CpG DNA	>25 μM	>25 μM	>25 μM	>25 μM

Results represent EC_{50} values for the indicated stimulus, promoter reporter, and cell line combination. Where appropriate, values are presented as the mean ± SEM.

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SEV infection activates PRR pathways in human neuronal cells

To determine whether neuronal PRR pathways are also activated in the context of a virus infection, we used SEV, which has been shown to potently induce innate immune responses in other cell types (18). We infected undifferentiated and differentiated BE (2)-C cells expressing an NF-κB promoter-driven reporter gene with increasing doses of recombinant GFP-tagged SEV and measured SEAP activity in tissue culture supernatants 30 h post-infection (hpi). We observed dose-dependent NF-κB responses only in differentiated BE(2)-C/m cells (Fig. 2*A*), which was not due to differences in SEV replication kinetics (Fig. 2*B*). Furthermore, SEV infection also induced endogenous IFN-β mRNA upregulation in both differentiated BE(2)-C/m cells (Fig. 2*C*) and primary rat cortical neurons (data not shown). However, the response in BE(2)-C/m cells was delayed until 20 hpi (Fig. 2*C*, compare *lanes 3* and *6*), whereas the transcriptional response to poly(I-C) stimulation was much more rapid (Fig. 2*C*, *lane 2*). The delayed IFN-β transcriptional response until 20 hpi corresponded with early logarithmic replication of SEV (Fig. 2*B*), suggesting that active viral replication was required for IFN-β mRNA induction. In support of this conclusion, UV inactivation of SEV abrogated IFN-β mRNA transcriptional responses (data not shown). Thus, both synthetic and natural PRR ligands were capable of activating innate immune pathways and IFN-β transcriptional upregulation in differentiated human neuronal cells.

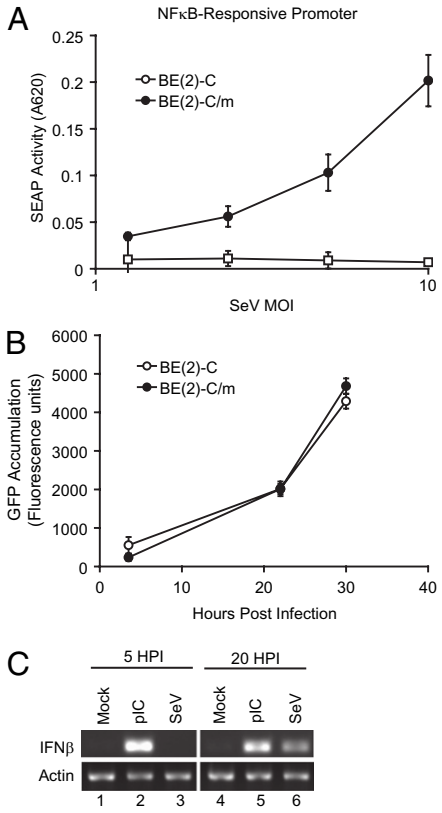

FIGURE 2. SEV infection induces a neuronal PRR response. *A,* NF-κB reporter cells were infected with GFP-tagged SEV using an increasing MOI, and SEAP reporter activity was assessed at 30 hpi. *B*, Cells were infected as in *A* and SEV replication, assessed by GFP fluorescence, was measured. *C*, BE(2)-C/m cells were stimulated with 100 μg/ml pIC (*lanes 2*, *5*) or infected with SEV at an MOI of 10 (*lanes 3*, *6*), and IFN-β mRNA or rRNA accumulation was assessed via RT-PCR 5 h (*lanes 1–3*) or 20 h (*lanes 4–6*) later. pIC, poly(I-C).

Human neuronal cells show restricted responses to PRR ligands

Poly(I-C) and SEV are stimuli that are frequently used to activate innate immune pathways via TLR3, MDA5, or RIG-I. To determine whether the differentiation-dependent responses of BE(2)-C cells to poly(I-C) and SEV extended to other stimuli, we examined several additional PRR ligands (Table I). We stimulated NF-κB or ISRE promoter-driven reporter cell lines with increasing concentrations of LPS, the imidazoquinoline compound derivative CLO97, or the CpG-containing oligonucleotide ODN2006, which are ligands for TLR4, TLR7/8, or TLR9, respectively (1). BE (2)-C cells showed a differentiation-dependent response to LPS using an NF-κB promoter-driven reporter, whereas the ISRE promoter-driven reporter was not stimulated by LPS regardless of cell differentiation. This observation was consistent with the differentiation-dependent expression of TLR4 and its coreceptor CD14 identified by microarray analyses (see below, Supplemental Tables I and II), and published studies demonstrating TLR4 expression in primary CNS neurons and neuronal cell lines (30, 48). Neither CLO97 nor ODN2006 stimulated reporter gene activity in BE(2)-C cells regardless of differentiation (Table I), even though these TLR ligands were able to activate an NF-κB promoter-driven reporter in differentiated U937 cells, a human macrophage cell line (49) (data not shown). We did not specifically examine TLR7/8 or TLR9 expression, and therefore cannot exclude the possibility that the inability of BE(2)-C cells to respond to CLO97 or ODN2006 was secondary to the absence of these TLRs. However, published data suggest that mRNAs for TLR7, 8, and 9 are present in some primary neurons and neuronal cell lines (30). Nevertheless, these results suggested that human neuronal cells possess restricted PRR-mediated responses, and ligands that stimulated predominantly antiviral innate immune pathways via TLR3-, MDA5-, or RIG-I–mediated responses were particularly active. Thus, we specifically focused subsequent studies on these pathways.

