Chapter 15

Evaluation of Innate Immune Signaling Pathways in Transformed Cells

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Abstract

Oncolytic viruses, the use of viruses to treat cancer, is emerging as a new option for cancer therapy. Oncolytic viruses, of both DNA and RNA origin, exhibit the ability to preferentially replicate in and kill cancer cells plausibly due to defects in innate immune signaling or translation regulation that are acquired during cellular transformation. Here, we review concepts and assays that describe how to analyze signaling pathways that govern the regulation of Type I IFN production as well as the induction of interferon-stimulated antiviral genes, events that are critical for mounting an effective antiviral response. The following procedures can be used to assess whether innate immune pathways that control antiviral host defense are defective in tumor cells – mechanisms that may help to explain viral oncolysis.

Key words: Innate immunity, Interferon, Oncolytic, Host defense, STING

1. Introduction

That natural virus infections occasionally coincided with the regression of certain types of tumors in patients suffering from such maladies is relatively well documented and has led to a spate of studies designed to utilize viruses as oncolytic agents for the treatment of malignant disease (1). However, the mechanisms that favorably enable the replication of viruses in tumor cells compared to normal cells still remain to be clarified. Such reasons could involve the accumulation of genetic mutations in innate signaling pathways important for mediating antiviral activity. In cancer cells, these defects can lead to a reduction or inactivation of the type 1 interferon (IFN-α/β) response, critical for upregulating numerous antiviral genes, making such cells highly susceptible to viral replication and

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subsequent cellular lysis (2). In order to fully explore the mechanisms of oncolytic virus action, it is necessary to understand the architecture of innate immune signaling cascades in normal cells and then evaluate their plausible deregulation in cancer cells. Only in the last few years has it become clear that the host has evolved a number of pathways to detect and respond to foreign pathogens such as viruses and bacteria (3–5). However, it is likely that important components of innate signaling still remain to be identified, which will make the reasons underlining oncolysis potentially elusive for some time. Nevertheless, progress has been made toward understanding these processes, and now these signaling cascades including their components can start to be analyzed in cancer cells. The reasons underlining viral oncolysis are particularly intriguing since numerous virus types replicate in a wide variety of cancer cells, making the responsible defective pathway potentially a major reason behind tumorigenesis (6). These recently unraveled pathways may include the Toll-Like Receptor (TLR) pathway, the RIG-I Like Helicase (RLH) pathway, and recently the STING regulated pathway (SRP), a brief description of which follows.

1.1. Toll-Like Receptor Signaling

The triggering of innate immune responses follows the detection of pathogen specific molecules (pathogen-associated molecular patterns- PAMPS) by cellular pattern-recognition receptors (PRRs). For example, bacteria have cell wall components comprising lipopolysaccharides that can trigger innate immune responses, while viral nucleic acids are also known to be potent PAMPs (5, 7). A key innate immune pathway that is highly conserved and able to recognize a wide array of pathogens is the Toll pathway, first identified in *Drosophila melanogaste*r. Activation of *D. melanogaster* Toll leads to the fat body cells triggering, via *dMyD88*, *tube*, and *pelle*, the production of antimicrobial peptides (AMPs) and the subsequent secretion of the peptides into the hemolymph. Another pathway, referred to as IMD (for *immunodeficiency*) utilizes Drosophila *RIP1* and *dFADD* to trigger the production of a different set of antimicrobial peptides (8–10). Both pathways culminate in the activation of NF-κB related transcription factors, Relish and Dorsal-related Immune Factor (DIF), respectively, and the transcription of hundreds of genes including those encoding the effector AMPs directed against the intruding microorganisms. However, these pathways are geared toward the recognition of bacteria, and almost nothing is known of how flies respond to virus infection (11). Nevertheless, these pathways have been preserved in mammalian cells and there are 13 TLRs currently known in human (12). Significantly, four TLRs (TLR 3, 7/8, and 9) are considered associated with the detection of viral infection (5). For example, TLR-3 is found in macrophages, myeloid dendritic cells, fibroblasts, and epithelial cells, is located in the endoplasmic reticulum, and senses extrinsic dsRNA species (13, 14). TLR-7/8 is similarly associated

with the endoplasmic reticulum, though predominantly in plasmacytoid dendritic cells (pDCs) and is responsible for detecting single-stranded RNA species (15, 16). By contrast, TLR 9 recognizes unmethylated DNA species (CpG DNA) and is similarly found in pDCs (15, 17). These TLRs reside in endosomes and can trigger the production of IFN-α/β in either a MyD88 dependent (TLR-7/9) or independent (TLR-3) manner (18). Upon recognition of extrinsic dsRNA captured in endosomes, TLR-3 activates the signal adaptor TIR domain-containing adapter inducing interferon-β (TRIF), which mediates association with TNF receptor-associated factor 6 (TRAF6) and 3 (19, 20). TRIF complexes then activate tank binding kinase 1 (TBK-1) leading to phosphorylation of interferon regulatory factor 3 (IRF-3) and IRF-7. IRF3 or 7 dimerize, facilitating translocation to the nucleus and activation of IFN-β transcription (19). TRIF also interacts with RIP1, which in association with TRAF6 activates the IKK complex, which signals through TGF-β activated kinase 1 (TAK-1, leading to dissociation of the IKK complex from NF-κB (21). IRF3/7 and NF-κB are both required for the robust transcriptional activation of type I IFN.

