

TLR3 Ligation Activates an Antiviral Response in Human Fetal Astrocytes: A Role for Viperin/cig5¹

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TLR3 functions as a viral nucleic acid sentinel activated by dsRNA viruses and virus replication intermediates within intracellular vesicles. To explore the spectrum of genes induced in human astrocytes by TLR3, we used a microarray approach and the analog polyriboinosinic polyribocytidylic acid (pIC) as ligand. As expected for TLR activation, pIC induced a wide array of cytokines and chemokines known for their role in inflammatory responses, as well as up-regulation of the receptor itself. The data also showed activation of a broad spectrum of antiviral response genes. To determine whether pIC induced an antiviral state in astrocytes, a pseudotyped HIV viral particle, vesicular stomatitis virus g-env-HIV-1, was used. pIC significantly abrogated HIV-1 replication, whereas IL-1, which also potently activates astrocytes, did not. One of the most highly up-regulated genes on microarray was the protein viperin/cig5. We found that viperin/cig5 expression was dependent on IFN regulatory factor 3 and NF- κ B signaling, and that repetitive stimulation with pIC, but not IL-1, further increased expression. Viperin induction could also be substantially inhibited by neutralizing Abs to IFN- β , as could HIV-1 replication. To explore a role for viperin in IFN- β -mediated inhibition of HIV-1, we used an RNA interference (RNAi) approach. RNAi directed against viperin, but not a scrambled RNAi, significantly inhibited viperin expression, and also significantly reversed pIC-induced inhibition of HIV-1 replication. We conclude that viperin contributes to the antiviral state induced by TLR3 ligation in astrocytes, supporting a role for astrocytes as part of the innate immune response against infection in the CNS. *The Journal of Immunology*, 2006, 177: 4735–4741.

Toll-like receptors constitute a family of type 1 transmembrane proteins that function as recognition receptors for common pathogens (1). The first described ligands for TLR were all of bacterial origin, but more recent data have shown that viruses also result in the activation of TLR. TLR1, 2, 4, 5, 6, and 9 respond to common microbial pathogens, with TLR2 together with TLR1 recognizing triacyl lipopeptides, TLR2 and TLR6 diacyl lipopeptides, TLR4 and CD14 recognizing the LPS of Gram-negative bacteria, TLR5 recognizing flagellin, and TLR9 recognizing unmethylated DNA sequences found in bacterial DNA, as well as in viruses. Following viral infections, TLR3 recognizes virus-derived dsRNA, and TLR7 and TLR8 virus-derived ssRNA (2). The four TLR that recognize nucleic acids (TLR3, 7, 8, and 9) are all thought to be expressed predominantly, if not exclusively, on membranes of intracellular vesicular compartments, but the exact nature of these vesicles remains unclear at the present time (3, 4).

Following interaction with ligand, a highly conserved cytoplasmic Toll-IL-1R domain is engaged that triggers the activation of the NF- κ B, JNK, and p38 MAPK signaling pathways via the recruitment of MyD88 (5). These transcription factors are known to

be required for the induction of cytokines and chemokines involved in the initiation of inflammation, host defense, and priming of the adaptive immune response. However, distinct signaling pathways have also been noted following activation of TLR particularly in the production of type 1 IFNs, which are detected following activation of TLR3, 4, 7, 8, and 9. For TLR3 and 4 this occurs via activation of IFN regulatory factor 3 (IRF3)⁴ through a MyD88-independent pathway (6, 7).

TLR3 is thought to be a major mediator of the cellular response to viral infection because it responds to dsRNA, a common by-product of viral replication. In the CNS, several studies have shown that both microglia and astrocytes express TLR3 and can be activated by the dsRNA analog polyriboinosinic polyribocytidylic acid (pIC) (4, 8–10). Astrocytes are the most abundant cell type in the brain. They maintain the homeostatic environment of the CNS and are essential components in the formation, maintenance, and restoration following injury of the blood-brain barrier (11). Recently, we showed that astrocytes signal via the IRF3-dependent pathway in response to IL-1, leading to the activation of genes involved in innate antiviral responses (12). In this study, we compared and contrasted the induction of antiviral response genes in human fetal astrocytes following stimulation with IL-1 or pIC. We show, for the first time, that pIC but not IL-1, induces antiviral activity in astrocytes, and that viperin/cig5 participates in this response.

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⁴ Abbreviations used in this paper: IRF3, IFN regulatory factor 3; pIC, polyriboinosinic polyribocytidylic acid; siRNA, small interfering RNA; Q-PCR, quantitative PCR; A.U., arbitrary unit; iNOS, inducible NO synthase; IHC, immunohistochemistry; OAS, oligoadenylate synthetase; CHX, cycloheximide; TTX-100, Triton X-100; VSV, vesicular stomatitis virus; NMMA, *N*-monomethyl L-arginine; PKR, dsRNA-dependent protein kinase; 2-AP, 2-aminopurine; WNV, West Nile virus; ISG, IFN-stimulated gene.

