MAJOR ARTICLE

Induction of Innate Immune Responses by SIV In Vivo and In Vitro: Differential Expression and Function of RIG-I and MDA5

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Interferon-β induction occurs during acute simian immunodeficiency virus (SIV) infection in the brain. We have examined expression and function of cytosolic RNA sensors, retinoic acid inducible gene I (RIG-I), and melanoma differentiation-associated protein 5 (MDA5), in vivo in the brain of our consistent, accelerated SIV-macaque model and in vitro in SIV-infected macaque macrophages to identify the pathway of type I interferon (IFN) induction. MDA5 messenger RNA (mRNA) and protein were expressed at higher levels in the brain than RIG-I, with protein expression correlating with the severity of disease from 42 until 84 days post-inoculation. The siRNA experiments reveal that mRNA expression of IFN-inducible gene MxA is dependent on MDA5, but not RIG-I. Finally, we demonstrate that SIV infection leads to the production of double-stranded RNA in vivo, which may act as the MDA5 ligand. We have shown for the first time to our knowledge the functional role of MDA5 in the innate immune response to SIV infection.

Although human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) cross the blood-brain barrier and establish central nervous system (CNS) infection early during acute infection, HIV-associated neurological complications usually only occur during late stage disease [1–5]. This delay between CNS infection and disease is partly due to the antiviral effects of type I interferon (IFN) β, a hallmark of virus infection [6–8]. Our consistent, accelerated SIV macaque model of HIV-associated neurological disease has been important in elucidating the role of viral and host factors in the pathogenesis of HIV infection in the CNS [9–14]. We have characterized the early infection of the CNS and demonstrated that the brain is infected by 4 days postinfection and shown that innate immune responses, particularly IFN-β and the type I IFN inducible gene MxA, are induced at this time in macrophages and microglial cells [10]. We have previously demonstrated that control of virus replication in macrophages and in brain is due, in part, to the induction of the IFN-β–induced dominant-negative isoform of the cellular transcription factor CCAAT/enhancer-binding protein beta (C/EBPβ). This isoform of C/EBPβ downregulates the transcription of SIV and HIV in macrophages in vitro, and in the brain and lungs of SIV-infected macaques in vivo [4, 15]. The pathway that is responsible for the induction of IFN-β by either SIV or HIV in macrophages or in the brain has not been identified.

The 2 major pathways for virus detection in the cell are differentiated mainly by subcellular localization of the receptors—*endosomal* Toll-like receptors (TLRs) or *cytosolic* RNA sensors—both of which trigger downstream innate immune responses. RIG-I and MDA5 are cytosolic RNA helicases that bind to ssRNA with 5′-triphosphates (RIG-I) or dsRNA (RIG-I and MDA5)

Received 2 March 2011; accepted 19 May 2011.

Potential conflicts of interest: none reported.

Presented in part: American Society for Virology Meeting, July 2009, Vancouver, British Columbia and International Society for Neurovirology Meeting, October 2010, Milan, Italy.

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The Journal of Infectious Diseases 2011;204:1104–14

0022-1899 (print)/1537-6613 (online)/2011/2047-0018$14.00

DOI: 10.1093/infdis/jir469

and function to enhance the detection of virus infections [16–19]. The 5′-triphosphates, a signature product of viral polymerase, and dsRNA are both non-self ligands, and the presence of either molecule is an indicator of ongoing viral infection. RIG-I and MDA5 signal through a mitochondria-bound adapter protein, IFN-β promoter stimulator 1 (IPS-1), ultimately activating an IRF-3-dependent type I IFN expression [20]. IFN in turn induces the expression of increased levels of RIG-I and MDA5 in a positive feedback loop. Although both RIG-I and MDA5 are IFN-stimulated genes (ISGs), some viruses are known to employ unique mechanisms to antagonize innate immune cellular defenses [21]. The roles of RIG-I and MDA5 in the context of SIV infection have not yet been investigated using infection experiments in naturally permissive cells such as macrophages.

Using our SIV macaque model of AIDS and HIV encephalitis, we examined expression of RIG-I and MDA5 mRNAs and proteins and report for the first time the induction of RIG-I and MDA5 mRNA and protein with different expression patterns in the brains of SIV-infected macaques. Additionally, gene silencing experiments using siRNA in SIV-infected macaque macrophages demonstrated that MDA5, but not RIG-I, contributed to the induction of IFN-β together with the endosomal TLR pathway.

MATERIALS AND METHODS

Animal Experiments and Viruses

Fifty-three pigtailed macaques (*Macaca nemestrina*) were intravenously inoculated with SIV/DeltaB670 ($50AID_{50}$) and SIV/17E-Fr (10 000 AID_{50}) and sacrificed at 4, 7, 10, 14, 21, 42, 56, and 84 days postinoculation as described elsewhere [12]. Procedural controls were sacrificed at 84 days post–mock inoculation. Many of these animals have been included in previous publications [10, 22]. All animal protocols in this study were approved by the Johns Hopkins University Institutional Animal Care and Use Committee in accordance with Animal Welfare Act regulations and the USPHS Policy on Humane Care and Use of Laboratory Animals.

Histopathology

Sections of CNS tissue were examined in a blinded fashion by microscope and the degree of encephalitis severity scored using a semiquantitative system as described elsewhere [11].

Antibodies

Antibodies to RIG-I (Alexis Biochemicals), MDA5 (Alexis Biochemicals), CD68 (DAKO), glial fibrillary acidic protein (GFAP) (DAKO), β3-tubulin (Promega), β-actin (Sigma), and SIV mac251 gp41 (NIH AIDS Research and Reference Reagent Program) were used. Double-stranded RNA-specific monoclonal antibody J2 (English & Scientific Consulting) recognizes the A-helix structure of dsRNA molecules at least 40bp long in a non–sequence-specific manner and do not crossreact with ssRNA, tRNA, rRNA, mRNA, or RNA-DNA hybrids [23].