In the case of TLR7 and 9, recognition of the appropriate PAMP leads to the recruitment of MyD88, initiating the phosphorylation of interleukin-1 receptor-associated kinase 4 (IRAK4) (22, 23). IRAK4 then activates both IRAK1 and TRAF-6 and 3, which triggers IRF7 leading to production of type I IFN (24). Experiments revealed using IRF-7$^{-/-}$ mice have emphasized that IRF-7 is a master regulator of type I IFN production. For example, fibroblasts and DC's from IRF-7$^{-/-}$ knockout mice have shown that such cells do not produce IFN-α/β in response to stimulation with poly(I:C) or virus infection underscoring the necessity of TBK-1 phosphorylation of IRF-7 in type I IFN signaling (25).

Of course, since TLR7/8 and 9 are predominantly associated with the production of type I in certain types of dendritic cells, it is unlikely that these PRRs are deregulated in cancers not originating from these types of cells. However, the TLR3 pathway is utilized in a wider range of cell types and may indeed be suppressed in various tumor types.

1.2. The RLH Pathway

A second key pathway that is responsible for producing type I IFN in response to RNA virus infection, in all cells but pDCs (which are TLR7 dependent), is referred to as the RLH (RIG-like helicase) pathway (26). Activation of this cascade relies on two DExD/H helicases referred to as retinoic acid inducible gene-I (RIG-I) and melanoma differentiation antigen 5 (MDA-5). RIG-I recognizes 5′-triphosphates on viral RNAs, such as those encoded by negative-stranded viruses such as vesicular stomatitis virus (VSV) and Newcastle Disease Virus (NDV). By contrast, MDA-5 recognizes longer RNAs (>1 kb) such as those encoded by positive-stranded viruses such as the picornavirus encephalomyocarditis virus

(EMCV) (27, 28). Binding of viral RNA leads to a conformational change in the helicases which exposes N-terminal Caspase Activation and Recruitment Domains (CARD), which facilitate a CARD-CARD interaction with a second CARD carrying mitochondrially associated protein referred to as Interferon-β Promoter Stimulator 1 (IPS-1/VISA/MAVS/CARDIF) (29, 30). IPS-1 is then able to mediate the phosphorylation of TRAF 3 and trigger phosphorylation of TBK1 and IKKi, leading to the phosphorylation of IRF-3 and 7 (20, 31). Concomitantly, IPS-1 also activates the NF-κB pathway through Fas Associated Death Domain (FADD) and Receptor Interacting Protein 1 (RIP-1) (32, 33). This is important, since as mentioned earlier, IRF-3, NF-κB, and AP-1 are required for maximal production of IFN-β. Even though the RLH and TLR pathways are activated by unique PAMPs, it is important to remember that they both signal through TBK-1 and IRF-3/7, as well as the NF-κB pathway. Thus, the RLH pathway is largely responsible for recognizing RNA viruses and triggering the production of type I IFN.

Despite this progress, little was known relating to the recognition of DNA pathogens, such as parasites and DNA viruses, by the host innate immune system. A number of DNA viruses are known to remain latent in the cell for many years and viruses such as human papilloma virus (HPV) and Epstein-Barr Virus (EBV) are known to contribute toward tumorigenesis (34). However, a molecule referred to as STING (Stimulator of interferon genes) has recently been shown to be responsible for regulating the production of type I IFN by intracellular DNA, as described next.

1.3. The STING Regulated Pathway

The Interferon stimulatory DNA (ISD) pathway is the least well characterized of the signaling pathways that lead to the production of type I IFN. ISD signaling plays a critical role in responding to DNA viral infections and may be defective in many types of cancer (35). Current data have indicated the existence of cytosolic DNA sensors such as a molecule referred to as DAI, which may indeed play a role in the production of type I IFN (36). However, a key intracellular DNA sensor(s) responsible for triggering the production of type I IFN has not yet been discovered. Nevertheless, a recently identified essential component for DNA-mediated IFN production has been found and is referred to as STING (Stimulator of Interferon Genes). STING is an endoplasmic reticulum-associated protein that is indispensible for activation of IFN-β in response to a variety of DNA pathogens such as herpes simplex virus (HSV) and some RNA viruses such as VSV (implicating a role for STING in regulating RIG-I activity). Upon activation STING associates with TBK-1, which can activate IRF-3, IRF-7, and NF-κB responsible for driving expression of Type I IFN (37, 38). Given the importance of STING in governing this pathway, it is possible that defects in the STING regulated pathway (SRP) could

explain the ability of DNA viruses such as vaccinia virus, HSV, and adenovirus to mediate viral oncolysis. Further research will undoubtedly reveal other components of this pathway that could be deregulated in a wide variety of tumors.

Needless to say, defects in these pathways may lead to the suppression of type I IFN production and other key antiviral genes activated through this primary innate immune signaling cascade. This may enable viruses to replicate in an uncontrolled way leading to cellular lysis. Presumably, viruses that infect normal cells trigger these potent host defense mechanisms and suffer deleterious consequences to their replication. However, it is plausible that the primary innate immune pathway remains intact in many cancer types and it may be that the JAK/STAT pathway responsible for triggering the production of antiviral genes may be defective (39). In addition, key IFN-inducible genes, such as PKR, themselves may be defective. In this light, many of the key components of the RLH and TLR as well as STING pathways, such as TLR3, RIG-I, MDA5, and IRF7, are IFN-inducible themselves (40). Thus, a defect in JAK/STAT signaling would prevent the production of such molecules that may enable the efficient production of type I IFN in the infected cell. Given this, we also include a section of the analysis of the JAK/STAT pathway.