Materials and Methods

Astrocyte cell culture and cytokines

Enriched human fetal brain astrocyte cultures were established from second trimester abortuses, as described previously (13). All tissue collection was approved by the Institutional Clinical Review Committee, Albert Einstein College of Medicine. rIL-1 was purchased from PeproTech, and diluted in medium containing 2 mg/ml endotoxin-free human serum albumin (Baxter Healthcare). Polyinosinic-cytidylic acid (Sigma-Aldrich) was diluted in endotoxin-free sterile PBS and used at 10 or 50 μ g/ml.

Human cDNA 28k microarrays

Three separate cases of human fetal astrocytes plated at 2×10^6 cells/dish were stimulated with 10 μ g/ml pIC or 10 ng/ml IL-1, respectively, for 6 or 24 h in DMEM containing 10% FCS. Cell harvest, RNA isolation, and microarrays obtained from the Albert Einstein College of Medicine cDNA Microarray Facility (relevant data available from <http://microarray1k.aecom.yu.edu/>) were performed as described previously (12). In brief, each slide contained an unbiased, random collection of 28k cDNA probe elements derived from the sequence-verified GEM1 clone set (Incyte Genomics). First-strand cDNAs were synthesized, labeled with Cy5 or Cy3 using a dendrimer method (3DNA Submicro; Genisphere), and hybridized to cDNA microarrays. Independent measurements of Cy5 and Cy3 signal intensity and background were generated for each cDNA element using a Genepix 4000B scanner (Axon Instruments) and Scanalyze software (<http://rana.lbl.gov/EisenSoftware.htm>). Cutoff values were established as Cy5:3 ratio >2.0 or <0.5 , and as net signal intensity more than three times background in one or both channels. All elements not satisfying both criteria were discarded. Controls included reversing the fluorochromes and labeling the same sample using both Cy3 and Cy5.

Immunocytochemistry

Astrocytes were plated on glass-bottomed microwell dishes (MatTek), treated with pIC for the indicated times, fixed with 4% freshly prepared paraformaldehyde for 20 min at room temperature, washed and blocked with 10% normal goat serum in 0.1% Triton X-100 (TTX-100) in PBS for 1 h at 25°C, and then incubated overnight at 4°C with rabbit anti-human viperin (14) diluted 1/200 in blocking solution. Cells were washed with 0.01% PBS-Tween 20 and incubated with goat anti-rabbit Alexa 488 (Molecular Probes) diluted 1/200 in PBS containing 2% BSA and 0.1% TTX-100. Cultures were washed in 0.01% PBS-Tween 20, nuclei were visualized with 4',6-diamidino-2-phenylindole, dihydrochloride (Molecular Probes), and mounted with aqueous mounting medium (Biomed). Control cultures were prepared by omitting the primary Ab.

Vesicular stomatitis virus (VSV) g-env-HIV infection of astrocytes and detection of HIV p55/24 gag expression

VSVg env-HIV was produced by cotransfecting 293T cell with VSVg env and pNL4-3 plasmids obtained from the National Institutes of Health AIDS Repository. Culture supernatant containing infectious virus was pretitrated and added to astrocyte cultures to produce $\sim 25\%$ p24⁺ cells. HIV-1 and pIC (or IL-1) were added to astrocyte cultures grown in DMEM with 5% FCS and antibiotics for 24 h. Input virus was washed out and pIC (or IL-1) was added back. Two to 3 days later, cultures were fixed with methanol for HIV-1 p24 immunocytochemistry to determine the number of productively infected cells as described (15). HIV p24 expression was also assessed by reading the immunoreactivity developed with NBT by an ELISA reader at 595 nm with the uninfected cell p24 levels as blanks. For immunoblotting, SDS-PAGE with 10% polyacrylamide gel was used and blots were probed with anti-HIV p24 (IgG1; DakoCytomation) that recognizes several forms of HIV gag proteins including p55 (16). Densitometry was performed using total-ERK kinase or vinculin as the protein loading control.

Viperin/cig5 small interfering RNA (siRNA) knockdown

Primary fetal astrocytes were plated in 10-mm dishes at a cell density of 1×10^5 in complete medium for 24 h. Cells were stimulated with 10 μ g/ml pIC for 16 h before transfection with 10 nM nontargeting or viperin/cig5 siRNA (Dharmacon). Transfection was conducted using transit-TKO (Mirus) per the manufacturer's instructions. Cells were incubated for 48 h posttransfection, infected with VSVg-env-HIV-1 for 48 h, and analyzed by immunoblotting.

Real-time quantitative PCR (Q-PCR)

Q-PCR was performed using the SYBR Green method, as described previously, in 384-well PCR plates (12). Plates were processed in an ABI

Prism 7000 light cycler (Applied Biosystems) using standard cycling conditions. All runs were accompanied by two internal control genes *GAPDH* and porphobilinogen deaminase (*PBDA*). Samples were normalized using a δ cycle threshold-based algorithm to give arbitrary units (A.U.) representing a ratio of experimental to control. IFN- β (forward: cagcagttccagaaggagga, reverse: agtctcattccagccagctgc); *PBDA* (forward: acgatcccgagactctgcttc, reverse: gcacggctactggcaccact); *GAPDH* (forward: tgcaccacc aactgcttagc, reverse: ggcattggactgtggctatgag); viperin (forward: tcaaaagctgaggaggtgt, reverse: gttccgctatgctctctcc).