Differentiated neurons express TLR3, MDA5, and RIG-I

We initially examined the expression of TLR3, MDA5, and RIG-I in neuronal cells by immunoblotting and immunofluorescence microscopy (Fig. 3). Previously published studies have demonstrated TLR3 expression in both cultured human and rodent neurons and CNS tissue sections (26–29, 48), and we also observed TLR3 expression in lysates from undifferentiated BE (2)-C cells (Fig. 3*A*, *lane 1*), differentiated BE(2)-C/m cells (Fig. 3*A*, *lanes 2–4*), and primary rat neurons (Fig. 3*A*, *lane 5*). To validate the specificity of TLR3 immunoblotting, we used lysates from BE(2)-C/m cells transfected with plasmids overexpressing either wild-type TLR3 (Fig. 3*A*, *lane 3*) or a dominant-negative mutant that contains a deletion of the TIR domain (Fig. 3*A*, *lane 4*). We also examined TLR3 expression in BE(2)-C/m cells by immunofluorescence microscopy, and observed a punctate cytoplasmic distribution (Fig. 3*B*, *upper right*), that was particularly evident at higher magnification (Fig. 3*B*, *lower left*). We observed a similar distribution pattern but increased TLR3 immunofluorescent signal intensity in cells transfected with a plasmid overexpressing wild-type TLR3 (Fig. 3*B*, *lower right*). These immunofluorescence results were consistent with the previously described endosomal localization of TLR3 in cultured human neuronal cells (29). Furthermore, immunoblot analysis revealed that both MDA5 and RIG-I were expressed in human BE (2)-C cells (Fig. 3*C*, *lanes 1–4*) and primary rat neurons (Fig. 3*C*, *lanes 5* and *6*). Interestingly, although RIG-I expression increased with BE(2)-C differentiation, probably due to the use of retinoic acid to induce neuronal maturation, MDA5 expression levels were

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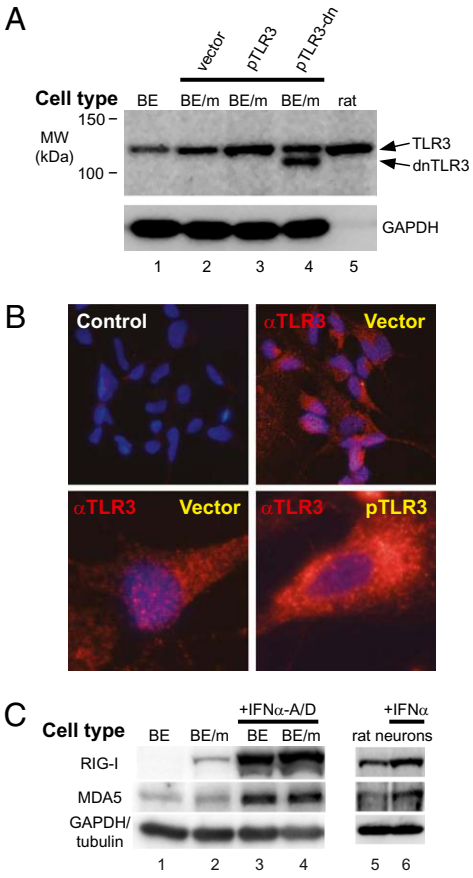

FIGURE 3. Human neuronal cells and differentiated rodent neurons express antiviral PRRs. *A*, Lysates from BE(2)-C cells (*lane 1*), differentiated BE(2)-C/m cells (*lanes 2–4*), or primary rat neurons (*lane 5*) were immunoblotted for TLR3 levels. To validate Ab specificity, BE(2)-C/m cells were transfected with either empty vector (*lane 2*) or plasmids expressing wild-type TLR3 (*lane 3*) or a dominant-negative TLR3 (*lane 4*) that contains a TIR domain deletion. The human TLR3 gene encodes a 904 aa protein with a predicted m.w. of 103 kDa, although it is heavily glycosylated. The TLR3 ΔTIR mutant contains a 162 aa deletion that reduces the predicted m.w. by ~18 kDa. The GAPDH-specific mAb used for immunoblotting cross-reacted poorly with the rat lysate (*lane 5*), but total protein staining showed that the rat lysate sample contained ~2- to 3-fold more total protein than the other lanes (data not shown). *B*, Immunofluorescent staining of TLR3 expression in BE(2)-C/m cells. The primary TLR3-specific Ab was excluded during incubation in control cells (*upper left image*). Nuclei were stained with DAPI (blue), whereas the punctate PE-staining (red) indicates TLR3 expression. Cells in the *upper right* and *lower left images* were transfected with empty vector, whereas cells in the *lower right image* were transfected with a plasmid expressing wild-type TLR3. We also saw a similar punctuate pattern but increased TLR3 signal intensity in cells transfected with the dominant-negative TLR3 expression plasmid (data not shown). Original magnification ×400. *C*, Lysates from BE(2)-C cells (*lanes 1*, *3*), differentiated BE(2)-C/m cells (*lanes 2*, *4*), or primary rat neurons (*lanes 5*, *6*), were immunoblotted for RIG-I, MDA5, and GAPDH (human) or tubulin (rat) levels. Lysates from cells treated with 100 IU/ml human IFN-α–A/D for 6 h (*lanes 3*, *4*) or 50 IU/ml rat IFN-α for 24 h (*lane 6*) were used as controls to validate the identity of RIG-I and MDA5 as IFN-stimulated genes.

independent of differentiation (Fig. 3*C*, *lanes 1* and *2*). However, the expression of both PRRs increased in response to type I IFN stimulation in both human BE(2)-C neuronal cells (Fig. 3*C*, *lanes 3* and *4*) and primary rat neurons (Fig. 3*C*, *lane 6*). These results suggested that the three PRRs associated with potent antiviral innate immune responses, TLR3, MDA5, and RIG-I, are expressed in human neuronal cells and differentiated neurons.