1.4. JAK/STAT Signaling

IFN-α/β can be secreted from most cell types, and TLR7-dependent pDCs are able to produce type I IFN in very high quantities. Type I IFN can act in an autocrine and paracrine manner and bind to and activate the IFN-α/β receptor (IFNAR), which is composed of two subunits IFNAR1 and IFNAR2 (41, 42). Activation of the IFNAR results in a triggering of the JAK-STAT signaling cascade. After engagement with type I IFN, JAK1 associates with the intracellular domain of IFNAR, as well as TYK2 (43). Additionally, JAK1 has tyrosine kinase activity, which allows it to phosphorylate the phosphotyrosine-binding domain (SH2) on SH2 containing STATs. Phosphorylated STATs then homo- or heterodimerize (42). Although STAT-1 and STAT-2 are commonly activated by type I IFNs, additional STAT molecules including STAT-3, -4, -5, and -6 can also be activated by this pathway (44). Predominantly, hetero- or homodimerized STAT-1/2 associate with an additional molecule, IRF9, prior to the complex (IFN-stimulated gene factor 3 – ISGF3) translocating into the nucleus. After translocation into the nucleus ISGF3 binds to IFN-stimulated response elements (ISRE) or IFN-activated sites (GAS) in the promoters of more than 100 IFN stimulated genes (ISGs) such as PKR, Mx, OAS, IFI6, and ISG15 which results in their transcriptional activation (45). Furthermore, IFN activated JAK-STAT signaling can activate additional pathways including the p38 mitogen activated protein kinase (MAP kinase) and the phosphatidylinositol 3 kinase (PI3K) pathways (45, 46). Thus, a defect in

this pathway would suppress the induction of type I IFN itself, since TLR3, RIG-I/MDA5, and IRF7 as well as IRF9 are IFN-inducible, as well as a plethora of important IFN-inducible antiviral genes such as PKR (40). While it is highly plausible that key IFN-regulated antiviral gene products could be mutated in numerous cancers, this prospect has been difficult to assess due to the large number of genes induced by IFN. As mentioned, key IFN-inducible genes that could be damaged in cancer include the following: PKR, 2,5 OAS, MX1/2, RNase L, ISG15, IFI6, CASP3, STAT-1, CFB, IRF7, TAP2, IL6, RANTES, and IL15 (40).

It is also noteworthy that other elements of the innate immune response that does not directly involve IFN could also be damaged in cancer. For example, all viruses require translation, and conceivably ribosomal checkpoints normally responsible for preventing the unscheduled translation of viral mRNAs could also be commonly defective in cancer cells. For example, Ras-transformed MEFs or telomerase-immortalized human fibroblasts are highly susceptible to VSV infection due to aberrant eIF2B-guanine nucleotide exchange rendering these cells insensitive to the phosphorylation of eIF2B by PKR in response to viral infection (47). Another defect in cancer cells that could facilitate increased viral replication may occur in RNA trafficking as shown by knockdown of NFAR, a molecule that is important for regulating RNA export from the nucleus (48). Additionally, cancer cells could also subvert the IFN response through translational repression of genes that lead to the expression of IFN such as IRF7, which can be repressed at the RNA level by 4E-BPs, a negative regulator of IFN signaling, leading to increased viral replication (49). Furthermore, IFN can activate transcription of p53 through an ISRE in its promoter and first intron. p53 is a putative regulator of cell cycle, apoptosis, and senescence and is commonly mutated in cancer, which can inhibit its function and lead to deregulated replication (50). p53 can also positively feedback and lead to an increase in IFN production by upregulating TLR3 (14). IFN can also regulate micro RNAs (miRNA) such as mir-122, which plays a role in the establishment of an antiviral state, or mir-21, which is downregulated by STAT3, and contributes to IFNs antitumor effects observed in glioma (51, 52). Moreover, cancer cells can inhibit the transcription of tumor suppressors and other genes by altering the methylation status of the promoters or genomic sequences of these genes, thereby preventing their production (53).

1.5. Innate Immune Defects in Cancer

A variety of evidence now indicates that innate immune pathways may be suppressed in cancer. For example, documented defects include but are not limited to alternative spliced isoforms of IRF-3 in hepatocellular carcinoma (54), CpG methylation of IRF-7 in gastric and lung cancers (55), mutations of CYLD in skin appendage tumors (56), reduced ability to phosphorylate STAT-1 and

activate transcription of IFN stimulated genes (ISGs) in melanoma (57, 58), mutations in JAKs leading to constitutive activation in childhood ALL (59), and TRAF-3 mutations in multiple myeloma and some B-cell lymphomas (60, 61). As a result, many cancer cells are unable to mount an effective immune response against infection while surrounding normal tissues are well protected by an intact innate immune system.

However, although TBK-1 is a critical regulator of type I IFN signaling this kinase also appears to have an unexpected role in the oncogenesis. Recently, it has been illustrated using siRNA knockdown that TBK-1 is required for KRAS-driven tumorigenesis in lung cancer. In this KRAS model, TBK-1 specifically activates NF-κB signaling leading to production of antiapoptotic proteins such as BCL-XL, which facilitates tumor formation (62). Thus, it appears that important components of the type I IFN pathway such as TBK-1 have multiple functions that can include the production of IFN or the targeting of other transcription factors that activate a different set of genes such as those involves with cell death. In any event, these findings have lead to the concept that TBK-1 targeting for suppression by drugs will prove to be an effective tumor therapy. While such data indicates that TBK-1 knockdown does inhibit KRAS-dependent tumor growth it may also suppress the general antiviral response in healthy cells. This would make normal cells highly susceptible to viral infection.