Western blotting

Cell lysates were resolved using SDS-PAGE, and immunoblotted as described previously (12). Primary Abs included: anti-phospho-Stat1 Y701, 1/1000, total Stat1, 1/1000, (Cell Signaling); anti-p24gag, 1/500 (Dako-Cytomation); anti-inducible NO synthase (iNOS), 1/500 (Santa Cruz Biotechnology), and rabbit anti-viperin/cig5, 1/2000. Membranes were incubated for 1 h with goat anti-rabbit or mouse HRP-linked secondary Abs (1/2000; Santa Cruz Biotechnology) in blocking buffer. Blots were washed and developed using the SuperSignal West Pico chemiluminescence ECL kit (Pierce). Blots were stripped and immunoreacted for vinculin or β -tubulin as loading controls and quantified using Scion NIH image software.

Results

To obtain an overall picture of gene expression induced in astrocytes in the presence of pIC, and to compare these data sets with astrocytes activated with IL-1, we performed a series of cDNA microarrays using a human 28K chip from cells that had been activated for 6 or 24 h with either 10 μ g/ml pIC or 10 ng/ml IL-1. The results showed that both pIC and IL-1 are potent activators of human fetal astrocytes in culture, but that pIC leads to a more robust response than IL-1 (Table I). They further showed that pIC induced a marked bias toward genes known for their role in host defense against viruses. As expected, and in agreement with previously published data (4, 17), pIC also induced a wide array of chemokines and cytokines characteristic of TLR activation.

The production of type I IFNs is considered a hallmark of the antiviral response and thus we sought to validate the results of the microarray data using Q-PCR for IFN- β . To control for the specificity of the reaction, we also tested the inactive analog poly(dI:dC). The data showed that pIC induced IFN- β mRNA in astrocytes in a dose- and time-dependent manner, whereas no response was detected in cells treated with poly(dI:dC) (Fig. 1A). Based on the recently elucidated crystal structure of TLR3 (18) and the fact that the receptor is largely expressed on intracellular vesicles (data not shown), it has been proposed that a low pH environment is required for the receptor to recognize, bind, and signal in response to ligand (19, 20). Therefore, we next attempted to inhibit TLR-3 signaling in our cells using the base acridine orange. Pretreatment of cells with acridine orange followed by a 6-h treatment with pIC completely blocked astrocyte expression of IFN- β (Fig. 1B). These data are consistent with localization of TLR3 to an acidic endosomal compartment in astrocytes.

It has now been well-documented that TLR expression is modulated by the activation and maturation state of the cell (4, 9). Consistent with this, the microarray data showed that activation of astrocytes with either IL-1 or pIC resulted in increased expression of TLR2 and TLR3, but not TLR4 (Table I). To investigate whether increased levels of TLR3 on astrocytes would result in increased signaling, primary cultures were pretreated for 24 h with IL-1, restimulated with pIC for 3, 6, or 24 h, and expression was assessed by Q-PCR for IFN- β . Cells restimulated with medium alone, or cells that had not been pretreated with IL-1 were tested in parallel. The data revealed that in IL-1-pretreated cells, expression levels of IFN- β were greatly enhanced over the levels observed in cells that had not been preactivated (Fig. 1C). Interestingly, the pattern of response did not change, showing a rapid

Table I. *Microarray analysis of poly(I:C) or IL-1 activated astrocytes*

Accession Number	Gene Name	6 h pI:C ^a	6 h IL-1	24 h pI:C	24 h IL-1
NM001548	<i>IFIT-1</i>	167	35	220	22
NM001547	<i>IFIT-2</i>	13	21	138	22
NM080657	<i>Viperin/cig5</i>	125	41	214	134
NM002462	<i>Mx1</i>	87	14	174	45
NM002201	<i>ISG-20</i>	33	17	78	94
NM002053	<i>GBP1</i>	19	12	25	11
NM001572	<i>IRF7</i>	14	6	41	23
NM016816	<i>2,5 OAS-1</i>	24	N/D	46	N/D
NM016817	<i>2,5 OAS-2</i>	6	N/D	17	12
AY302136	<i>PKR</i>	6	2	10	5
NM001511	<i>CXCL1/GROα</i>	10	170	133	160
NM000584	<i>IL-8/CXCL8</i>	20	197	326	217
NM002985	<i>CCL5/RANTES</i>	1.5	2	50	2
NM000600	<i>IL-6</i>	4	64	88	186
NM003264	<i>TLR2</i>	1	2	7	17
AL570789	<i>TLR3</i>	15	2.6	21	7
AF177765	<i>TLR4</i>	1	1	1	1

^a Data are expressed as fold increase over controls.

