The IFN-Independent Response to Virus Particle Entry Provides a First Line of Antiviral Defense That Is Independent of TLRs and Retinoic Acid-Inducible Gene I[1]

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The innate immune system responds to pathogen infection by eliciting a nonspecific immune response following the recognition of various pathogen-associated molecular patterns. TLRs and the RNA helicases retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 recognize foreign nucleic acid within endosomal and cytoplasmic compartments, respectively, initiating a signaling cascade that involves the induction of type I IFN through the transcription factors IFN regulatory factor (IRF) 3 and NF-κB. However, a recent paradigm has emerged in which bacterial DNA and double-stranded B-form DNA trigger type I IFN production through an uncharacterized TLR- and RIG-I-independent pathway. We have previously described a response in primary fibroblasts wherein the entry of diverse RNA- and DNA-enveloped virus particles is sufficient to induce a subset of IFN-stimulated genes and a complete antiviral response in an IRF3-dependent, IFN-independent manner. In this study, we show that the innate immune response to virus particle entry is independent of both TLR and RIG-I pathways, confirming the existence of novel innate immune mechanisms that result in the activation of IRF3. Furthermore, we propose a model of innate antiviral immunity in which exposure to increasing numbers of virus particles elevates the complexity of the cellular response from an intracellular, IFN-independent response to one involving secretion of cytokines and activation of infiltrating immune cells. *The Journal of Immunology,* **2006, 177: 8008–8016.**

The cellular innate immune response to invading pathogens constitutes an early defense mechanism aimed at controlling the spread of infection. Central to this response is the induction and secretion of type I IFN including IFNα and IFNβ. The IFN family comprises a group of cytokines that mediate a multitude of cellular and immunomodulatory actions including host defense responses against viruses (1, 2) and bacteria (3). IFNs do not possess direct anti-pathogen activity but instead mediate these actions via the induction of IFN-stimulated genes (ISGs)[3]. In fibroblast and epithelial cells, the induction and secretion of type I IFN is multiphasic (4, 5) and involves the activation of a group of transcription factors known as IFN regulatory factor (IRF) (5, 6). During the initial wave of IFN production, constitutively expressed transcription factors such as IRF3, NF-κB, and activating transcription factor-2/c-Jun are activated and translocate to the nucleus where they interact with the coactivator CREB binding protein/p300 to form an enhanceosome complex on the IFN-stimulated response element within the promoter region of IFNβ and ISGs (7–10). A small amount of IFNβ is subsequently synthesized and acts in a paracrine/autocrine manner through the type I IFN receptor, which results in the induction of other ISGs such as IRF7. In concert with IRF3, IRF7 facilitates the expression of all type I IFN species as well as the full complement of ISGs (5, 11).

The activation of IRF3 is critical during the initial induction of IFN and ISGs. A great deal of effort has been committed to understanding the signaling events that lead to the activation of IRF3. In response to virus infection, cytoplasmic IRF3 is activated following the phosphorylation of C-terminal serine/threonine residues (7, 10, 12) by the noncanonical IκB kinase homologues, TANK-binding kinase (TBK), and IκB kinase ε (13–15). These events cause conformational change that allow IRF3 to homodimerize and translocate to the nucleus where it initiates the transcription of IFN and ISGs (7, 10, 16, 17). An additional phosphorylation event, mediated by the PI3K pathway, is required for the full activation of IRF3 in response to dsRNA (18). To date, TLRs and retinoic acid-inducible gene I (RIG-I) are two recognized mediators that activate IRF3 upon pathogen detection. These mediators function independently of one another but have complementing roles in innate immunity as TLRs recognize both viral and bacterial ligands (19) whereas RIG-I combats intracellular infection via the recognition of dsRNA (20–22).

All identified TLR family members can signal through NF-κB to induce the expression of proinflammatory cytokines and chemokines (23, 24), whereas only TLR3 and TLR4 have been shown to signal through IRF3 to induce the expression of ISGs and IFNβ in response to viral dsRNA and bacterial LPS, respectively (25–28). Several TLR family members have been associated with recognizing and mediating IFN responses against viruses (29, 30). For instance, TLR3, TLR7/8, and TLR9 respond to genomic dsRNA (25), ssRNA (31–33), and unmethylated CpG DNA (34, 35), respectively. In addition, TLR4 has been shown to respond to the wild-type

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[3] Abbreviations used in this paper: ISG, IFN-stimulated gene; IRF, IFN regulatory factor; TBK, TANK-binding kinase; WT, wild type; SeV, Sendai virus; DC, dendritic cell; HEL, human embryonic lung; HCMV, human CMV; VSV, vesicular stomatitis virus; poly(I:C), polyinosinic/polycytidylic acid; L-Gln, L-glutamine; MEF, murine embryonic fibroblasts; MOI, multiplicity of infection; 1°, primary; RIG-I, retinoic acid-inducible gene I.

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(WT) measles virus hemagglutinin protein (36) and respiratory syncytial-virus F protein (37, 38) whereas TLR2 responds to human CMV glycoprotein B (39). A link between HSV-1 infection and TLR-mediated antiviral responses has been demonstrated by the attenuation of proinflammatory cytokine expression in the absence of TLR2 and TLR9 (40, 41).