Immunohistochemical Analysis

Paraffin-embedded tissue sections of macaque basal ganglia were processed, probed, photographed, and analyzed as described elsewhere [11]. For fluorescent images, iVision (BioVision Technologies) software was used.

Cell Culture

Blood from adult pigtailed macaques was processed using a Percoll gradient protocol, and PBMCs were maintained in RPMI 1640 (Invitrogen) supplemented with 20% (non-autologous) pigtail serum, 200ng/mL M-CSF (R&D Systems), 10 mmol/L HEPES (Invitrogen), 2 mmol/L L-glutamine (Invitrogen), and 0.5 mg/mL gentamicin (Invitrogen) (MDM20) for 7 days until differentiation into monocyte-derived macrophages (MDM). Thereafter, cells were maintained in RPMI with 10% pigtail serum (MDM10).

Gene Silencing of RIG-I and MDA5

Cells were treated with 100 nmol/L of RIG-I–specific (5′-CUGAAUAUACUGCACCUCUTT-3′), MDA5-specifc (5′-UAUCAUUCGAAUUGUGUCATT-3′), or a nonspecific siRNA control (5′-UUCUCCGAACGUGUCACGUTT-3′) (Integrated DNA Technologies) using Hiperfect Transfection Reagent (Qiagen) according to the manufacturer's protocol. Cells were transfected 24 hours prior to infection. Uridines had 2′-O-methyl modifications to minimize induction of IFN and ISGs as a result of transfection [24].

Chloroquine Treatment

Primary macaque macrophages were pretreated with 50 umol/L of the endocytosis inhibitor chloroquine (Invivogen) 30 minutes before infection. Cells were maintained in the presence of chloroquine throughout the experiment until the indicated time points.

SIV Infection of Primary Macrophages

Mock- or siRNA-transfected primary macrophages were infected with SIV/17EFr [25] at a multiplicity of infection of 0.05 for 6 hours at 37°C. After infection, cells were washed 5 times with phosphate-buffered saline (PBS) and cultured in MDM10 until collection of samples at various time points.

RNA Extraction and qRT-PCR Analysis

Total RNA from basal ganglia tissue, as well as infected and/or transfected cells was extracted, purified, and run in quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) conditions as described elsewhere [10]. Specifc primer and probe sets to RIG-I (Fwd:5′-GTGCAAAGCCTTGGCATGT-3′,

Rev:5′-TGGCTTGGGATGTGGTCTACTC-3′, Probe:5′-AGAG GAATGCCATTACACTGTGCTTGG-3′), MDA5 (Fwd:5′-GTT TGGCAGAAGGAAGTGTC-3′, Rev:5′-GCTCTTGCTGCCA-CATTCTC-3′, Probe:5′-AACAGCAACATGGGCAGTGATTC AGG-3′), MxA (Fwd:5′-AGGAGTTGCCCTTCCCAGA-3′, Rev:5′-TCGTTCACAAGTTTCTTCAGTTTCA-3′, Probe:5′-ACCAGCGGGCATCTGGTCACGA-3′, 18S (Fwd:5′-AGTCCC TGCCCTTTGTACACA-3′, Rev:5′-GATCCGAGGGCCTCAC-TAAAC-3′, Probe:5′-CGCCCGTCGCTACTACCGATTGG-3′), and SIV RNA (Fwd:5′-GTCTGCGTCATCTGGTGCAT TC-3′, Rev:5′-CACTAGGTG TCTCTGCACTATCTGTTTTG-3′, Probe:5′-CTTCCTCAGTGTGTTTCACTTTCTCTTCTG-3′) were used. Fold-change calculations were performed using the ΔΔCt method, which involved an initial normalization against an internal gene (18S ribosomal RNA), and a second normalization against RIG-I/MDA5 expression in uninfected control animals [26].

Protein Isolation and Western Blot Analysis

Cells were lysed with ice-cold radioimmunoprecipitation assay buffer containing Protease Inhibitor Cocktail III (Calbiochem) as described elsewhere [27]. Forty micrograms of protein was resolved using a 10% polyacrylamide gel and probed with RIG-I, MDA5, and β-actin antibodies.

Statistical Analysis

Spearman′s rank correlation test was used to determine correlations of RIG-I and MDA5 mRNA and protein levels with the largely previously published levels of SIV RNA, IFN-β mRNA, and MxA mRNA as shown in Supplemental Figure 1 [10]. Mann–Whitney test was used to determine statistical differences between 2 different time points and 2 different severity groups in macaque brain samples. A 2-sample *T* test with equal variances was used to analyze significance between siRNA- or chloroquine-treated cells versus untreated samples in vitro.

RESULTS

RIG-I and MDA5 mRNA Are Induced in the Brain During SIV Infection

We examined the expression of RIG-I and MDA5 mRNA in the brains of SIV-infected macaques at different stages of infection by quantitative real time RT-PCR. Values were reported as fold-change in RNA. At 4 days postinfection, both RIG-I and MDA5 mRNA expression levels increased in the SIV-infected brain. RIG-I levels increased 4.2-fold, while MDA5 levels increased 20.7-fold (Figure 1*A* and *B*). MDA5 reached its highest level at 4 days postinfection, in contrast to RIG-I which peaked at 7 days postinfection, with a 5.4-fold increase versus control. At 7 days postinfection the increase in MDA5 was only 13.3-fold compared with the peak of 20.7-fold at 4 days postinfection. By 10 days postinfection, both RIG-I and MDA5 mRNA expression were dramatically reduced to levels comparable to those in uninfected control. During acute infection (4–21 days postinfection), both RIG-I and MDA5 expression negatively correlated with SIV RNA, and positively correlated with IFN-β and MxA mRNA (Table 1; Supplementary Table S1).