Specific PRRs are required for poly(I-C)– and SEV-mediated activation of innate immune pathways in human neuronal cells

We next examined the functional impact of PRR expression on neuronal innate immune responses using genetic disruption of receptor function (Fig. 4). To disrupt TLR3- or RIG-I–mediated pathway activation in BE(2)-C/m cells, we used stable cell lines expressing specific dominant-negative mutants. Initial experiments using transient transfection with the TLR3 ΔTIR mutant described previously showed an ~50% reduction in extracellular poly(I-C)–stimulated ISRE-SEAP activity (data not shown). However, we were unable to generate stable cell lines constitutively

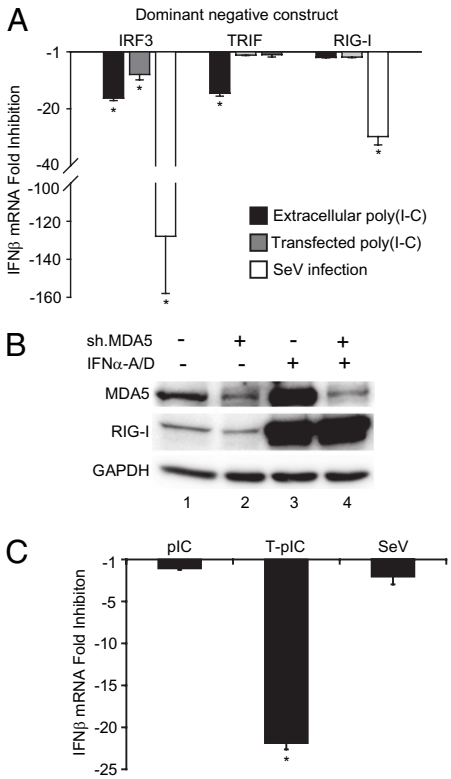

FIGURE 4. Human neuronal cells possess functional PRR-mediated innate immune pathways. *A*, BE(2)-C/m cells stably overexpressing dominant negative forms of IRF3, TRIF, or RIG-I were stimulated with 100 μg/ml extracellular poly(I-C) or 700 ng/ml transfected poly(I-C) for 10 h, or infected with SEV for 30 h, and IFN-β mRNA levels were measured by quantitative RT-PCR using rRNA transcript levels as the loading control. Results are expressed as the fold-change compared with similarly stimulated cells stably transfected with an empty vector. *B*, Lysates from BE(2)-C/m cells stably transfected with plasmids expressing shRNAs targeted against either a control protein (*lanes 1*, *3*) or MDA5 (*lanes 2*, *4*) were immunoblotted for MDA5, RIG-I, and GAPDH expression levels. The level of MDA5 suppression in cells expressing an MDA5-specific shRNA was 44.5 ± 7.4% compared with control cells. Live-cell imaging of differentiated cells also demonstrated that greater than >95% of cells expressed the control GFP reporter gene encoded on the shRNA expression plasmid (data not shown). Lysates from cells treated with 1000 IU/ml IFN-α–A/D for 12 h (*lanes 3*, *4*) served as positive controls to validate the specificity of shRNA-mediated knockdown of MDA5 under enhanced expression levels. *C*, IFN-β mRNA levels in BE(2)-C/m cells stably expressing an MDA5-targeted shRNA after stimulation with extracellular pIC, T-pIC, or infected with SEV as described previously. Transcript levels were determined by quantitative RT-PCR and results are expressed as the fold-change compared with similarly stimulated cells stably transfected with an shRNA-encoding vector targeting an irrelevant control protein. *p value <0.05. pIC, poly(I-C); T-pIC, transfected poly(I-C).

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expressing this construct, and therefore we subsequently targeted a downstream signaling molecule. Because TLR3 is the only known dsRNA-sensing PRR to use the adaptor protein TRIF for signal transduction (2), we used a TRIF mutant that contains only the TIR domain (50) to disrupt TLR3 function. In contrast, both RIG-I and MDA5 use the adaptor protein IFN-β promoter stimulator protein 1 (2), and we therefore used an *N*-terminal RIG-I deletion mutant (51) to disrupt RIG-I function. As a positive control we used a dominant-negative IRF3 mutant (52), because this transcription factor is a central regulator of innate antiviral responses (2). We generated BE(2)-C cell lines stably transfected with individual constitutive expression plasmids encoding the dominant-negative mutants described previously, differentiated cells with retinoic acid, stimulated with either extracellular or transfected poly(I-C) or infected with recombinant SEV, and measured IFN-β mRNA induction by quantitative RT-PCR (Fig. 4*A*). Dominant-negative IRF3 expression inhibited the IFN-β transcriptional responses to all three stimuli, where the largest decrease (~130-fold) was seen with SEV infection. In contrast, dominant-negative TRIF expression specifically inhibited extracellular poly(I-C)–stimulated responses, whereas dominant-negative RIG-I expression specifically inhibited SEV-stimulated responses.