One of three independent experiments is shown. N/D, Not detected.

(within 3 h) up-regulation followed by an equally rapid down-regulation of gene expression. These data show that pretreatment with IL-1 resulted in increased expression of TLR3 that had functional consequences.

One of the most highly up-regulated genes noted by microarray in the dsRNA-treated astrocytes was viperin/cig5 (Table I). To validate this observation, we studied viperin expression in astrocytes using a combination of Q-PCR, immunoblotting, and immunohistochemistry (IHC). Viperin mRNA and protein levels were elevated rapidly in response to dsRNA with maximal expression occurring around 3 and 6 h poststimulation (Fig. 2A). IHC showed that viperin/cig5 was localized to the endoplasmic reticulum and to budding vesicles associated with the Golgi complex (Fig. 2B), as has been described previously (14). To determine the effect of restimulation with ligand on viperin expression, cells were treated daily with IL-1 or pIC and viperin expression was studied by immunoblotting. Consistent with the microarray data, pIC more potently induced viperin than IL-1 (Fig. 2C). Furthermore, repeated stimulation with pIC resulted in increased expression of viperin whereas IL-1 did not.

In many cell types, the induction of antiviral proteins such as the type I IFNs by ligands for TLR3 has been shown to require activation of IRF3 (6), and components of the NF- κ B-signaling pathway. Thus, we next sought to determine whether these signaling pathways were required for pIC-induced production of viperin/cig5 in astrocytes. To do this, astrocytes were infected with adenoviral vectors containing either a proteolysis-resistant I κ B α superrepressor (S32, S36E, AdI κ B α (21)), or an IRF3 construct in which the N-terminal DNA-binding domain had been deleted, which functions as a dominant-negative form of IRF3 (Δ IRF3) (12, 22). Cells were incubated for 48 h with three different concentrations (1, 3, and 10 \times 10³ particles/ml) of each of the adenoviral constructs, along with appropriate controls, activated with pIC at 10 μ g/ml for 24 h, and levels of viperin/cig5 were determined by Western blotting (Fig. 3). The data showed that both the I κ B α super repressor and the Δ IRF3 constructs dose-dependently blocked pIC induction of viperin/cig5, whereas empty vector controls were without effect. Two bands were detected by SDS-PAGE in our IRF3 and I κ B α dominant-negative studies, with the top band migrating at the predicted molecular mass of 43 kDa and the lower band resolving at 35 kDa. As postulated by previous studies, the lower band is likely a proteolysis product or a cell case-specific

phenomenon (14). In contrast, inhibitors for components of the MAPK, p38, or JNK pathways were without effect (data not shown).

In cells of the monocyte/macrophage series viperin is induced by IFNs, with IFN- β providing the most potent stimulus (14, 23). Because pIC induced IFN- β mRNA in human fetal astrocytes, we then tested whether viperin was a direct target of pIC activation of astrocytes, or resulted from autocrine/paracrine signaling by IFN- β . To do this, neutralizing or nonneutralizing Abs to IFN- β derived from multiple sclerosis patients receiving IFN- β therapy (a gift from Dr. J. Oger, Multiple Sclerosis Clinic, University of British Columbia, Vancouver, Canada) were tested. The data showed that neutralizing Abs, but not nonneutralizing Abs, to IFN- β inhibited expression of viperin at 24 h. To confirm that these Abs did, or did not, inhibit autocrine signaling via IFN- β , the blots were stripped and probed for expression of phospho-STAT1 (Fig. 4A), which showed loss of STAT1 phosphorylation in cultures treated with neutralizing but not nonneutralizing Abs to IFN- β . However, the low-level detection of viperin at 6 h post-pIC activation in cells treated with the neutralizing Ab suggested that some induction of viperin may represent a direct activation of the gene by pIC. To test for this, cells were first treated with cycloheximide (CHX) then with pIC and viperin expression was determined by Q-PCR. A marked reduction in viperin expression was noted at 6 h, indicating that de novo protein expression was required for pIC induction of viperin (Fig. 4B). These results strongly implicate IFN- β signaling via an autocrine/paracrine pathway as the major mediator of viperin induction in astrocytes.

The high level induction by pIC of antiviral proteins in astrocytes raised the question as to whether this response induced an antiviral state in these cells. Astrocytes are generally considered to be resistant to productive infection by HIV-1, but can be readily infected with VSV-G *env*-pseudotyped HIV (VSVg *env* HIV-1) through an endocytic pathway, resulting in a single round of HIV-1 replication (24). As such, this system permitted us to determine whether pIC activation resulted in inhibition of intracellular viral replication. Because we have shown previously that IL-1 can also activate a host of antiviral genes (12), astrocytes were activated with IL-1 in parallel. Cells were cotreated with HIV-1 and pIC or IL-1 for 24 h, followed by a complete medium change and read-dition of pIC or IL-1. Cultures were examined by IHC or by Western blot analysis after 3 additional days of infection with an Ab to

