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RNA species generated in vaccinia virus infected cells activate cell type-specific MDA5 or RIG-I dependent interferon gene transcription and PKR dependent apoptosis

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ABSTRACT

RNA species produced during virus replication are pathogen-associated molecular patterns (PAMPs) triggering cellular innate immune responses including induction of type I interferon expression and apoptosis. Pattern recognition receptors (PRRs) for these RNAs include the retinoic acid-inducible gene I (RIG-I) like receptors (RLRs) RIG-I and melanoma differentiation associated gene 5 (MDA5) and the dsRNA dependent protein kinase (PKR). Currently, poxvirus PAMPs and their associated PRRs are not well characterized. We report that RNA species generated in vaccinia infected cells can activate MDA5 or RIG-I dependent interferon-β (IFN-β) gene transcription in a cell type-specific manner. These RNA species also induce the activation of apoptosis in a PKR dependent, but MDA5 and RIG-I independent, manner. Collectively our results demonstrate that RNA species generated during vaccinia virus replication are major PAMPs activating apoptosis and IFN-β gene transcription. Moreover, our results delineate the signaling pathways involved in the recognition of RNA-based poxvirus PAMPs.

Introduction

Recognition of pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs) activates innate immune responses. Double-stranded RNA (dsRNA) is a PAMP which can be generated inside virus infected cells during viral replication. Several cytosolic host PRRs have been identified for these dsRNA species including the retinoic acid-inducible gene I (RIG-I) like receptors (RLRs) RIG-I and melanoma differentiation associated gene 5 (MDA5) and the dsRNA dependent protein kinase (PKR) (Mogensen, 2009).

DsRNA binding to the C-terminal of RIG-I and MDA5 unmasks the N-terminal caspase recruitment domain (CARD), stimulating CARD-CARD interactions with IFN-β promoter stimulator 1 (IPS-1) (also known as MAVS, Cardiff and VISA) (Kawai et al., 2005; Mogensen, 2009). Downstream signaling results in the activation of the transcription factors NF-κB and IRF3 to coordinate transcription of genes encoding anti-viral cytokines (Kawai et al., 2005; Meylan et al., 2005; Paz et al., 2006). MDA5 and RIG-I differentially respond to RNA virus infection, with MDA5 recognizing picornaviruses and RIG-I recognizing influenza viruses and paramyxoviruses (Kato et al., 2006). MDA5 also recognizes synthetic polyinosinic acid-polycytidylic acid (pIC), while *in vitro* transcribed dsRNA is recognized by RIG-I. The actual ligand recognized by RIG-I may be the 5′-tri-phosphate moiety of short, blunt dsRNAs (Schlee et al., 2009). RIG-I, MDA5 and IPS-1 have also been reported to regulate apoptosis in a variety of systems (Besch et al., 2009; Lei et al., 2009).

PKR represents another cytosolic dsRNA sensor capable of activating innate immune responses. Following binding to dsRNA, PKR dimerizes and undergoes auto-phosphorylation (Garcia et al., 2006). PKR then phosphorylates the serine-51 residue of the alpha subunit of eukaryotic initiation factor 2 (eIF2α) resulting in a global halt to translation. Infection of cells with a mutant vaccinia virus lacking the E3L gene results in PKR dependent eIF2α phosphorylation (Zhang et al., 2008). Similar to RIG-I and MDA5, PKR can also mediate NF-κB activation and the induction of apoptosis (Bonnet et al., 2006;

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Table 1
Cytokine induction by RNA species generated in vaccinia infected cells. HeLa cells were either mock transfected or transfected with 3 μg of early, late or late + araC RNA preparations (as described in Materials and methods). Cells were collected at 6 h post-transfection and 0.75 μg of RNA was reverse transcribed from each sample and real-time PCR arrays were performed to detect common human cytokines. GAPDH and β-actin were used as loading controls for normalization. Cytokines displaying a Ct value of ≥35 were defined as not significantly detected and excluded from the analysis. Results are shown as the fold change in expression of transfected cells in comparison to mock transfected cells.