To disrupt MDA5-mediated pathway activation in BE(2)-C/m cells, we depleted receptor levels through stable expression of a plasmid encoding a shRNA specifically targeting MDA5 (Fig. 4*B*, 4*C*). We initially optimized conditions and obtained a 40–50% reduction in MDA5 expression levels in BE(2)-C/m cells without significantly altering expression levels of the related RLR, RIG-I (Fig. 4*B*, compare *lanes 1* and *2*). Depletion of MDA5 inhibited the IFN-β transcriptional response to stimulation with transfected poly(I-C) but not with extracellular poly(I-C) or SEV infection (Fig. 4*C*). These results indicated that human neuronal cells possess functional TLR3-, MDA5-, and RIG-I–activated pathways that respond to specific stimuli.

Table II. *Signaling pathways preferentially upregulated in differentiated human BE(2)-C/m neuronal cells*

Signaling Pathway[a]	No. of Genes	*p* Value
14-3-3–mediated signaling	21	0.0016
α-Adrenergic signaling	13	0.0089
Axonal guidance signaling	38	0.0044
cAMP-mediated signaling	18	0.0162
Cardiac β-adrenergic signaling	15	0.0129
Caveolar-mediated endocytosis	11	0.0155
Ephrin receptor signaling	23	0.0010
ERK/MAPK signaling	20	0.0158
FcγR-mediated phagocytosis in macrophages and monocytes	12	0.0269
G-protein coupled receptor signaling	24	0.0025
Hepatic fibrosis/stellate cell activation	21	0.0001
IGF-1 signaling	17	0.0001
IL-8 signaling	20	0.0098
Insulin receptor signaling	15	0.0209
Integrin signaling	24	0.0029
Leukocyte extravasation signaling	19	0.0457
Neuregulin signaling	15	0.0006
Neurotrophin/TRK signaling	10	0.0112
Notch signaling	8	0.0037
p53 signaling	11	0.0355
PI3K/AKT signaling	15	0.0102
PTEN signaling	15	0.0008
PXR/RXR activation	9	0.0417
RAR activation	25	0.0002
Synaptic long-term potentiation	14	0.0115
TGF-β signaling	14	0.0007
Tight junction signaling	18	0.0174
VDR/RXR activation	13	0.0021
Wnt/β-catenin signaling	22	0.0011

Signaling pathways associated with innate immunity in the literature are shown in bold. These pathways were identified by the presence of at least 10 Medline coreferences with the keywords "innate" and "immunity."

[a]The listed pathways were identified as significantly associated with BE(2)-C differentiation using Ingenuity Pathway Analysis software and data sets derived from both Genomatix and Affymetrix Bioconductor analyses of microarray data. Only those pathways identified with both data sets are shown, where the number of genes and *p* values listed are from the Genomatix dataset analysis.

Neuronal cell differentiation modulates innate immune signaling pathway component expression

There are multiple signal transduction events that occur between PRR interaction with its ligand and downstream antiviral effector production. To identify potential neuronal components involved in these events, we used genome-wide transcriptional microarray results combined with pathway analyses and compared BE(2)-C cells before and after retinoic acid-mediated differentiation. This approach was feasible because BE(2)-C cells showed minimal responsiveness to select PRR ligand stimulation (Figs. 1, 2, Table I) prior to differentiation. We identified 1002 upregulated and 863 downregulated genes in differentiated BE(2)-C/m cells. The complete list of differentially regulated genes is provided in Supplemental Table I.

We subsequently conducted an in silico analysis with upregulated genes that were assigned to known cellular pathways using Ingenuity Pathway software to identify potential innate immune networks active in neuronal PRR signaling. We identified 29 canonical signaling pathways preferentially upregulated in differentiated BE(2)-C/m cells, nine of which have been linked with innate immunity (Table II). We were particularly interested in the identification of the PI3K/AKT signaling pathway, as PI3Ks have been implicated as positive and negative regulators of TLR3-mediated signaling events (53–58), TLR3 expression and function have been implicated in neuronal antiviral responses (26–29, 31), and the extracellular poly(I-C) stimulation experiments implicated an active TLR3-mediated pathway in differentiated BE(2)-C/m cells (Figs. 1, 4). To validate the microarray results with a particular focus on the PI3K/AKT pathway, we used a microplate-based quantitative RT-PCR array that included 71 genes associated with this pathway. Using this targeted array, we validated the transcriptional upregulation of 19 genes in differentiated BE(2)-C/m cells that are associated with PIK3 signaling, including *AKT3, APC, CD14, CTNNB1, FOXO1, FOXO3, FRAP, GSK3B, ITGB1, JUN, MAPK8, PAK1, PDPK1, PI3KCA, PI3KR1, RASA1, TLR4, TSC2*, and *YWHAH* (complete quantitative results provided in Supplemental Table II). Furthermore, we validated increased protein expression levels of the PI3K regulatory subunit isoform p85α encoded by the *PI3KR1* gene in differentiated BE(2)-C/m cells by immunoblotting (data not shown). These results suggested that canonical PI3K/AKT pathway components were involved in neuronal innate immune responses.