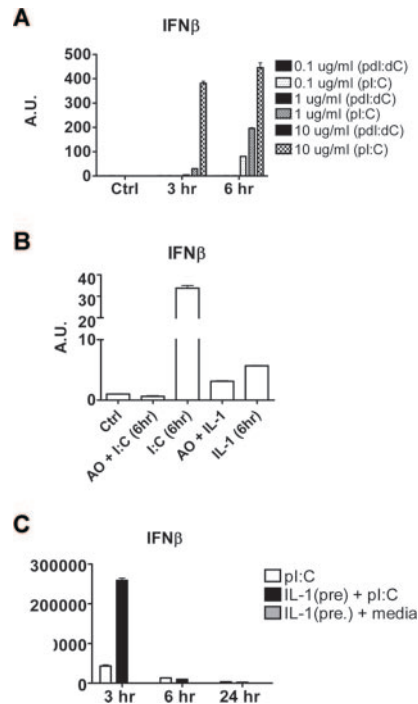


FIGURE 1. Responsiveness to the TLR3 ligand pIC in astrocytes is dose dependent and potentiated in IL-1-activated cells. *A*, Astrocytes stimulated for 3, 6, or 24 h with increasing dosages of pIC (0.1, 1, 10 $\mu\text{g}/\text{ml}$) were harvested for total RNA, and Q-PCR performed for IFN- β . Poly(dI: dC) was used as a control for receptor specificity. *B*, Astrocytes were treated for 30 min with 10 μM acridine orange (AO), activated with 10 $\mu\text{g}/\text{ml}$ pIC or 10 ng/ml IL-1 for 6 h, and Q-PCR was performed for IFN- β . *C*, Astrocytes were stimulated with IL-1 (10 ng/ml) or medium for 24 h and then stimulated for 3, 6, or 24 h with pIC (10 $\mu\text{g}/\text{ml}$) or medium alone. Total RNA was isolated and Q-PCR for IFN- β performed. Data are representative of three or more independent experiments and presented as the normalized fold increase over controls (A.U.).

HIV-1 p24 *gag* protein. IHC for p24 (Fig. 5A) showed scattered productively infected astrocytes in HIV-1-exposed cultures only. The number of p24⁺ cells was dramatically reduced in pIC-treated cultures, but not in IL-1-treated cultures. This observation was further supported by Western blot data. Results from two different cases are shown (Fig. 5B).

To investigate the mechanism of pIC-induced antiviral effect in astrocytes, we examined the dsRNA-dependent protein kinase (PKR) inhibitor, 2-aminopurine (2-AP), the NOS inhibitor *N*-monomethyl *L*-arginine (NMMA), and a neutralizing Ab to IFN- β . All reagents were added simultaneously with HIV-1 and results were evaluated using a cell-based ELISA for HIV-1 p24 after 60 h (Fig. 5C). The results again showed that pIC significantly reduced p24 expression whereas IL-1 did not. This reduction was completely reversed by 2-AP, but not NMMA. In addition, a rabbit neutralizing Ab to IFN- β substantially reversed the pIC effect, whereas a control serum did not.

Finally, we sought to investigate a possible role for viperin in the pIC-induced inhibition of viral replication using a siRNA approach. Preliminary experiments showed that transient transfection of astrocytes with viperin-targeted siRNA at 10 nM inhibited viperin expression whereas a scrambled siRNA was without effect (Fig. 6A). To determine whether the knockdown of viperin reversed the inhibitory effects of pIC for VSVg-env-HIV replication, cells were activated with pIC before viperin siRNA transfection and chimeric HIV infection. Cell homogenates were prepared and subjected to immunoblotting and densitometry for HIVp24gag, vi-

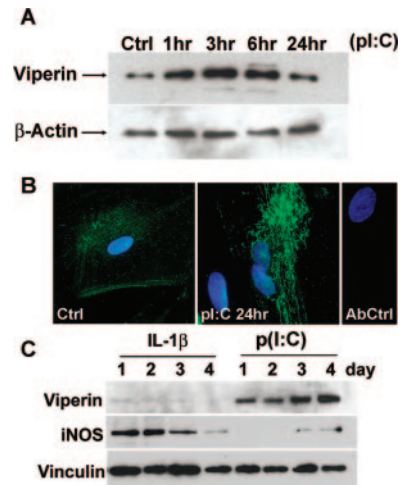


FIGURE 2. Poly(I:C) up-regulates the antiviral protein viperin/cig5. *A*, Astrocytes were stimulated for the times indicated with 10 $\mu\text{g}/\text{ml}$ pIC, total cell lysates were isolated, resolved using 10% SDS-PAGE, and immunoblotted for viperin. Blots were stripped and reprobed for β -actin as a loading control. *B*, Astrocytes were activated with 10 $\mu\text{g}/\text{ml}$ pIC for 24 h, fixed with 4% paraformaldehyde, blocked, and permeabilized in 10% goat serum and 0.01% TTX-100 in PBS, and stained overnight using a polyclonal Ab raised against viperin (1/200). Cultures were washed, immunoreacted with an Alexa-488 conjugated goat anti-rabbit secondary Ab (1/500), followed by staining with 4',6'-diamidino-2-phenylindole. Primary Ab was omitted as a negative control. Cells were viewed and imaged using a cooled CCD camera. *C*, Astrocytes were stimulated daily with 10 ng/ml IL-1 or 10 $\mu\text{g}/\text{ml}$ pIC for 4 days, total protein was isolated, resolved using SDS-PAGE, and immunoblotted for viperin, iNOS, and vinculin as a loading control.