The assays described in this chapter can be used to determine where defects in type I IFN signaling occur though the pathways emphasized above. These assays include IFN-α/β luciferase assays used to assess activation of the IFN-α/β promoter, western blotting for native and active phosphorylated forms of signaling proteins, ELISA to determine if IFN-α/β proteins are being produced and secreted, qRT-PCR to evaluate whether transcription of downstream targets regulated by type I IFN is occurring, and immunostaining for IRF-3 or 7 to determine if these transcription factors are translocating to the nucleus. Furthermore, it is possible establish if defects occur up or downstream of TBK-1 or IFN-α/β production by activating each pathway specifically. This can be done by transfecting cDNA plasmids; TRIF (TLR), IPS-1 (RLH), STING (ISD), TBK-1(IRFs, JAK-STAT), nucleotide analogs; Poly (I:C) (TLR), Poly (dA:dT) (ISD), pretreating cells with recombinant IFN-α/β protein (JAK-STAT), or infecting cells with DNA or RNA viruses.

2. Materials

2.1. Activation of Innate Signaling Pathways

1. 293-T cells (or other cancer cell line).
2. Opti-MEM reduced serum media (GIBCO).
3. Lipofectamine 2000 (INVITROGEN).

4. Plasmids: pCDNA-3.1 (vector control), pCDNA-3.1-IPS-1, pCDNA-3.1-TRIF,pCDNA-3.1-RIG-I,pCDNA-3.1-STING, pCDNA-3.1-TBK-1, poly (I:C), poly (dA:dT).
5. Recombinant Vesicular Stomatitis Virus expressing Green Fluorescent Protein (VSV-GFP).

2.2. Cell Culture

1. 293-T cells: Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% antibiotic/antimycotic solution (Gibco).
2. 0.05% trypsin-EDTA (Gibco).
3. Sterile phosphate buffer saline (PBS) (Gibco).

2.3. SDS-Polyacrylamide Gel Electrophoresis

1. RIPA Buffer: 50 mM Tris–HCl pH 7.8, 150 mM NaCl, 5 mM EDTA, 1% NP-40 (IGEPAL), 0.1% SDS, 0.75% sodium deoxycholate, 1 mM DTT, 10 mM NaF, deionized water, and protease inhibitor tablet (complete) 1 tablet per 50 mL (Roche), .1 mM PMSF (add PMSF just before use) store aliquots at –20°C.
2. Separating buffer 1.5 M Tris–HCl pH 8.8.
3. Stacking buffer 0.5 M Tris–HCl pH 6.8.
4. 40% Stock (19:1 Ratio) acrylamide/bis-acrylamide (Sigma).
5. 10% SDS solution (Gibco).
6. *N*,*N*,*N*,*N*′-Tetramethyl-ethylenediamine (TEMED) (EM Science).
7. 10% ammonia persulfate (APS) in water (Store at –20°C).
8. dH_2O.
9. 100% isopropanol.
10. 6× SDS sample loading buffer: 1.21 g SDS, 6 mg bromophenol blue, 4.7 mL glycerol, 1.5 mL 0.5 M Tris–HCl pH 6.8, 2.1 mL dH_2O; shake and warm until dissolved, then add 0.93 g DTT, aliquot and store at –20°C.
11. Running buffer (10×): Tris–HCl 121 g, HEPES 238 g, SDS 10 g, dH_2O to 1 L.
12. Precision plus protein standard kaleidoscope (Bio-Rad).
13. Coomassie plus protein assay reagent (Thermo Scientific).
14. Albumin standard (Thermo Scientific).

2.4. Western Blotting for Innate Signaling Proteins

1. Transfer buffer (1×): Tris Base 3 g, glycine 14.4 g, 200 mL methanol, dH_2O to 1 L.
2. BioTrace polyvinylidene fluoride transfer membrane (PVDF) 0.45um (Pall Corp.).
3. 100% methanol.

4. PBS-T: PBS (Gibco) with 0.1% TWEEN-20.
5. Blocking buffer: 5% (w/v) nonfat dry milk in PBS-T.
6. Primary antibody dilution buffer: PBS-T with 1%(w/v) bovine serum albumin (BSA) (Sigma).
7. Primary antibodies: see Table 1 below.
8. Secondary antibody: goat anti-mouse IgG or goat anti-rabbit IgG (per the primary antibody) conjugated to horseradish peroxidase (Santa Cruz).
9. Detection reagents: SuperSignal West Pico stable peroxide solution and luminol enhancer solution (Thermo Scientific).
10. X-ray film (Kodak).
11. X-ray film cassette (BIO-RAD).

2.5. IFN-α/β Luciferase Reporter Assay

1. Opti-MEM reduced serum media (GIBCO).
2. Lipofectamine 2000 (INVITROGEN).
3. Firefly luciferase reporter plasmid (pIFN-α/β-luc).
4. Renilla luciferase transfection control plasmid (pRLTK).
5. pCDNA-3.1, pCDNA-3.1-IPS-1, pCDNA-3.1-TRIF, pCDNA-3.1-RIG-I, poly (I:C), poly (dA:dT).
6. Recombinant vesicular stomatitis virus expressing green fluorescent protein (VSV-GFP).
7. Luciferase assay system (PROMEGA).
8. Luminometer (Turner Designs).

2.6. ELISA for hIFN-α/β

1. Verikine human IFN-α/β ELISA kit (PBL-Interferon Source).
2. Supernatants from treated cells.
3. Microplate reader.

2.7. qRT-PCR for mRNA Levels of Type I IFN-Induced Proteins

1. RNeasy kit (Qiagen).
2. QIAshredder (Qiagen).
3. Thin-walled PCR tubes.
4. AMV reverse transcriptase (Promega).
5. 10× reverse transcriptase buffer (Roche).
6. dNTP (Promega).
7. 25 mM $MgCl_2$.
8. Random hexamer (or oligo d(T)) (Promega).
9. RNase inhibitor (Promega).
10. PCR-grade H_2O.
11. Taqman Probe (ABI).