From 21 to 42 days postinfection, RIG-I and MDA5 continued to increase with the highest levels of expression of both RIG-I and MDA5 at 42 days postinfection. The level of expression of both reflected the severity of CNS disease, with macaques exhibiting moderate/severe disease having higher fold-change gene induction (median expression: RIG-I=12.5; MDA5=23.5) compared with macaques with none/mild disease (median expression: RIG-I=6.2; MDA5=7.1). A significant difference between the medians of both severity groups (moderate/severe vs. none/mild) was observed on 42 days postinfection for MDA5 but not RIG-I ($P = .0476$ and $P = .3810$, respectively). Levels for the moderate/severe animals stabilize from 42 to 84 days postinfection, while RIG-I and MDA5 mRNA expression in the none/mild group dramatically decrease to levels comparable to uninfected control animals at 56 days postinfection and maintains those levels until 84 days postinfection. During the late stage of infection (42–84 days postinfection), RIG-I and MDA5 correlated with brain SIV RNA, IFN-β mRNA, and MxA mRNA (Table 1; Supplementary Table S1). It should be noted that during the later stages of infection, IFN-β levels do not strongly parallel MxA levels (Supplementary Table S1). Specifically, IFN-β levels between none/mild versus moderate/severe groups become quite close (56 and 84 days postinfection dpi), while ISGs such as MxA, RIG-I, and MDA5 are 10-fold different between these 2 severity groups (Supplementary Table S1). This discrepancy is likely due to the brain switching to IFNα (instead of IFN-β) production during the later stages of infection [22].

RIG-I and MDA5 Protein Expression Are Differentially Regulated in the Brain of SIV-Infected Macaques

In order to examine the location and temporal pattern of RIG-I (Figure 1*C*, *E*, and *G*) and MDA5 expression (Figure 1*D*, *F*, and *H*), immunohistochemical analysis was performed and protein expression was quantitated in basal ganglia of uninfected control and SIV-infected macaques from 4 to 84 days postinfection. Increased expression of RIG-I and MDA5 proteins were found in white matter of infected macaques compared with uninfected controls (Figure 1*C* and *D*). However, the increases in RIG-I expression were much less significant compared with MDA5 (Figure 1*G* and *H*). In contrast, gray matter had higher basal levels of expression of both proteins, but there was little change in expression of either protein during SIV infection (Figure 1*E* and *F*). In the white matter, there was a modest 1.7-fold increase in RIG-I protein expression at 7 days postinfection compared with controls, and returned to uninfected levels at 10 and