perin and vinculin as a loading control. The targeting siRNA, but not the nontargeting siRNA, again led to reduced expression of viperin, and also substantially reversed the pIC induced down-regulation of p24gag (Fig. 6, *B* and *C*). These data indicate that viperin expression forms part of the antiviral response induced by pIC in astrocytes.

Discussion

In this study, we characterized the response to the TLR3 ligand pIC in human fetal astrocytes in culture, and the possible role that secondary factors induced through TLR3 signaling might have on astrocytic viral interactions. Using microarray technology, we found, in agreement with others (4, 10, 17), that pIC induced a wide range of cytokines and chemokines characteristic of an innate immune response, but we also noted a marked bias toward genes characterized for their role in antiviral responses when results were compared with similar data sets produced in response to the cytokine IL-1 (Table I).

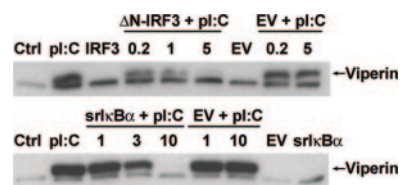


FIGURE 3. Poly(I:C)-induced expression of viperin is dependent on the NF- κ B and IRF-3 pathways. Primary astrocytes were infected for 48 h with increasing dosages of an adenoviral vector expressing a dominant-negative IRF3 (Δ IRF3), an $\text{I}\kappa\text{B}\alpha$ superrepressor (sr $\text{I}\kappa\text{B}\alpha$), or an empty vector (EV). Cells were then stimulated with 10 $\mu\text{g}/\text{ml}$ pIC for 24 h, total cell homogenates were isolated and immunoblotted for viperin.

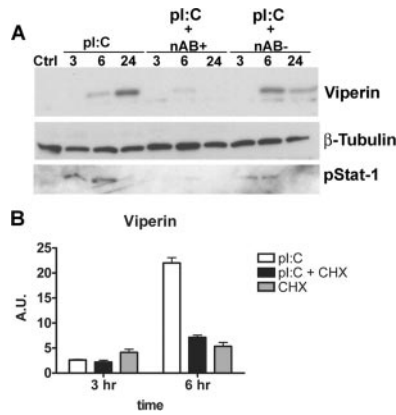


FIGURE 4. Viperin expression is secondary to primary induction of IFN- β . *A*, Astrocytes were pretreated for 30 min with neutralizing and nonneutralizing Abs to IFN- β (1/20), stimulated for 3, 6, or 24 h with pI:C (10 μ g/ml), and immunoblotted for viperin and β -tubulin as a loading control. Immunoblots were stripped and reprobed for pStat-1 (Y701). Results reflect the use of two independent neutralization Abs. *B*, Astrocytes were pretreated for 30 min with 10 μ M CHX, activated for 6 h with 10 μ g/ml pI:C, total RNA were isolated, and Q-PCR was performed for IFN- β . CHX treatment of nonactivated astrocytes was performed as a control.

The only known natural ligand for TLR3 is dsRNA, which is found in some RNA viruses such as reovirus. However, it has also been proposed that TLR3 may be activated by dsRNA intermediates that form as a consequence of viral replication, indicating that TLR3 may function as a universal receptor for activation of the innate immune response to viral infections (25). The localization of TLR3 to endosomal compartments and the bias toward antiviral response genes noted following activation would be consistent with such a role for TLR3. It is also possible that TLR3 may be activated by cellular RNA released as a result of tissue destruction

caused by inflammatory and immune-mediated events occurring within the CNS (26).

Using Q-PCR, we showed that pI:C induced a robust up-regulation of genes known to require the transcription factors NF- κ B and IRF3. In agreement with others (4, 8–10), we also showed that activation with either pI:C or IL-1 led to increased mRNA for TLR3 in astrocytes, and further demonstrated that this up-regulation was functionally active by studying expression of mRNA for IFN- β and IL-8. The rapid and robust expression of chemokines and cytokines in astrocytes in response to pI:C would implicate this receptor in the induction of inflammatory/immune responses to virus infection. Consistent with this are the studies in mice in which the gene for TLR3 has been inactivated (TLR3^{-/-} mice). These data show that following infection with West Nile virus (WNV) TLR3 plays a critical role in facilitating viral entry into the CNS resulting in a lethal encephalitis in mice through the formation of a TNF-dependent inflammatory response, loss of blood-brain barrier function and access of inflammatory cells to the CNS (27). Thus, in this model, loss of TLR3 resulted in impaired cytokine production and was protective against inflammation associated with viral infection, even though viral burden in the periphery was significantly elevated. These data indicate that viral pathogenesis in this model is mediated by the inflammatory response in the brain (27). TLR3 deletion has also been found to alter the immune environment in the lung following infection with respiratory syncytial virus (28). In TLR3^{-/-} mice, viral growth was unaffected but lung pathology was more severe, an effect linked to an increase in Th2-type responses in the TLR3^{-/-} mice, thus reflecting a role for TLR3 in promoting Th1-type immune responses. A similar role for TLR has been noted following activation of dendritic cells (29).