PI3K inhibition blocks poly(I-C)–mediated innate immune system activation in neuronal cells

To examine the potential functional role of PI3K in neuronal PRR pathway signaling, we initially used the universal PI3K inhibitor LY294002 (59). We incubated differentiated BE(2)-C/m cells expressing ISRE or NF-κB promoter-driven reporter genes with increasing concentrations of LY294002, stimulated with extracellular or transfected poly(I-C), LPS, IFN-α–A/D, or TNF-α, and measured SEAP activity in tissue culture supernatants after 20 h (Fig. 5*A*). Initial viability studies showed minimal cytotoxicity with up to 25 μM LY294002 in BE(2)-C/m cells (data not shown). LY294002 potently inhibited both extracellular and

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transfected poly(I-C) stimulation in ISRE reporter cells with an IC_{50} of ~7 μM (Fig. 5*A*, *top graph*), but had no effect on NF-κB promoter activation in response to poly(I-C), LPS, or TNF-α (Fig. 5*A*, *bottom graph*). The inhibition of LY294002 on the IRSE promoter-driven reporter gene was due to disruption of autocrine IFN-β production rather than feedback signaling and amplification, as LY294002 had no effect on exogenous IFN-α–A/D stimulation of ISRE promoter reporter cells (Fig. 5*A*, *top graph*) but did suppress poly(I-C)–stimulated IFN-β mRNA transcriptional upregulation (see below, Fig. 6*B*). Furthermore, LY294002 suppressed poly(I-C)–stimulated IFN-β mRNA transcriptional upregulation in primary rat cortical neurons (Fig. 5*B*). These results suggested that PI3K is involved in NF-κB–independent neuronal PRR pathways stimulated by poly(I-C) and mediated through TLR3 and MDA5.

To gain further insight into the signaling molecules involved in neuronal PRR pathway activation, we used a defined library of kinase inhibitors and examined their effects on poly(I-C)–mediated activation of differentiated BE(2)-C/m cells expressing an ISRE promoter-driven reporter. This library contains 99 inhibitors targeting 48 different kinases, including several involved in canonical PI3K/AKT signaling networks (complete inhibitor list provided in Supplemental Table III). Each inhibitor was serially diluted in duplicate from 100 to 0.8 μM, incubated with reporter cells stimulated with extracellular or transfected poly(I-C), and SEAP activity was measured after 20 h. To control for nonspecific cytotoxicity, we conducted parallel viability assays. We identified 23 kinase inhibitors that blocked either extracellular or transfected poly(I-C)–mediated activation of an ISRE promoter-driven reporter gene in differentiated BE(2)-C/m cells (Table III). Interestingly, there was not a complete overlap between the lists of inhibitors that disrupted extracellular versus transfected poly(I-C) stimulation. For example, inhibitors of epidermal growth factor receptor kinase were more active against transfected poly(I-C) (Supplemental Table III), suggesting that further studies using these pharmacologic probes may provide additional information regarding potential divergences in neuronal PRR signaling pathways.

One noteworthy observation from the kinase inhibitor library studies in the context of our previous results was the identified activity of several PI3K inhibitors (Table III). However, these active compounds were either general PI3K inhibitors or targeted to the PIK3 p110α subunit, where compounds targeted to the PI3K p110β or p110γ subunits were not active in this medium throughput assay. The PI3K complex consists of a receptor subunit that binds activated membrane-associated receptors and recruits a p110 catalytic subunit (α, β, or γ) that mediates the conversion of phosphatidylinositol (4, 5)-bisphosphate to phosphatidylinositol (3–5)-trisphosphate, which is generally required for downstream signaling (60). To validate the kinase inhibitor library results, we purchased new inhibitors specifically targeting p110α (PI3K p110α Inhibitor 2), p110β (TGX-221), or p110γ (AS-252424), and used these compounds in detailed dose-titration studies with the same reporter cell line used for the kinase inhibitor library medium throughput assays (Fig. 6*A*). The PI3K p110α-specific inhibitor blocked both extracellular and transfected poly(I-C)–activated ISRE reporter activity with IC_{50} values of 0.5 and 1.6 μM, respectively (Fig. 6*A*, *upper graph*). In contrast, neither the p110β-specific (Fig. 6*A*, *middle graph*) nor p110γ-specific (Fig. 6*A*, *lower graph*) inhibitor significantly suppressed poly(I-C)-stimulated reporter gene activity until reaching concentrations >10 μM, at which point their subunit specificity decreases significantly (61, 62). To verify the ability of PI3K subunit-specific inhibitors to block the induction of endogenous IFN-β mRNA, we