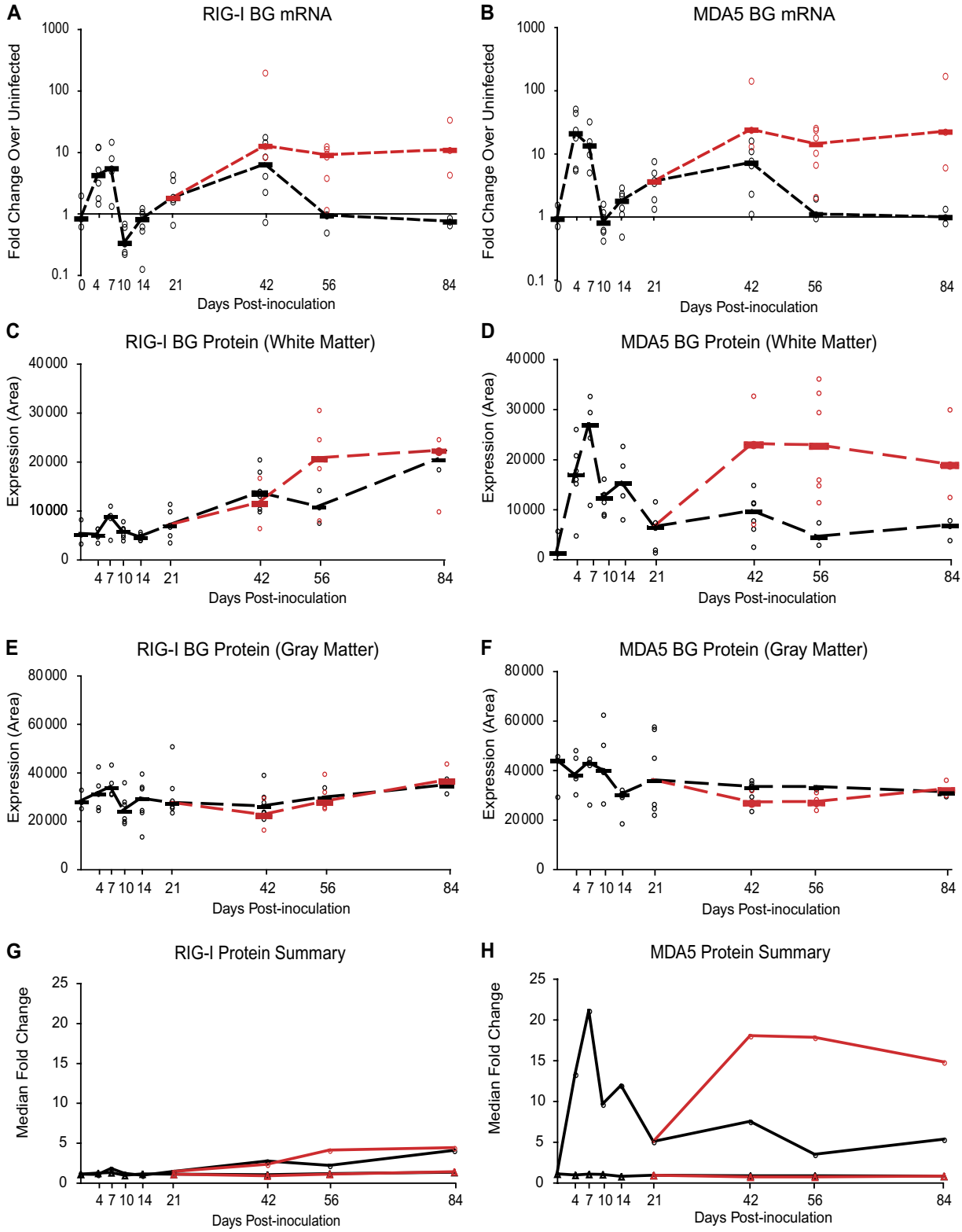

Figure 1. Relative induction of retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) mRNA and protein in brain of simian immunodeficiency virus (SIV)-infected macaques. *A*, RIG-I and (*B*) MDA5 mRNA were measured by quantitative RT-PCR from basal ganglia sections of uninfected and SIV-infected macaques sacrificed at various time points (4, 7, 10, 14, 21, 42, 56, or 84 days post inoculation). The number of animals used for each time point are: 6 (4 days), 5 (7 days), 6 (10 days), 6 (14 days), 6 (21 days), 9 (42 days), 9 (56 days), 6 (84 days). The mRNA levels are represented as fold-change over the average of 3 uninfected brain RNAs, calculated using the $\Delta\Delta$Ct method. Medians (*black bars*) are indicated for each experimental group. Each circle represents one animal, color-coded according to severity of CNS disease (*red*, moderate/severe; *black*, none/mild). The

Table 1. Correlation of RIG-I and MDA5 mRNA With SIV RNA, IFN-β, and MxA mRNA.

	Acute stage			
	RIG-i		MDA5	
	r[a]	P[b]	r	P
SIV RNA	−0.3539	.0597	−0.4974	.0061
IFN-β mRNA	0.444	.0158	0.4398	.017
MxA mRNA	0.8131	<.0001	0.8166	<.0001
	Terminal stage			
SIV RNA	0.6271	.001	0.7964	<.0001
IFN-β mRNA	0.4063	.0488	0.4097	.0468
MxA mRNA	0.7145	<.0001	0.8317	<.0001

[a] Correlation coefficient.

[b] Significance.

21 days postinfection (Figure 1*C*). In comparison, median MDA5 expression was up-regulated by 13.2-fold at 4 days postinfection, reaching a peak of 21-fold induction by 7 days postinfection, before adjusting to 9.5-fold by 10 days postinfection (Figure 1*H*). This pattern of MDA5 expression parallels the expression of IFN-β and MxA mRNA in the brain of these macaques peaking at 7 days postinfection, followed by decreased expression by 10 days postinfection [10]. SIV RNA expression in brain also increases from 4 to 7 days postinfection, followed by decline from 10 to 14 days postinfection [10]. The control of exponential virus replication at 10 and 14 days postinfection is in part due to the expression of IFN-β and ISGs in the brain during acute infection [10]. A negative correlation was observed between MDA5 (but not RIG-I) protein expression in the white matter during acute stages of infection (4–21 days postinfection) and SIV RNA ($r = -0.4860$; $P = .0102$).

A bifurcating pattern of expression for MDA5 at 42, 56, and 84 days postinfection was observed (Figure 1*H*). SIV-infected macaques with moderate to severe encephalitis had higher median levels of MDA5 protein compared with none to mild disease in macaque brain at 42, 56, and 84 days postinfection There is a statistically significant difference between medians of both severity groups (moderate/severe vs none/mild) at 56 days postinfection ($P = .0238$). The bifurcating pattern is markedly less distinct for RIG-I protein expression, as is the overall induction of RIG-I (Figure 1*G*). MDA5 protein expression in the white matter strongly correlated with SIV RNA and MxA mRNA (r = 0.7426, $P < .0001$ and r = 0.7704, $P < .0001$, respectively), but not IFN-β (r = 0.1861, $P = .384$). There were no correlations between the expression of RIG-I protein with SIV RNA, IFN-ß mRNA, or MxA mRNA.

RIG-I and MDA5 Protein Is Induced in Perivascular Macrophages and Astrocytes of SIV-Infected Macaques

In order to determine the specific cells in the brain that expressed RIG-I and MDA5 proteins, immunohistochemistry using fluorescent probes was done on uninfected and SIV-infected brain (basal ganglia) from 10 days postinfection. We demonstrated that in uninfected brain, protein expression for both RIG-I (Figure 2*A*) and MDA5 (Figure 2*B*) was undetectable in perivasular macrophages and astrocytes, while being constitutively expressed in neurons. In contrast, in SIV-infected brain, protein levels for both RIG-I (Figure 3*A*) and MDA5 (Figure 3*B*) were clearly induced in perivascular macrophages and astrocytes. Expression of both proteins was maintained in neurons of infected animals.

SIV-Infection Produces Double-Stranded RNA in Brain Macrophages

We examined whether there was double-stranded RNA (dsRNA) in the brain of SIV-infected macaques by performing immunohistochemistry using an antibody capable of recognizing dsRNA helices longer than 40bp [28]. The dsRNA was detected in SIV-infected brain in perivascular macrophages and colocalized with detection of SIV gp41 protein in the same cell (Figure 4*B*). There was no dsRNA detected in the brain of uninfected animals (Figure 4*A*). This provides evidence that dsRNA is produced in SIV-infected macrophages in brain and can be recognized by either RIG-I or MDA5.

SIV Infection Induces MDA5-Dependent, but Not RIG-I–Dependent, Type I IFN Response in Macrophages

In order to determine whether RIG-I or MDA5 (or both) played a role in inducing the type I IFN response to SIV infection, primary pigtail macaque macrophages were used and RIG-I and MDA5 silenced using small interfering RNA (siRNA). Macrophages were transfected with either a scrambled siRNA control, or a gene-specific RIG-I- and MDA5-siRNA, in at least 3 independent experiments. The levels of both proteins were analyzed at 24 and 48 hours postinfection; and while protein levels decreased at 24 hours, there was no significant effect on function until 48 hours. The reduction of RIG-I protein was ∼40% at 48 hours postinfection in this experiment (Figure 5*A*), but

medians for both moderate/severe (*red dashed line*) and none/mild (*black dashed line*) animals are represented in the 42-, 56-, and 84-day groups. For protein expression, basal ganglia tissue sections from SIV-infected macaque brains were singly probed using either RIG-I or MDA5-specific antibodies and stained with liquid diaminobenzidine (DAB) and digitally quantified. Protein expression of RIG-I in white (*C*) and gray matter (*E*), as well as MDA5 in white (*D*) and gray matter (*F*) was quantified using measured area. These results are represented as median fold-change for RIG-I (*G*) and MDA5 (*H*), with white matter (*circles*) and gray matter (*triangles*) color-coded according to severity of CNS disease (*red*, moderate/severe; *black*, none/mild).