However, this clear involvement of TLR3 in inflammatory/immune-mediated events contrasts with results from studies that have examined the effect of TLR3 deletion on viral clearance. Thus, in the TLR3^{-/-} mice no effect on viral pathogenesis was noted for

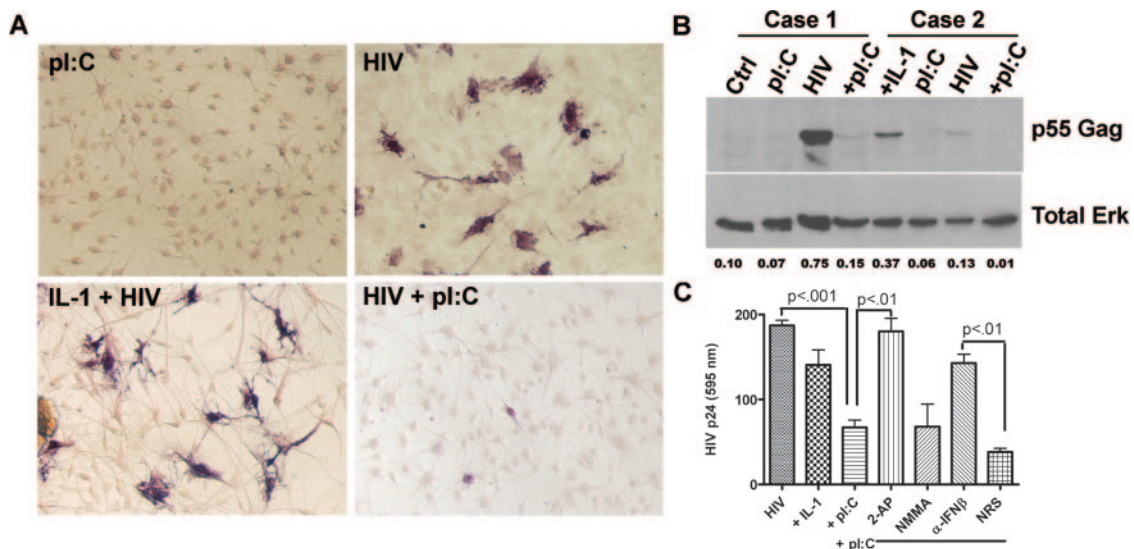


FIGURE 5. Poly(I:C) blocks VSVg-env-HIV-1 replication in human primary fetal astrocytes in culture. *A*, Astrocytes cultured in 96-well plates were infected with VSVg-env pseudotyped HIV-1 and stimulated with 10 μ g/ml pI:C or 10 ng/ml IL-1 for 3 days, as described in *Materials and Methods*. Cells were fixed with methanol and stained for p24 immunoreactivity. Poly(I:C)-stimulated and HIV-infected cells alone were used as positive and negative controls. *B*, Experiments were performed on two separate cases of human fetal astrocytes as described above. Cell homogenates were prepared and samples immunoblotted for p25/p55 gag, stripped, and reprobed for total ERK as a loading control followed by densitometric analysis. *C*, Astrocytes were infected with VSVg-env pseudotyped HIV-1 and treated with either 10 ng/ml IL-1 or 10 μ g/ml pI:C. Samples were also cotreated with either pI:C, 2-AP (5 mM), NMMA (100 μ M), a polyclonal neutralizing Ab to IFN- β (anti-IFN- β , 6×10^3 U/ml), or normal rabbit serum (NRS). Cells were stained for p24 and immunoreactivity was assessed at 595 nm. Significant *p* values were based on an *n* = 3 using one-way ANOVA.