Table 1
Primary antibodies for Western blotting

Antibody	TLR pathway	RLH pathway	SRP pathway	Jak/Stat	ISGs	Company
TLR 3	✓					ebiosciences
TLR 7	✓					Alexis
TLR 8	✓					Abcam
TLR 9	✓					Abcam
MyD88	✓					Stressgen
IRAK 1	✓					Santa Cruz
IRAK 4	✓					Upstate
TRAF 6	✓					Santa Cruz
RIP-1	✓	✓				Abcam
p38 MAPK	✓			✓		Santa Cruz
IKKi	✓	✓	✓			Calbiochem
IkBa	✓	✓	✓			Santa Cruz
pIkBa	✓	✓	✓			Cell Signaling
IkBb	✓	✓	✓			Santa Cruz
NF-kB	✓	✓	✓			Cell Signaling
TBK-1	✓	✓	✓			Abcam
IRF 3	✓	✓	✓			Zymed
pIRF 3	✓	✓	✓			Abcam
IRF 7	✓	✓	✓		✓	Abcam
RIG-I		✓	✓		✓	Cell Signaling
MDA-5		✓				Abcam
IPS-1		✓				Alexis
TRAF 3		✓				Abcam
TANK		✓				Santa Cruz
FADD		✓				Upstate
pFADD		✓				Cell Signaling
Fas		✓				Upstate
TRIF	✓					Alexis
STING			✓			IMGENEX
JAK 1				✓		Biosource

(continued)

Table 1 (continued)

Antibody	TLR pathway	RLH pathway	SRP pathway	Jak/Stat	ISGs	Company
STAT 1				✓	✓	Upstate
pSTAT-1				✓	✓	Upstate
STAT 2				✓		Upstate
pSTAT-2				✓		Upstate
STAT 3				✓		Millipore
STAT 5				✓		Millipore
IRF 9				✓		Abcam
Tyk2				✓		Sigma
Gab				✓		Santa Cruz
PI3K				✓		Santa Cruz
CBP/p300				✓		Upstate
IFN-α	✓	✓	✓	✓		Abcam
IFN-β	✓	✓	✓	✓		Abcam
p53					✓	Santa Cruz
PKR					✓	Santa Cruz
MX1					✓	Santa Cruz
RNase L					✓	Millipore
ISG15					✓	Cell Signaling
IFIT3					✓	Abcam
RANTES					✓	Abcam

12. LightCycler FastStart DNA master hybridization probes (Roche).
13. 18S RNA probe.
14. DNA-free (Ambion).
15. Capillary cooling block.
16. 20 μl light cycle capillaries (Roche).
17. PCR machine (Eppendorf).
18. qRT machine (Light Cycler) (Roche).
19. Light cycler carousel centrifuge (Roche).

2.8. Immunostaining for IRF-3, -7 Nuclear Translocation

1. Sterile PBS.
2. Bovine serum albumin (BSA) (Sigma).
3. Blocking buffer 1% BSA in PBS.
4. Triton X-100 0.2% in PBS.
5. Formaldehyde 4% in DMEM.
6. Primary antibody: IRF-3, -7 (Santa Cruz).
7. Fluorophore-conjugated secondary antibody (Sigma).
8. 4′,6-diamidino-2-phenylindole (DAPI).
9. Microscope slides (VWR).
10. Mounting media: ProLong gold antifade reagent (Invitrogen).

3. Methods

3.1. Activation of Innate Signaling Pathways

It is possible to activate these pathways either by transfection (step 2), pretreatment with recombinant IFN-α/β (step 3), or VSV infection (step 4).

1. In a 12-well cell-culture plate seed 2×10^5 HEK-293 T cells (or other cancer cell line) and allow to adhere overnight (O/N).
2. Prepare the transfection in 1.5-mL eppendorf tubes. Each transfection will require two tubes. In the first tube add 250 μL serum free Optimem and the needed amount of Lipofectamine 2000 (~1 μl/μg DNA). In the second tube, add 250 μL serum free Optimem and the plasmid cDNA or the DNA or RNA analog to be transfected (pCDNA3.1-IPS-1, TRIF, STING, RIG-I, TBK, poly (I:C), poly (dA:dT)). Allow the tubes to sit separated at room temperature (RT) for 10 min. Then, combine and mix the tubes by inverting and incubate at room temperature for 20 min. After the incubation, remove the media from the cells and wash one time with PBS. Then, plate the transfection mix and incubate for 3–4 h at 37°C. Following this incubation, remove the transfection from the cells and add complete media.
3. To activate cells by pretreating with IFN-α/β prepare a stock of complete media with IFN-α/β add such that the concentration is 100–500 U/mL. Remove media form the plate and add 1 mL of IFN-α/β complete media.
4. In order to prepare the virus for infection first count one well of cells. This will allow calculation of the multiplicity of infection (MOI), which generally ranges from 0.01 to 10 for VSV. After counting the cells remove a stock of purified, titered virus from −80°C and calculate the amount of viral stock needed (see Note 1). Add the amount of virus stock to 300 μl serum

free media. Remove the complete media from the cells, wash one time with PBS, and plate the infections. Incubate for 1 h at 37°C. Then, remove the virus infection wash two times with PBS and add complete media.