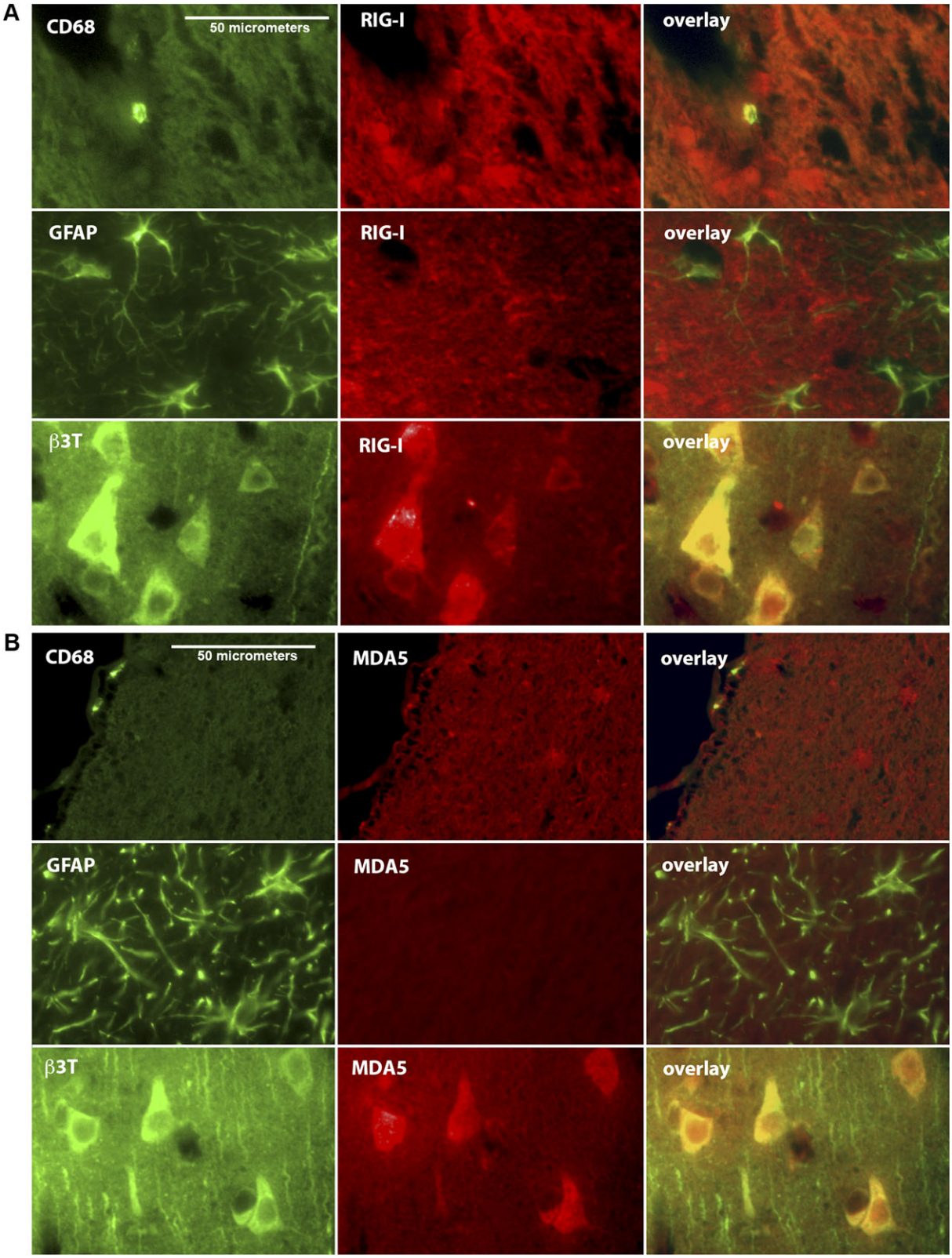

Figure 2. Retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) expression in specific brain cells in uninfected macaques. RIG-I (*A*) and MDA5 (*B*) protein expression were investigated in uninfected basal ganglia brain tissue sections by dual staining with specific brain markers for macrophages (CD68), astrocytes (glial fibrillary acidic protein [GFAP]), and neurons (ß3-tubulin), as well as RIG-I and MDA5 specific antibodies. Fluorescent secondary antibodies were used and overlays analyzed using a fluoresent microscope. It is shown that RIG-I and MDA5 are both hardly expressed in perivascular macrophages and astrocytes, while being constitutively expressed in neurons.