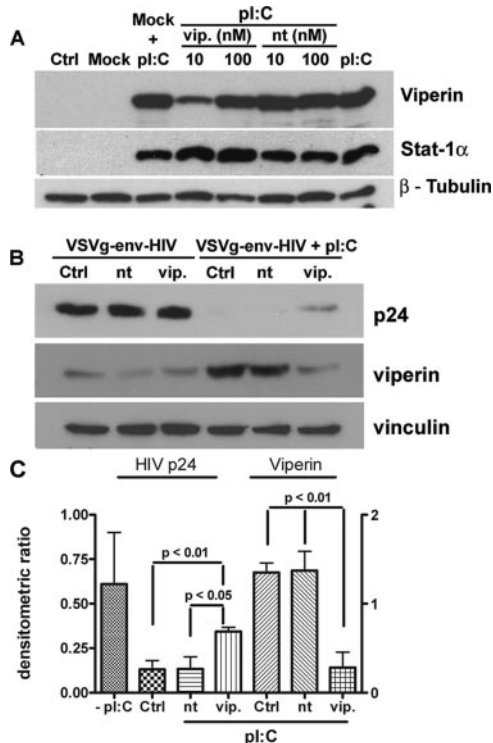


FIGURE 6. RNAi-mediated knockdown of viperin/cig5 partially reverses the pIC induced inhibition of pseudotyped HIV-1 replication in primary astrocytes. *A*, Astrocyte cultures were transfected with 10 nM or 100 nM of nontargeting or targeting viperin siRNA for 48 h as described in *Materials and Methods*. Cells were then stimulated for 24 h with 10 μ g/ml pIC. Cell homogenates were isolated, resolved using SDS-PAGE, and immunoblotted for viperin and total Stat-1 α . Blots were stripped and reprobed for β -tubulin to assess equivalence of loading. *B*, Astrocyte cultures were stimulated with 10 μ g/ml pIC, transfected with 10 nM of either viperin siRNA or nontargeting siRNA, and then infected with VSVg-env pseudotyped HIV-1 alone or VSVg-env pseudotyped HIV-1 together with pIC stimulation for 48 h. Cell homogenates were prepared and immunoblotted for viperin and p24, stripped, and reprobed for vinculin as a loading control. *C*, Densitometric analysis was performed on the Western blots described in *B*. The unpaired Student *t* test was used to determine significant *p* values.

lymphocytic choriomeningitis virus, VSV, murine CMV, or reovirus (30). Furthermore, no effect was detected in the formation of an adaptive immune response, even when mice were challenged via different routes of inoculation and different doses. It is important to note, however, that most pathogenic viruses have evolved multiple mechanisms to evade the innate immune response. So, for example, WNV interferes with pIC-induced activation of IRF3 (31), and the susceptibility of laboratory mice to WNV, as opposed to wild mice that are resistant, is linked to a polymorphism within the 2'5' oligoadenylate synthetase (OAS) family of IFN-stimulated genes (ISG) (32). A similar occurrence of polymorphisms within the 2'5' OAS genes has been noted in patients hospitalized with WNV (33). The NS3/4A protein complex of hepatitis C virus also blocks phosphorylation of IRF3 (34).

Among the ISG, the antiviral activity of 2'5' OAS has been well-characterized (35, 36), however, the complete spectrum of ISG with potential antiviral activity remains to be defined. In this context, it is of interest to note that the microarray analysis of our pIC-activated astrocytes identified viperin/cig5 as one of the most highly expressed genes. Viperin/cig5 was first cloned from human CMV-infected fibroblasts (37), and subsequently identified as an IFN- γ -induced gene in primary macrophages (14). However, in

other cell types IFN- α /IFN- β , rather than IFN- γ , proved to be the more effective stimulus. Stable expression of the protein inhibited human CMV production in fibroblasts (14). Viperin/cig5 is also highly expressed in liver tissue from patients chronically infected with hepatitis C virus, and again stable transfection of the protein was able to significantly decrease hepatitis C virus replication in a replicon model (23). Using neutralizing Abs to IFN- β , our data strongly support the conclusion that in astrocytes viperin is predominantly a type 1 ISG. However, a small subset of genes that are directly activated by IRF3 have been identified (38). To determine whether viperin/cig5 represented such a gene in our system, we pretreated cells with CHX. The data indicated that viperin/cig5 is predominantly a secondary response gene in this system, most likely induced by IFN- β .

We then sought to determine whether pIC-induced ISG contributed to an antiviral response in astrocytes. To do this, we used a VSVg-env pseudotyped single-cycle HIV-1 (24). It is well-known that HIV-1 infectivity of astrocytes is low, with minimal production of viable particles (39, 40). In contrast, the VSVg-env-mediated entry is efficient resulting in a high percentage of infectivity in our astrocytes permitting us to directly address intracellular antiviral mechanisms. These data showed that pIC markedly down-regulated HIV-1 p24 expression, an effect that could be reversed by neutralizing Abs to IFN- β and the PKR inhibitor 2-AP, suggesting that in astrocytes, PKR activation is upstream of pIC-induced IRF3 activation. To determine whether viperin also contributed to the antiviral effects of pIC as a downstream target of IFN- β , we used a siRNA approach. This viperin-targeted siRNA showed a reversal of pIC-induced p24 inhibition, as well as pIC-induced viperin expression. Therefore, our findings that viperin knockdown partially reversed p24 inhibition further supports the idea that viperin is a component of the ISG that is involved during antiviral responses mounted in the CNS. From these results, we conclude that activation of astrocytes with pIC induces a potent antiviral effect which, in combination with the induction of proinflammatory genes, indicates that TLR3 expression by astrocytes could play a major role in initiating immune responses to a wide range of infectious agents in the CNS.

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Disclosures

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