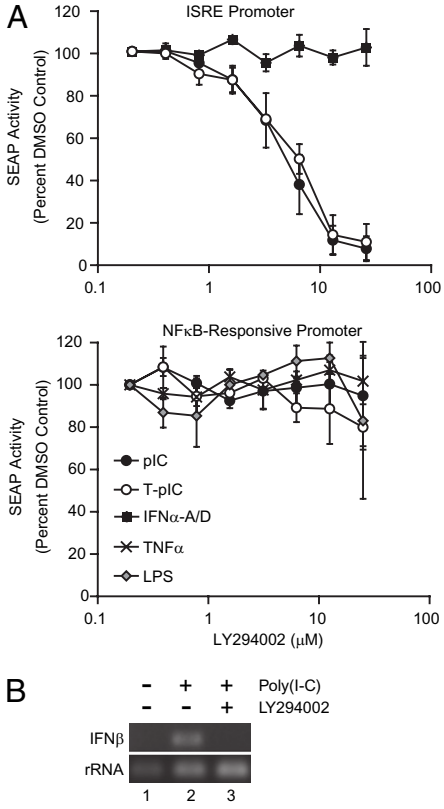

FIGURE 5. Neuronal response to poly(I-C) is mediated by PI3K. *A*, BE(2)-C/m ISRE (*upper graph*) and NF-κB (*lower graph*) promoter-driven reporter cells were treated with an increasing concentration of LY294002, stimulated with 100 μg/ml extracellular pIC, 700 ng/ml T-pIC, 100 IU/ml IFN-α–A/D, 50 ng/ml TNF-α, or 500 ng/ml LPS, and SEAP reporter activity was measured 24 h later. Results are expressed as the percentage of SEAP activity compared with DMSO-treated controls. TNF-α and LPS were used as controls only with NF-κB promoter-driven reporter cells as the ISRE reporter cells did not respond to either stimuli even in the absence of LY294002 (Table I and data not shown). *B*, Primary rat cortical neurons were treated with either DMSO (*lanes 1*, *2*) or 10 μM LY294002 (*lane 3*), stimulated with 50 μg/ml of extracellular poly(I-C) for 8 h (*lanes 2*, *3*), and IFN-β mRNA levels were assessed by RT-PCR. pIC, poly(I-C); T-pIC, transfected poly(I-C).

examined the effects of LY294002, p110α Inhibitor 2, and AS-2552424 on IFN-β mRNA transcription after poly(I-C) stimulation in BE(2)-C/m cells (Fig. 6*B*). We found that both LY294002 and the PI3K p110α-selective inhibitor significantly suppressed IFN-β mRNA transcriptional activation when stimulated with either extracellular or transfected poly(I-C), whereas the p110γ-selective inhibitor had no effect.

Finally, to provide genetic validation for the inhibitor studies, we depleted protein levels through stable shRNA expression targeted against the PI3K p110α subunit (Fig. 7). We obtained an ~60% reduction in PI3K p110α levels in differentiated BE(2)-C/m cells (Fig. 7*A*), which resulted in significant inhibition of extracellular poly(I-C)–mediated stimulation of IFN-β mRNA transcription (Fig. 7*B*). However, in contrast to results with p110α-specific inhibitors (Fig. 6), shRNA-mediated knockdown of p110α protein levels did not suppress the ability of transfected poly(I-C) to stimulate IFN-β mRNA transcription (Fig. 7*B*). The ability of the p110α-depleted neuronal cells to remain responsive to transfected poly(I-C) may have been due to an insufficient depletion of p110α levels, which is consistent with a reproducible 3-fold higher IC_{50} of the p110α-specific inhibitor for transfected versus extracellular poly(I-C)–mediated neuronal responses (Fig. 6*A*). Nevertheless,

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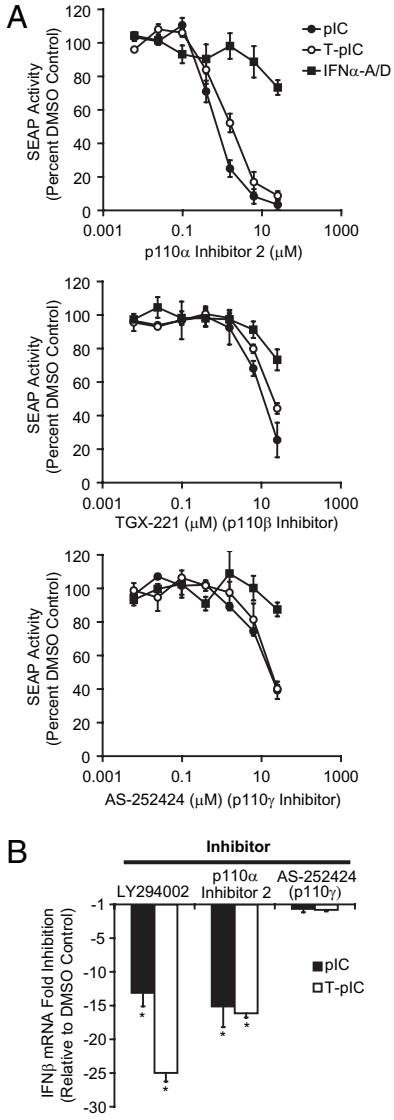

FIGURE 6. PI3K catalytic subunit p110α mediates human neuronal cell responses to poly(I-C). *A*, BE(2)-C/m ISRE reporter cells were treated with increasing concentrations of a selective PI3K p110α (p110a Inhibitor 2), p110β (TGX-221), or p110γ (AS-252424) catalytic subunit inhibitor, stimulated with 100 μg/ml extracellular pIC, 700 ng/ml T-pIC, or 100 IU/ml IFN-α–A/D, and SEAP reporter activity was measured 20 h later. Results are presented as the percent reporter gene activity compared with DMSO-treated controls. *B*, BE(2)-C/m cells were treated with 10 μM LY294002, 5 μM p110α Inhibitor 2, or 5 μM AS-252424, stimulated with poly(I-C) as described previously, and IFN-β mRNA levels were measured 4 h later by quantitative RT-PCR. $*p$ values <0.05. pIC, poly(I-C); T-pIC, transfected poly(I-C).

these results indicated that PI3K, and in particular the p110α subunit, modulates TLR3- and possibly MDA5-dependent innate immune pathway activation in human neuronal cells.