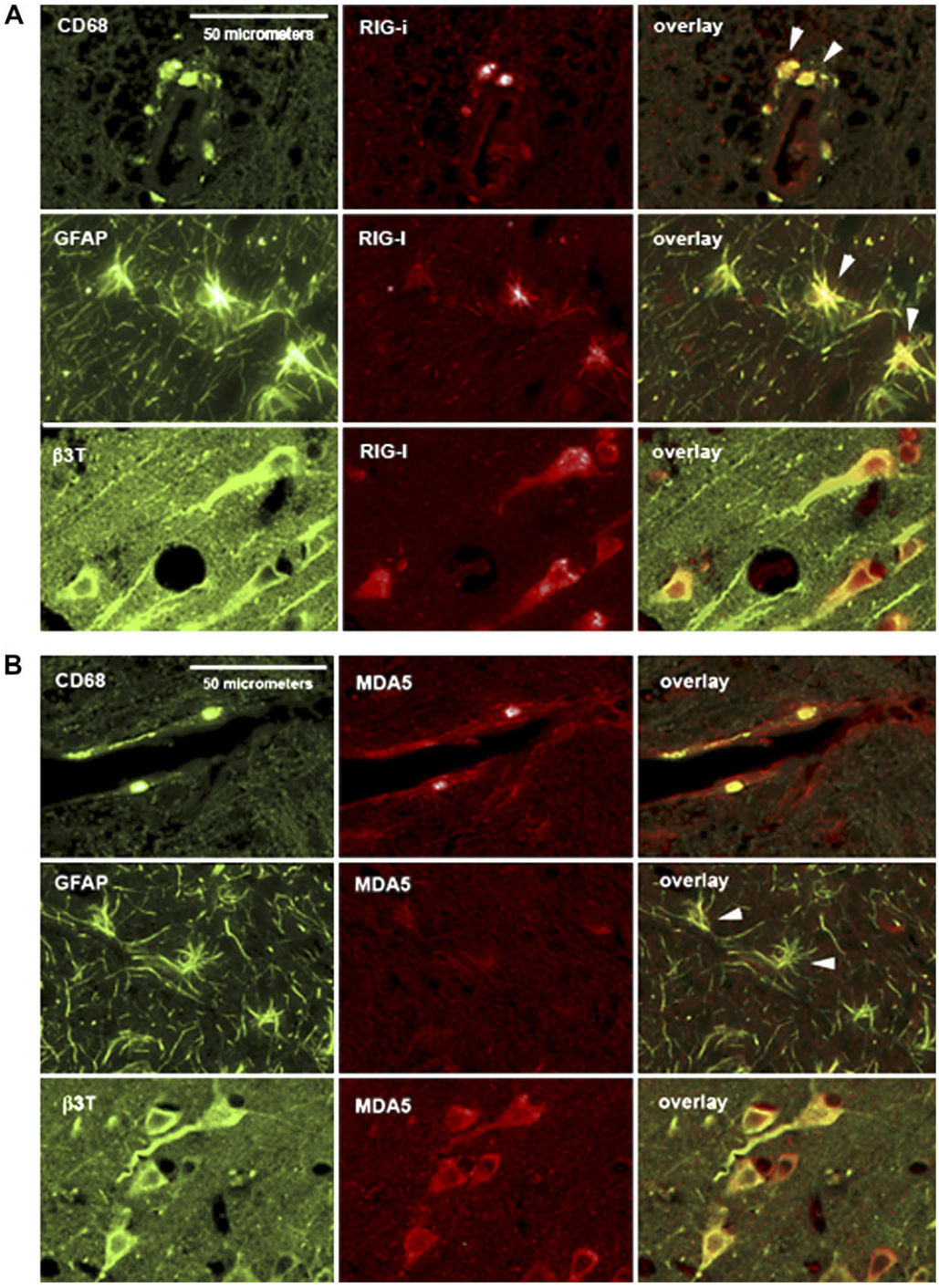

Figure 3. Retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) expression in specific brain cells in SIV-infected macaques. RIG-I (*A*) and MDA5 (*B*) protein expression were investigated in infected basal ganglia brain tissue sections by dual staining with specific brain markers for macrophages (CD68), astrocytes (glial fibrillary acidic protein {GFAP}), and neurons (ß3-tubulin), as well as RIG-I and MDA5 specific antibodies. Fluorescent secondary antibodies were used and overlays analyzed using a fluoresent microscope as described in Materials and Methods. White arrowheads indicate colocalization of RIG-I and CD68, and RIG-I and GFAP in (*A*), and MDA5 and GFAP in (*B*). It is shown that RIG-I and MDA5 are both induced in perivascular macrophages and astrocytes upon infection, while being constitutively expressed in neurons.

knockdown ranged from ~40% to 70% in experimental replicates. On the other hand, MDA5 protein was reduced by ~74% at 48 hours post infection (Figure 5*B*). Type I IFN induction, which was measured using the induction of the ISG MxA mRNA, was significantly reduced by 14.7-fold in MDA5 siRNA-treated cells at 48 hours postinfection ($P = .0035$) compared with controls (Figure 5*D*). Despite the variable 40%–70% reduction in RIG-I, MxA levels never decreased in the experiments

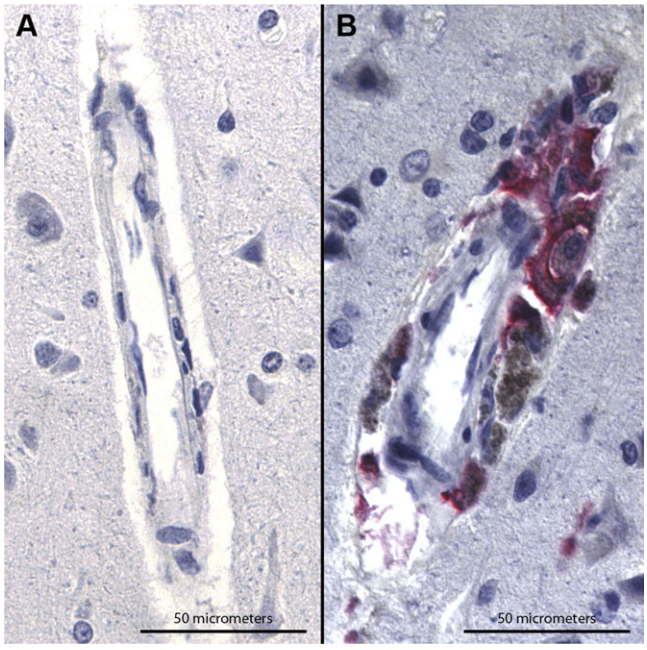

Figure 4. Double-stranded RNA (dsRNA) is detected in simian immunodeficiency virus (SIV)–infected perivascular macrophages. Uninfected (*A*) and SIV-infected (*B*) basal ganglia tissue sections from macaques were dual probed with antibodies against SIV gp41 and dsRNA and stained using vector red and liquid diaminobenzidine (DAB), respectively. Colocalization of SIV gp41 protein and dsRNA in infected perivascular macrophages indicates that SIV infection produces detectable amounts of dsRNA.

but surprisingly increased ($P = .0136$) (Figure 5*C*). Thus, the intracellular signaling induced by SIV infection of macrophages that leads to type I IFN response and ISGs appears to be MDA5-dependent.