5. Incubate for desired time 4–48 h at 37°C and use for the following assays.

3.2. Preparation of Samples for Western Blotting

1. Seed 293 T cells in 12-well plates at a density of 2×10^5 cells/well and allow to adhere O/N.
2. Activate the cells as previously described in Subheading 3.1. Transfect with cDNA, nucleotide analogs, or infect with virus or treat with 100–500 U/mL of recombinant human IFN-α/β.
3. After desired incubation period generally 4–24 h prepare cells for lysis by collecting the supernatant in a 1.5-mL tube and freezing at –80°C washing one time with cold PBS (see Note 2).
4. Lyse the cells by adding 50–100 μl (depending on protein concentration desired and number of gels to run) of ice-cold RIPA buffer. Rock plates for 5 min at 4°C and transfer lysate to 1.5-mL eppendorf tube and centrifuge for 2 min at 13,000 ×*g*. Keep samples on ice.
5. Quantify the amount of protein in each sample using Coomassie Plus Protein Assay reagent and a spectrophotometer reading at 595 nm absorbance. First prepare a standard curve using albumin standard. Make six serial dilutions from a beginning stock of 2 μg/mL. Add 2 μl of each dilution to 1 mL Coomassie and read at 595 nm absorbance (see Note 3). After generating the standard curve, read each sample by adding 2 μl of sample to 1 mL of Coomassie reagent, vortex briefly, add to cuvette, and read at 595 nm absorbance.
6. Prepare the samples by adding 20 μg of total protein to 5 μl of loading buffer. Boil the sample at 100°C for 5 min. Then, centrifuge the sample from 2 min at 13,000 RPM. The samples are now ready for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

3.3. SDS-PAGE

1. This protocol is designed for use with the BIORAD MINI-Protean gel system. It is, however, easily adaptable to other formats including precast and larger gels. It is essential that the gel casting plates are cleaned prior to use with dH_2O and 70% ethanol. Dry the plates with a Kimwipe.
2. Prepare a 1.0-mm thick 10% gel by mixing 4.8 mL dH_2O with 2.5 mL 40% acrylamide/bis-acrylamide solution, 2.5 mL 1.5 M Tris–HCl pH 8.8, 100 μl 10% SDS, 100 μl 10% APS, and 10 μl TEMED for a total of 10 mL, enough for two gels. Pour the gel leaving space for the stacking gel and overlay with 100% isopropanol. The gel should polymerize in 15–20 min.

3. Pour off the isopropanol, and wash the top of the gel with water. Remove the remainder of the water using a kimwipe or blotting paper.
4. Prepare a 5% stacking gel by mixing 3 mL dH_2O with 625 µl 40% acrylamide/bis-acrylamide solution, 1.25 mL 0.5 M Tris–HCL pH 6.8, 50 µl 10% SDS, and 50 µl 10% APS, 8 µl TEMED for a total of 10 mL, enough for two gels. Pour the stacking gel to the top of the glass plate and insert the comb. The stacking gel should polymerize in 15–20 min.
5. Prepare the running buffer by diluting 100 mL of the 10× running buffer with 900 mL dH_2O.
6. Once the stacking gel has set, carefully remove the comb and use a squirt bottle to wash the wells and remove bubbles with dH_2O.
7. Assemble the gel unit. Be sure to check that the casting plates form a good seal with the rubber insert.
8. Add the running buffer to the inner and outer chambers of the gel unit and load the sample to each well. Include one well for prestained molecular weight marker.
9. Complete the assembly of the gel unit and connect to a power supply. Set the power supply to constant volts and run the gel at 80–100 V. The dye front (blue) can be run off the gel if desired.

3.4. Western Blotting for Innate Immune Proteins

1. After the samples have been separated by SDS-page the protein needs to be transferred to a PVDF membrane electrophoretically. Cut pieces of PVDF membrane just larger than the gel 8 mm × 6 mm and soak in 100% methanol for at least 30 min (see Note 4).
2. These directions assume the use of a BIO-RAD transfer system. Set up a tray of transfer buffer that is large enough to accommodate the transfer cassette with the foam pad and two pieces of blotting paper submerged on the anode (clear plastic) side. Place the PVDF membrane on top of the submerged blotting paper.
3. Disconnect and disassemble the gel unit from the power supply and remove and discard the stacking gel. Rinse the separating gel with 1× transfer buffer (in the tray) and place on top of the PVDF membrane. Check to make sure there are no bubbles between the gel and the membrane. If bubbles are present they can be rolled out using a sterile serological pipette or by gently guiding them using a finger.
4. Carefully place two more pieces of blotting paper that has been prewet in 1× transfer buffer on top of the gel, taking care to not introduce bubbles into the resulting sandwich. Place the second foam pad on top of the blotting paper and close the transfer cassette.

5. Place the cassette into the transfer tank such that the PVDF membrane is between the gel and the anode. This orientation is critical for the capture of proteins, if placed in backward proteins will be run into the buffer and be lost.
6. Place an ice block into the transfer box and fill with 1× transfer buffer (see Note 5).
7. Put the lid on the tank and set the power supply for constant amperes. Run the transfer at 240 mA for 90–110 min depending on the protein target size.
8. Once the transfer is complete and the cassette has been removed and disassembled remove the PVDF from the gel. The molecular weight ladder should be clearly visible on the membrane.
9. Place the membrane in 10 mL of blocking solution and shake for 1 h at RT on a rocking or shaking platform.
10. Discard the blocking solution and wash the membrane 3× for 5 min with PBS-T.
11. Add the primary antibody in PBS-T/1% BSA. Incubate overnight at 4°C on a rocking platform. Dilutions generally range from 1:250 to 1:1,000 depending on which primary antibody is being used.
12. Remove the primary antibody and wash the membrane 3× for 5 min with 10 mL PBS-T.
13. Prepare the secondary antibody in blocking buffer at a 1:5,000 dilution. After the final wash add 10 mL to the membrane and incubate for 1 h at RT on a rocking or shaking platform.
14. Discard the secondary antibody and wash the membrane 3× for 10 min each with PBS-T.
15. After the final wash prepare the ECL reagents by mixing equal volumes of substrate and luminol enhancer. Remove excess PBS-T by removing the membrane and blotting one corner with a paper towel. Add the mixed ECL reagents to the membrane and shake by hand for 1 min to ensure complete coverage.
16. Remove the blot from the ECL reagents and blot onto a paper towel to remove excess reagent. Place the membrane into the leaves of a plastic sheet protector and place into a film cassette.
17. Take the cassette and X-ray film to a dark room for developing. Place a piece of X-ray film into the cassette for a suitable exposure time (see Note 6). After the exposure develop the film.