Discussion

The innate immune system plays a critical role in both the initial response to an invading pathogen, which frequently limits or contains pathogen replication and dissemination, and the induction of an effective adaptive immune response, which is most often the primary mechanism for pathogen clearance. The characteristics of the innate immune response are determined in part by the pathogen initiating the response but can also be influenced by the type of cell in which the response is generated. In this report, we examined the functional PRR-mediated pathways present in human neuronal cells and differentiated primary rat neurons, with a particular focus on those pathways previously identified as being important for antiviral innate immune responses in other cell types. We drew four main conclusions. First, human neuronal cells possess functional TLR3-, TLR4-, RIG-I–, and MDA5-mediated PRR pathways whose activity was maturation-dependent. Second, both extracellular and transfected poly(I-C) induced potent IFN-β induction in neurons that resulted in autocrine ISRE activation. Third, the neuronal antiviral innate immune pathways mediated by TLR3, RIG-I, and MDA5 are nonredundant and preferentially respond to distinct ligands. Fourth, TLR3- and possibly MDA5-mediated neuronal responses are positively regulated by the PI3K pathway, and in particular the PI3K p110α subunit. These results indicate that human neuronal cells possess a relatively broad complement of PRR-mediated innate immune pathways, and that those pathways typically stimulated by viral pathogens via nucleic acid recognition are particularly active.

Previous studies on PRR pathways in CNS neurons have focused predominantly on TLR-mediated pathways and have examined their impact on multiple aspects of brain physiology, including development and regeneration (63). Several studies have examined the role of TLR3 in response to CNS viral infections (26–29, 31), although the potential antiviral role of TLR3-mediated pathways is controversial and may be pathogen-specific (64). For example, humans with a TLR3 deficiency have a genetic predisposition to HSV encephalitis (65), but $TLR3^{-/-}$ mice have decreased susceptibility to rabies virus encephalitis (29). Furthermore, $TLR3^{-/-}$ mice have been shown to have both increased (66) and decreased (31) susceptibility to WNV encephalitis. However, these studies cannot fully separate the neuron-specific activity of TLR3 from other cell types, including professional immune cells, such as macrophages and dendritic cells. Although CNS neurons from $TLR3^{-/-}$ mice have a modest increase in WNV production when infected in culture (31), suggesting that neuronal TLR3-mediated responses can have antiviral effects, further studies in mice with conditional cell-specific TLR3 deletions will be required to fully delineate the potential antiviral activity of TLR3-activated innate immune pathways in neurons and their role in viral pathogenesis.

In contrast to TLR3, neuronal innate immune responses mediated by the cytosolic PRRs RIG-I and MDA5 have been less well studied. The expression of both RIG-I or MDA5 could be induced by IFN-β or WNV infection in cultured mouse cortical neurons, but basal expression was not detected by immunoblotting (19). Although we also found that both RLRs were upregulated with type I IFN treatment of human neuronal cells, we did detect basal expression, especially in differentiated cells. Furthermore, we found that both RIG-I– and MDA5-mediated pathways were active in human neuronal cells. The presence of functional cytosolic PRR-activated innate immune pathways in neurons is not surprising, as the innate antiviral responses to several neurotropic viruses has been shown to involve RIG-I– and/or MDA5-mediated pathways in non-neuronal cells (67, 68). However, the outcome of these responses may differ significantly between neuronal and non-neuronal cells, where mature CNS neurons are essentially irreplaceable and therefore initiation of an altruistic apoptotic cascade may result in irreversible damage to the host, despite simultaneously preventing virus spread. One intriguing hypothesis is that neuronal PRR responses, potentially augmented or modulated by PRR-initiated responses in other CNS-resident cells, such as astrocytes or microglia, control virus replication *and* promote neuronal survival through either previously unrecognized or uncharacterized pathways. Consistent with this hypothesis, preliminary results suggest that the neurotropic alphavirus Western

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Table III. *Kinase inhibitors that suppressed poly(I-C)–mediated ISRE promoter stimulation in human neuronal cells*

Inhibitor[a]	Target(s)
BML-257	AKT
Triciribine	AKT
Terreic acid	BTK
Flavopiridiol	CDK 1,2,4
Apigenin	CK-II
PI-103	DNA-PL, PI3K p110α, mTOR
Tyrphostin 25	EGFRK
Tyrphostin 51	EGFRK
BML-265	EGFRK
5-Iodotubercidin	ERK2, adenosine kinase, CK1, CK2
PK412	FTL3, Src, ABL
GSK3 Inhibitor XIII	GSK3
Purvalanol A	GSK3-α/β, CDKs
ZM 449829	JAK-3
Rapamycin	mTOR
2-Aminopurine	P58 PITSLRE-β1
LY294002	PI3K
Quercetin dehydrate	PI3K
PI3Kα Inhibitor 2	PI3K p110α
H8	PKA, PKG
Palmitoyl-DL-carnitine Cl	PKC
HBDDE (2,2′,3,3′,4,4′-Hexahydroxy-1,1′-biphenyl-6,6′-dimethanol dimethyl ether)	PKCα, PKCγ
Rottlerin	PKCδ
Tyrphostin AG 1288	Tyrosine kinases

[a]The listed inhibitors suppressed ISRE promoter-driven reporter gene activity in BE(2)-C/m cells with IC_{50} values <20 μM for either extracellular or transfected poly (I-C) and had CC_{50} values >20 μM. Inhibitors with activity against PI3K are shown in bold and italics.