Cytokines	*HeLa cell RNA*	*Early RNA*	*Late RNA*	*Late RNA + araC*
Interferon family				
IFN-β1	5.79	10.37	108.04	5.79
IFN-γ	1.73	4.44	2.21	2.91
IFN-κ	2.02	2.41	2.72	2.14
IFN-α8	2.13	1.62	2.66	−1.06
IFN-α2	1.73	1.76	2.21	6.29
IFN-α1	1.76	2.20	1.91	1.50
Interleukin family				
IL19	9.07	19.64	19.43	5.47
IL20	1.65	5.92	14.37	3.69
IL1F7	2.0	6.44	11.78	5.28
IL6	3.58	6.15	11.55	2.27
IL8	2.70	4.05	10.9	1.90
IL1A	2.32	3.99	10.46	1.67
IL22	1.73	9.55	4.36	2.91
IL13	1.54	1.05	8.46	1.75
IL1B	1.83	4.11	4.18	1.34
IL12A	1.46	1.67	2.86	3.29
IL5	1.73	1.76	3.21	2.91
IL1F9	1.73	2.39	2.21	2.91
IL17C	1.82	1.76	2.21	2.91
IL11	1.52	1.96	2.66	1.64
IL21	−1.16	2.16	1.09	2.26
IL7	1.46	2.24	2.09	1.18
IL24	1.63	2.16	2.10	1.70
IL15	1.46	1.85	2.12	1.64
IL10	1.29	1.56	1.83	1.42
IL18	1.26	1.42	1.66	1.33
TXLNA	1.26	1.61	1.41	1.24
IL12B	−1.15	1.07	1.40	1.08
IL17B	−1.34	−1.32	−1.05	1.24
TNF family				
TNF	3.15	6.19	35.98	1.58
LTB	1.73	1.76	3.68	2.91
TNFSF11	2.09	1.23	1.37	3.27
LTA	−1.05	1.92	1.20	3.07
TNFSF10	2.74	1.99	2.45	1.35
TNFSF12	−1.18	1.76	1.90	2.43
TNFSF13	1.26	1.81	1.36	1.52
TNFSF13B	1.35	1.10	−1.21	1.40
CD70	1.05	1.74	1.40	1.31
TNFRSF11B	1.04	1.16	1.20	−1.07
TNFSF14	1.00	−1.10	−1.17	−1.18
Bone Morphogenic Proteins				
BMP4	−6.5687	1.6756	2.1871	1.4573
INHBA	1.6308	2.6851	4.0668	1.6479
INHA	1.8753	4.6244	2.2144	2.9163
GDF9	3.5394	3.3533	2.1177	1.6969
MSTN	1.7374	1.76	2.2144	3.2197
GDF5	1.7374	1.76	2.2144	3.1688
NODAL	1.0862	1.1004	1.3844	3.0559
BMP3	1.7267	1.7047	2.1447	2.8245
BMP2	1.4102	2.5841	1.8519	1.142
GDF2	2.5123	1.1983	1.662	1.9855
TGFA	1.2609	1.5492	1.4066	2.1818
TGFB2	1.4298	1.7659	2.1221	1.6079
BMP5	−1.3169	−1.3	1.0459	1.8488
GDF11	1.2716	1.6474	1.1479	1.1133
TGFB1	1.2518	1.573	1.3315	1.216
TGFB3	1.1649	1.4571	1.5324	1.2867
BMP8B	−1.043	−1.0518	1.4677	1.3471
BMP1	−1.0938	1.212	1.0013	−1.0602
BMP6	−1.1333	−1.5552	−1.0742	1.0288
PDGF/VEGF Family				
FIGF	1.3004	1.3324	2.0243	2.3656
PDGFA	1.3219	1.5483	1.5148	1.3317
Others				
FAM3B	1.7374	2.299	13.1574	2.9163
CSF2	1.6068	2.5954	8.0172	1.1546
CSF1	1.2198	1.6594	1.735	1.3139

Ishii, et al., 2001; Zamanian-Daryoush et al., 2000; Gil and Esteban, 2000). Recently, both PKR and MDA5 were shown to regulate IFN-β expression in response to infection with a Sindbis virus with a point mutation in the nsP2 protein (Burke et al., 2009).

While the interactions between RNA-based PAMPs of RNA viruses and their cellular PRRs are well characterized, comparatively less is known about the role of RNA species generated by DNA viruses as PAMPs. In the case of poxviruses, dsRNA is thought to be generated primarily during convergent transcription of intermediate and late class genes (Boone, et al., 1979; Colby et al., 1971; Ludwig et al., 2006). Previously, the effect of vaccinia dsRNA species on the induction of cytokines and apoptosis has been inferred from the phenotypes of recombinant vaccinia viruses lacking the dsRNA binding protein E3 (vvΔE3L) or expressing E3 mutants lacking dsRNA binding capacity. Few studies have directly assessed the immuno-stimulatory activity RNA species generated during vaccinia replication in the absence of other viral factors such as viral DNA or proteins. It was recently shown that the dsRNA binding domain of E3, as well as single amino acids essential for dsRNA binding, also account for the ability of E3 to inhibit ssRNA and dsDNA induced signaling (Marq et al., 2009). Thus, although the induction of cytokine expression and apoptosis during vvΔE3L infection is speculated to be stimulated by viral RNA species, it is possible that an alternative viral PAMP(s) could be the stimulatory molecule. Therefore, a more direct model is required to address the specific role of RNA species generated in vaccinia infected cells in the absence of other potential viral PAMPs. To address this, we previously demonstrated that RNA species generated in vaccinia infected cells are capable of activating PKR and IFN-β and TNF-α expression (Myskiw et al., 2009). Subsequently, it was reported that these vaccinia RNA species activate IFN-β expression in an MDA5 dependent manner in murine cells (Pichlmair et al., 2009).

Here we extend our previous findings by profiling the global cytokine response resulting from treatment of cells with these RNAs. We report that in human cells, RIG-I, in addition to MDA5, regulates cell type-specific IFN-β gene transcription in response to RNAs generated in vaccinia infected cells. Furthermore, we demonstrate that these RNA species induce PKR dependent, but MDA5 and RIG-I independent, apoptosis. These results provide the strongest evidence to date that RNA species generated in vaccinia infected cells act as PAMPs triggering cytokine expression and apoptosis.

Results

RNA species generated during vaccinia virus replication activate expression of IFN-β and a select group of pro-inflammatory cytokines

Previously we reported that RNA species associated with vaccinia virus replication can activate IFN-β and TNF-α expression and PKR phosphorylation (Myskiw et al., 2009). In this study, we sought to further characterize the cellular response to these RNA species. First, the global induction of cytokines following transfection of these RNA species into HeLa cells was examined using real time PCR arrays. To prepare RNA samples for transfection, HeLa cells were mock infected (HeLa cell RNA) or infected with vvΔE3L-Rev (Arsenio et al., 2008) and collected at 2 (early RNA) and 8 (late RNA) h post-infection (hpi).