3.5. IFN-α/β Luciferase Assay

1. In a 12-well cell-culture plate, seed 2×10^5 HEK-293-T cells allow to adhere overnight (O/N).
2. Treat cells as previously described in Subheading 3.1.

3. After the desired incubation time prepare a 1× solution of Cell Culture Lysis Reagent (CCLR) (PROMEGA) from the 5× stock solution with PBS. Remove the media from the cells and wash one time with PBS (see Note 7). Add 100 µl 1× CCLR to each well and rock the plate for 5 min at 4°C. Then, transfer the lysate to 1.5-mL eppendorf tubes and centrifuge for 2 min at 13,000 RPM.
4. After preparing the sample turn on the luminometer (Turner Designs TD 20/20) to run the luciferase assay. First read the firefly luciferase by adding 10 µl of the cell lysate with 10 µl firefly luciferase substrate in a 0.5-mL tube. Mix by pipetting up and down three to four times and then placing the tube in the luminometer. Set the luminometer to wait for 2 s and then integrate (read) the signal for 10 s. The value it returns represents the amount the IFN-β promoter was induced. Next, read the renilla luciferase by adding 10 µl of lysate to 10 µl renilla luciferase substrate and mix by pipetting three to four times. Then, read the as described above. This value represents the transfection control (see Note 8).

3.6. ELISA for IFN-α/β

1. Treat the cells as previously described in Subheading 3.1. After the desired incubation time remove and collect the supernatant into 1.5-mL eppendorf tubes. Centrifuge the supernatants to remove any cells. These can then be stored at −80°C if the ELISA is not being run immediately after collection.
2. Prepare 1× wash buffer from the 10× wash concentrate and prepare a 1:10 working stock of the mouse IFN-β standard by pipetting 10 µl mouse IFN-β standard (Vial B) in 90 µl of sample diluent (Bottle C). From this working stock (50,000 pg/mL) prepare the standard curve, with the first dilution being 1,000 pg/mL and serial diluting 1:1 in assay diluent to 15.6 pg/mL and one blank. Prepare fresh dilutions of the standard curve for each assay run.
3. Determine the number of precoated wells needed to run the assay. Add precisely 100 µl of interferon samples and standard curve prepared in the previous step in individual wells of the microplate in duplicate (see Note 9).
4. Cover the plate with a plastic plate sealer (included in the kit) and incubate for 60 min at RT. After the 60 min incubation was the plate 3× with at least 250 µl wash solution blotting well after the final wash.
5. During the incubation, prepare the antibody solution. Dilute the Antibody Concentrate with Concentrate Diluent, refer to the Certificate of Analysis (COA) to determine the correct amounts of each reagent to use. Store unused undiluted antibody concentrate at 2–8°C for future use.

6. Add 100 µl of the antibody solution prepared using the COA to each well. Cover the plate with a plastic plate sealer (included in the kit) and incubate for 60 min at RT. After the 60 min incubation was the plate 3× with at least 250 µl wash solution blotting well after the final wash.
7. During the incubation prepare the HRP solution. Dilute the HRP Concentrate with HRP conjugate Diluent, refer to the Certificate of Analysis (COA) to determine the correct amounts of each reagent to use. Store unused undiluted antibody concentrate at 2–8°C for future use.
8. Add 100 µl of the HRP solution prepared using the COA to each well. Cover the plate with a plastic plate sealer (included in the kit) and incubate for 60 min at RT. After the 60 min incubation was the plate 3× with at least 250 µl wash solution blotting well after the final wash.
9. During the HRP incubation period warm the TMB substrate solution to RT. Add 100 µl of the TMB substrate solution to each well. Incubate for 15 min at RT in the dark. Do not seal the plate during this step.
10. Add 100 µl of Stop solution to each well. Mix gently using caution to avoid bubbles as this can affect results. Using a microplate reader, determine the absorbance at 450 nm within 5 min of adding the stop solution. Unknown sample concentrations can be determined by extrapolation off the standard curve.

3.7. qRT-PCR for mRNA Levels of Type I IFN-Induced Proteins

1. Treat the cells as previously described in Subheading 3.1.
2. Harvest the mRNA using the mRNeasy kit form Qiagen. Determine the yield using a spectrophotometer (see Note 10).
3. First cDNA must be synthesized from the RNA template. Label the required number of thin walled PCR tubes on a cooling block or ice. Add up to 1 µg of total RNA in a total volume of 5 µl. Include one tube with only water as a negative control (see Note 11). Denature the RNA for by placing the tubes in a thermocycler at 65°C for 10 min.
4. During the RNA denaturation prepare the cDNA master mix. For 20 µl of cDNA (enough for 10 qRT reactions) add 3.2 µl PCR-grade water, 2 µl 10× RT buffer, 0.8 µl AMV RT, 2 µl dNTP, 4 µl 25 mM $MgCl_2$, 2 µl Random hexamer (or oligo d(T)), and 1 µl RNase inhibitor (see Note 12).
5. Spin PCR tubes quickly in a bench top centrifuge and add 15 µl of the master mix to each tube. Mix by pipetting up and down a few times, do not vortex. Perform the cDNA synthesis in a thermocycler with the following program. 10 min 25°C, 60 min 42°C, 5 min 99°C (denature), Hold at 4°C. Use 2 µl of the reaction product as template for qRT-PCR in the light cycler.