Induction of the Type I IFN Response in SIV-Infected Macrophages Is Not Exclusively Through Cytosolic Sensors

In order to determine whether type I IFN response also signaled through endocytosis-dependent pathways such as the TLR response, macrophages were pretreated with chloroquine 30 minutes before infection. MxA was downregulated in SIV-infected, chloroquine treated cells by 5.1-fold at 48 hours ($P = .0077$) compared with untreated, infected cells (Figure 5*E*). When cells were pretreated with both MDA5 siRNA and chloroquine, MxA expression was down-regulated by 12.4-fold ($P = .0027$), compared with controls (Figure 5*F*). These data suggest that intracellular signaling induced by SIV infection of macrophages that leads to the type I IFN response occurs through both MDA-5 and the endocytosis-dependent TLR pathway.

DISCUSSION

The innate immune response to SIV infection in the CNS is critical for controlling viral replication during acute infection and preserving the neurological function within the immune-privileged site of the brain. We have previously shown coordinated regulation of innate immune responses, including IFN-β and MxA, which function to control viral replication during the early stages of infection in SIV-infected macaque brains [10]. In this study, we examine the pathways in macaque brain and in primary macrophages that induce IFN-β. We demonstrate for the first time to our knowledge that both cytosolic RNA sensors RIG-I and MDA5 are significantly induced in the brains of SIV-infected macaques at the mRNA level. Although both RIG-I and MDA5 proteins increase upon SIV infection, only MDA5 upregulation was statistically significant. The increased expression of RIG-I and MDA5 protein was found in astrocytes and perivascular macrophages, while constitutive expression was observed in neurons. Additionally, SIV-infected macrophages induce the type I IFN response in an MDA5-, but not a RIG-I-dependent manner. Finally, we demonstrate that signaling in response to SIV is not exclusively through the cytosolic sensors, as treatment with chloroquine also decreased the levels of the ISG MxA, implicating a role for membrane-bound TLR sensors as well.

Unlike other RNA viruses that use viral polymerase to synthesize their genome in the cytosol, HIV and SIV exclusively use the cellular transcriptional machinery in the nucleus. Correspondingly, the newly synthesized viral genome is also subject to posttranscriptional modifications. This results in masking of the 5′-triphosphate, the ligand of RIG-I in the viral genome, by a methylated guanosine cap similar to cellular mRNA, resulting in the viral RNA appearing to be "self" RNA [29]. In addition to the absence of a 5′-triphosphate, retroviruses do not possess a dsRNA intermediate step in their replication cycles. However, the HIV genome does form complex secondary structures that result in long stretches of dsRNA, the ligand for MDA5 [30–35]. In fact, the architecture and extensive secondary structure of an entire HIV-1 RNA genome was demonstrated using high-throughput RNA analysis [36]. These secondary structures constitute previously unrecognized regulatory motifs and are an important and *conserved* part of the genetic code. In this respect, the HIV genome with its long stretches of dsRNA regions could trigger MDA5. We have found that SIV infection in brain leads to significant amounts of >40bp long dsRNA molecules, particularly in perivascular macrophages, the main site of SIV infection. In contrast, dsRNA was not detected in astrocytes, probably because SIV replication is restricted in these cells in vivo [37]. In vitro, MxA expression is reduced in SIV-infected macrophages that have significantly reduced expression of MDA5 protein. This effect is not seen when RIG-I expression is reduced. The lack of a 5′-triphosphate "viral signature" in retroviruses also suggests that the cytosolic sensor RIG-I is not used for antiretroviral IFN-β induction.

Introduction of RNA molecules to silence genes in in vitro experiments causes unwanted stimulation of the type I IFN response [38]. Optimization of gene silencing experiments was