BTK, Bruton's tyrosine kinase; CDK, cyclin-dependent kinase; CK, creatine kinase; EGFRK, epidermal growth factor receptor kinase; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G.

equine encephalitis virus activates an IRF3-dependent prosurvival pathway in human neuronal cells (D. Peltier and D. Miller, unpublished results). Detailed studies are currently in progress to examine the roles of TLR3-, RIG-I–, and MDA5-activated pathways in human neurons in response to a variety of neurotropic viruses to more fully explore this hypothesis.

The signal transduction pathways that mediate PRR-activated immune responses are complex and interconnected at multiple levels. Several steps in these pathways are mediated by cellular kinases, and we found that the PI3K pathway was important for optimal TLR3- and possibly MDA5-activated responses in human neuronal cells. The PI3K pathway has previously been implicated in innate immune pathway regulation, and in particular TLR3-mediated signaling (69). However, both stimulatory (53, 54) and inhibitory (55) effects on TLR3-mediated signaling have been observed, which may be explained in part by cell type differences. Our results suggest that the PI3K pathway plays a stimulatory role in neuronal TLR3-mediated responses, and is necessary for full IFN-β mRNA induction. Furthermore, both pharmacologic and genetic approaches identified a potential role for PI3K, and in particular the PI3K p110α subunit, in neuronal MDA5-mediated signaling. Although the PI3K pathway has been tentatively implicated in cytoplasmic RLR-initiated signaling (53), the majority of work thus far has focused on its effects during TLR-initiated signaling (69). Cellular PI3K/AKT pathways are essential for neuronal development and survival (70–72), suggesting a potential link between antiviral PRR pathway activation and the ability of neurons to overcome an infection until an adaptive immune response can be fully established. Additional studies will be required to further delineate the precise PI3K pathway components involved in neuronal antiviral PRR activation, but our kinase inhibitor library studies suggest that protein kinase B (AKT), mammalian target of rapamycin (mTOR), and glycogen synthase kinase 3 (GSK3) may be involved. All of these kinases participate in PI3K signaling and have been implicated in innate immunity in non-neuronal model systems (73–78).

Innate immune pathway stimulation via TLRs or RLRs eventually results in the activation of multiple genes involved in immune responses (1, 2). For antiviral pathway stimulation via TLR3, RIG-I, or MDA5, one important group of upregulated genes are type I IFNs, and in particular IFN-β in nonprofessional immune cells (7). Indeed, IFN-β mRNA upregulation is a convenient marker of innate antiviral pathway activation, which we used to monitor responses to TLR3, RIG-I, and MDA5 ligands. Furthermore, we found that BE(2)-C/m human neuronal cells are capable of synthesizing and excreting IFN-β in response to specific PRR stimulation, consistent with previously published studies in other cultured human neuronal cell lines (27) and rodent neurons both in vitro (19, 20, 31) and in vivo (33). However, whether neuronal IFN-β production in vivo in response to neurotropic pathogens or other CNS inflammatory conditions plays an important role in either the amelioration or augmentation of disease is controversial. Studies in conditional knockout mice that have disrupted type I IFNR expression in neuroectodermal cells, which includes neurons, indicate that responses to type I IFNs are important to control virus spread within the CNS (79) but not in the progression of experimental autoimmune inflammatory disease (80). Similar studies with mice containing conditional disruption of IFN-β production in CNS-resident cells will be needed to definitively examine the potential importance of neuronal type I IFN production in vivo.

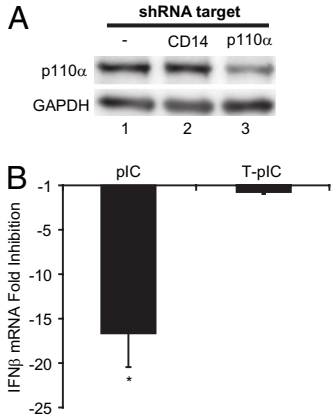

FIGURE 7. The PI3K p110α catalytic subunit mediates a TLR3-dependent response in neuronal cells. *A*, Lysates from BE(2)-C/m cells stably transduced with lentiviruses expressing an empty vector (*lane 1*) or shRNAs targeted against either a control protein (CD14, *lane 2*) or PI3K p110α (*lane 3*) were immunoblotted for p110α and GAPDH expression levels. The level of suppression in cells expressing a p110α-specific shRNA was 60.4 ± 4.9% compared with control cells. Live-cell imaging of differentiated cells also demonstrated that >95% of cells expressed the control GFP reporter gene encoded on the shRNA expression plasmid (data not shown). *B*, IFN-β mRNA levels in BE(2)-C/m cells stably expressing a p110α-targeted shRNA after stimulation with extracellular pIC or T-pIC as described in Fig. 6. Transcript levels were determined by quantitative RT-PCR and results are expressed as the fold-change compared with similarly stimulated cells stably transfected with an shRNA-encoding vector targeting an irrelevant control protein. *p value <0.05. pIC, poly(I-C); T-pIC, transfected poly(I-C).

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Disclosures

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