6. If using Taqman probes, use the LightCycler FastStart DNA Master Hybridization Probes from Roche to set up the PCR mix. Each kit contains three tubes labeled 1b and one tube labeled 1a. To make the PCR mix add 10 μl of enzyme (tube 1a) to a tube of buffer mix (tube 1b). Make the PCR master by mixing the following components: PCR mix 4 μl, taqman probe 1 μl, 18S rRNA probe 1 μl, and H_2O 12 μl (see Note 13).
7. Take the capillary cooling block out of the refrigerator and place the required number of 20 μl light cycler capillaries in to the slots. Add 2 μl cDNA sample to each capillary tube, taking care not to jam the tip in too far and break the capillary. Apply 18 μl of the PCR master mix to each capillary and mix by pipetting. Place the capillaries onto the carousel provided for the light cycler and spin down the capillaries to get the samples to the bottom using the light cycler the LightCycler carousel centrifuge.
8. Load the carousel in the LightCycler and execute the program. The run takes approximately 1.5 h.

3.8. Immunostaining for IRF-3, -7 Nuclear Translocation

1. On the day before treatment seed cells in slide chamber plates or on 15 mm cover slips in a 10-cm cell-culture plate (Confocal microscope). Alternatively, this can be done in 12-well plates (Fluorescent microscope).
2. Treat the cells as previously described in Subheading 3.1. After the desired incubation time (1–4 h), wash the cells 2× for 5 min with PBS.
3. Fix the cells with 4% formaldehyde for 15 min at 37°C. Wash 3× for 5 min with PBS.
4. Permeabilize the cells with 0.2% Triton X100 for 5 min at 4°C. Wash the cells 3× with PBS.
5. Add blocking buffer and incubate overnight at 4°C. Remove the blocking buffer and add primary antibody (mouse anti-IRF-3) in PBS with 1% BSA and incubate for 1 h at RT. During this incubation remove the mounting media from the freezer.
6. After the primary antibody incubation wash 3× for 5 min with PBS. Next add a secondary antibody that is conjugated to a fluorophore (mouse anti-FITC green or CY3 red) Incubate at RT for 30 min. Wash 3× for 5 min with PBS.
7. Add DAPI 1 μg/mL to stain the nucleus. Incubate for 10 min at RT. Wash 3× for 5 min with PBS.
8. Mount slides and allow to dry for ~12 h (see Note 14).
9. Store in the dark (or wrap in tin foil) to prevent photobleaching and analyze by confocal microscopy within 24 h of staining.

3.9. Conclusion

In conclusion, it is highly plausible that defects in innate immune signaling pathways involving the production of type I IFN, or JAK/STAT pathway, or in key IFN-inducible genes themselves, may enable the replication of viruses to proceed. Research into these processes will undoubtedly shed light into mechanisms of oncolysis, which may be frequently defective in cancer, and so bring to light important causes of tumorigenesis. Understanding these processes will also enable the design of new oncolytic agents that could exhibit more specific and enhanced antitumor activity (6). Further unraveling of these important signaling cascades, thus, affords a multitude of opportunities to combat malignant disease.

4. Notes

1. The equation used to determine the amount of virus needed for infection of cells (# of cells × Multiplicity of Infection (MOI))/Virus Titer = mL viral stock needed.
2. If cells were infected with virus many cells may be floating in supernatant at later time points. These supernatants should be spun down to pellet and remove the cells prior to storage.
3. It is best to add the 2 μl of sample to the Coomassie just prior to mixing and reading. As samples sit for a longer period of time in the Coomassie reagent, the color change becomes more pronounced and higher OD readings are returned.
4. This can be done while the SDS-page gel is running.
5. Alternatively, place the transfer box in a bucket and surround it with ice or run the transfer in a 4°C cold room.
6. Exposure times can range from 5 s to 10 min depending on the strength of signal Additionally, if the has a strong signal creating a "dirty" blot insert two pieces of film (one on top of the other) and expose. This can reduce the background observed on the film. Furthermore, to maintain the orientation of the film make a crease in the top left hand corner of the film.
7. If cells were infected with virus many cells may be floating in the supernatant. These cells should also be pelleted and lysed.
8. To normalize the data, divide the firefly value by the renilla value. You can then determine the fold induction by dividing the normalized values by either the mock infected or vector transfected control.
9. Samples may need to be diluted 1:5 or 1:10 with assay diluent so that upon completion of the assay the reading will fall within the detectable range of the standard curve. Values can then be multiplied by the dilution factor to determine the concentration.

10. If the RNA will be used to assess IFN-β mRNA levels the samples need to be treated with DNase I (DNA-free kit Ambion) to remove the genomic DNA. This will greatly reduce the background during assessment of IFN-β mRNA.
11. As low as 0.1 μg of RNA is acceptable to use for the cDNA synthesis reaction and in some cases can yield better results.
12. This mix is for 1 cDNA reaction and can be scaled up for as many reactions as needed. If preparing master mix for multiple reactions make 5–10% additional mix to prevent running out when aliquotting it to tubes.
13. This master mix is for one reaction but can be scaled up for as many reactions as needed with a particular probe.
14. More than one primary antibody can be used, useful for studying colocalization, as long as the primary antibodies come from different species and each secondary has a different fluorophore.

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