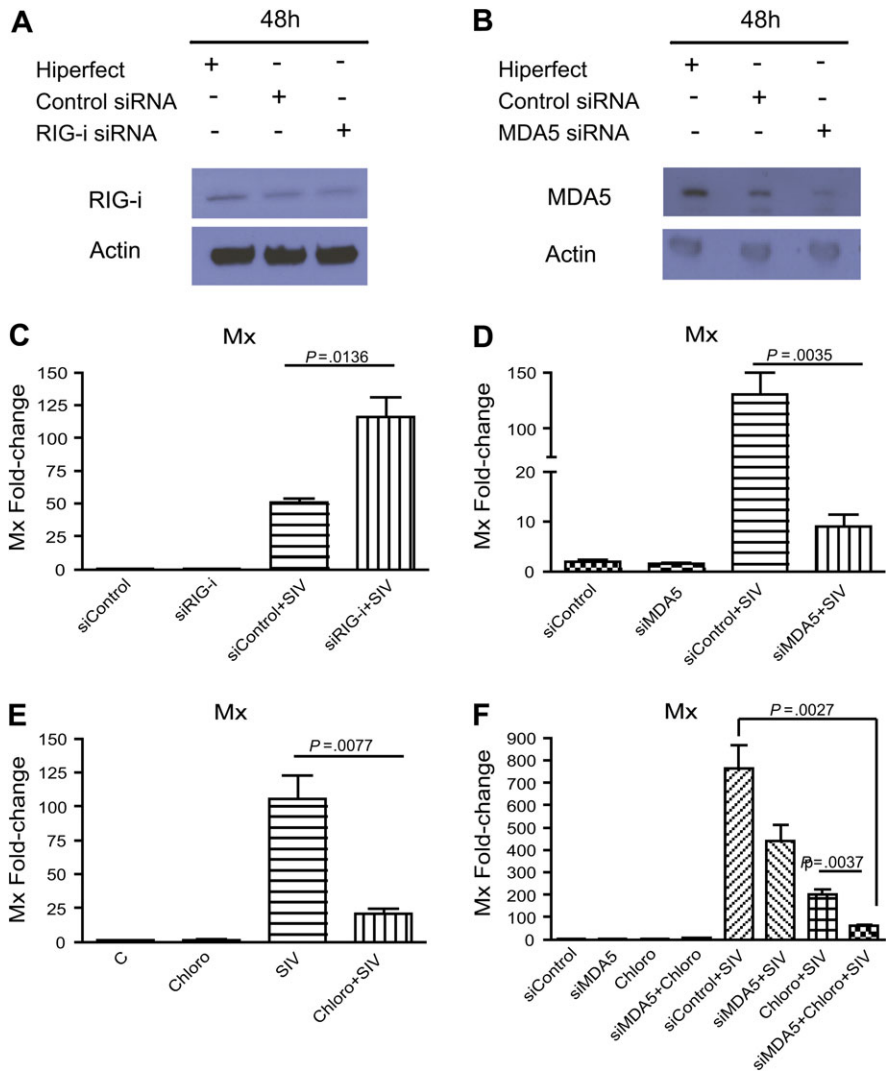

Figure 5. Type I interferon (IFN) stimulated gene MxA is induced in a melanoma differentiation-associated protein 5 (MDA5)- and Toll-like receptor (TLR)–dependent manner. RIG-I (*A*) and MDA5 (*B*) were silenced with either a scrambled control sequence or gene-specific siRNA containing 2′-O-methyl uridine modifications in order to prevent immune stimulation during transfection of macaque monocyte-derived macrophages. Knockdown efficiency was evaluated at 48 hours post-infection using western blot by first normalizing protein levels against ß-actin and then comparing against Hiperfect-only treated cells. MxA mRNA (*C–F*) was measured by quantitative RT-PCR from total RNA of monocyte-derived macrophages pretreated with (*C*) 100 nmol/L RIG-I siRNA, (*D*) 100 nmol/L MDA5 siRNA, (*E*) 50 umol/L of chloroquine, or (*F*) both MDA5 siRNA and chloroquine, before SIV infection. The mRNA levels are represented as fold-change over uninfected macrophage RNA and calculated using the ΔΔCt method. Data are representative of at least 3 independent experiments. A 2-sample *T* test with equal variances was used to analyze significance between treatments.

hindered by IFN responses to siRNA treatment; thus, silencing of the cytosolic sensors resulted in upregulation of ISGs and was a challenge for our studies. To minimize this effect, siRNAs with uridine 2′-O-methyl modifications were used because these modifications enable binding of the siRNA to RIG-I–like receptors and TLR7/8, but do not trigger downstream signaling or interfere with silencing efficiency [24, 39].

The protective antiviral effects of type I IFN have been demonstrated during acute SIV infection [4, 5, 27], whereas IFN expression during the later stages of infection appears to contribute to the increase in inflammatory cytokines and CNS disease during chronic and late stages of HIV infection [40]. In this study, MDA5 protein expression negatively correlated with SIV RNA during the *acute* infection, suggesting that MDA5 expression negatively impacts SIV replication. Conversely, MDA5 protein *positively* correlated with SIV RNA and MxA mRNA during the *terminal* phase of infection, suggesting that during later stages of disease, MDA5 and MxA are associated with the inflammatory environment that underscore the uncontrolled innate immune responses in the brain.

It is interesting to speculate why RIG-I did not increase to the same extent as MDA5 in response to IFN-β in the brain, since both are considered ISGs and signal through the same adaptive molecule, IPS-1. However, there are important differences

between the 2 proteins. Recent studies suggest that RIG-I is a more versatile pattern recognition receptor, recognizing viral RNA as well as bacterial DNA [41, 42]. MDA5 appears to be specialized for viral dsRNA detection. Thus, MDA5 may be upregulated via pathways specific for viral infection. Furthermore, retroviral proteins could act to antagonize RIG-I in a direct or indirect manner, as seen in other virus-cell interactions [21].

TLR and cytosolic sensors may have a similar role in macrophages as they do in plasmacytoid dendritic cells (pDCs). In pDCs, TLR-dependent viral recognition and type I IFN feedback signaling masks the requirement of viral replication for IFN-α production; thus, cytosolic sensors are dispensable. However, if the positive feedback loop is disabled, then IFN-α production in pDCs becomes viral replication-dependent and signals through the RIG-I/MDA5-IPS1 pathway [43]. In a similar manner, HIV-infected macrophages probably signal through both TLR- and cytosolic-sensing paths, depending on the extent of infection as well as the strength of the primary IFN response. Since the IFN response in pDCs is significantly stronger than in macrophages, it is possible that cytosolic sensors play a greater role in these cells than their corresponding roles in pDCs. Unlike HIV infection of T cells that occurs exclusively through direct fusion of viral and cellular membranes, HIV infection in macrophages occurs through both endocytosis and fusion [44, 45]. These 2 routes of HIV infection in macrophages further support a dual method of viral detection: by the cytosolic sensor MDA5 and membrane-bound TLRs.

Supplementary Data

Supplementary Data are available at *The Journal of Infectious Diseases* online.

Funding

This work was supported by National Institutes of Health grants to J. E. C. [MH070306, NS047984, NS055648].

Acknowledgments

We thank medical editor Michael Linde for helping with the organization and editing of the manuscript, Brandon Bullock and Ming Li for technical assistance, Dr Patrick Tarwater for statistical advice, and Dr David Graham and the entire Retrovirus Laboratory at Johns Hopkins for insightful discussion.

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