Although the impact of TLRs on the innate antiviral response has been well documented, there are also studies that suggest the presence of TLR-independent pathways. For instance, a cellular immune response was observed following Sendai virus (SeV) infection in the absence of TLR3, TLR7, and TLR8 (42). Furthermore, the expression of TLR3, TLR7/8, and TLR9 was not required for dendritic cell (DC) maturation in response to SeV infection (43). Malmgaard demonstrated virus-mediated activation of macrophages by both TLR-dependent and independent pathways (44). Most recently, the IFN-inducible RNA helicase, RIG-I, has been shown to induce the expression of IFN in response to intracellular viral dsRNA in an IRF3- and NF-κB-dependent manner but independent of TLRs (22, 45). The importance of RIG-I during the host response to virus infection has also been suggested by the observations that RIG-I regulates cellular permissiveness to hepatitis C virus replication (46) and is the target of the hepatitis C virus NS3/4A protease (47). Moreover, several studies have demonstrated the importance of RIG-I, both in vitro and in vivo, during innate immune responses against a number of different ssRNA viruses (20, 21).

The intricacy of the innate immune response has recently been augmented by the discovery of TLR- and RIG-I-independent mechanisms of pathogen recognition. Recent studies with the bacterium *Listeria monocytogenes* have revealed a novel pathway in which IFN and ISGs are synthesized in response to cytoplasmic DNA following the activation of the TBK/IRF3 pathway independent of TLRs, RIG-I, NF-κB, and mitogen-activated protein kinases (48, 49). Similar results were obtained with DNA isolated from viruses (50), although NF-κB and mitochondrial antiviral signaling but not RIG-I were involved during this antiviral response, thereby indicating a level of distinction between the cellular innate response against bacterial vs viral DNA. In both cases, however, the cellular receptor mediating these innate responses has yet to be identified. Nevertheless, these studies support the essential role of IRF3 during innate immunity and indicate that there are several mediators capable of activating this transcription factor, some of which remain to be described.

In contrast to the IFN-dependent antiviral response described above, studies from our laboratory have identified an IFN-independent, IRF3-dependent antiviral response against enveloped viruses that requires virus particle entry but occurs in the absence of virus replication (51, 52). These studies were conducted in nontransformed and nonimmortalized cell cultures to avoid the defects in IFN signaling that exist in transformed and immortalized cells (53, 54). Using UV radiation to inhibit replication, microarray analysis of 19,000 human genes demonstrated an antiviral response in human embryonic lung (HEL) fibroblasts against nonreplicating HSV-1 virions that did not require IFN (52). In contrast to IFN-mediated ISG induction, the cellular response to virus particles resulted in the expression of a defined subset of ISGs (52). In support of this response, other studies have also demonstrated the induction of ISGs in human fibroblasts in response to HSV-1 (55) and human cytomegalovirus (HCMV) (56–58) in the absence of virus replication. However, a similar gene expression pattern was not observed with replicating HSV-1 because the expression of viral proteins, such as infected cell protein 0 and viron host shutoff, inhibits this response (52, 59). Characterization of the IFN-independent antiviral response demonstrated a requirement for IRF3 and specificity toward enveloped viruses but not nonenveloped viruses such as adenovirus (51). Moreover, this response was observed against viruses with either a RNA or DNA genome, although the RNA viruses tested (SeV, vesicular stomatitis virus (VSV), and NewCastle Disease virus) were also capable of eliciting an ISG response following virus replication (51, 60). In contrast, ISG induction was not observed upon treatment with replicating DNA viruses because these viruses encode proteins that inhibit IRF3 activation and IFN production (59, 61–64). Virus binding and entry are required for the IFN-independent antiviral response following HSV-1 (52, 60) and HCMV (60, 65) infection, which suggests that the interaction with a cell surface receptor is insufficient to trigger the response. However, replication of viral nucleic acid was not required for the induction of this response. Furthermore, ISG and antiviral state induction is observed using both HSV mutants that fail to release their genomes from protein capsids (K. Mossman, unpublished data) and preparations of defective light particles that contain the HSV envelope but neither capsid nor genome (C. M. Preston, personal communication), indicating that this innate immune response does not rely upon recognition of a specific form of nucleic acid. Most recently, a novel PI3K family member has been implicated in this response downstream of IRF3 (66). However, the factors responsible for initiating this response are unknown.

In this study, we examined the role of TLRs and RIG-I in response to virus particle entry. Although TLRs and RIG-I can recognize viral components and signal through IRF3, their involvement in the innate cellular response against enveloped virus particle entry remains unclear. For instance, the expression of proinflammatory genes was not observed in fibroblasts in response to nonreplicating HSV-1 particles in the aforementioned microarray analyses (52), suggesting an absence of NF-κB activation. Conversely, TLR-mediated recognition of HCMV triggered a proinflammatory response in immune cells in the absence of both IFN and ISG induction, suggesting the activation of NF-κB but not IRF3 (39). In light of these data, we initiated a study to investigate the role of TLRs and RIG-I during the host innate immune response to virus particle entry in nontransformed epithelial and fibroblast cells, as these cells represent the first physical barrier to many clinically relevant viral infections.

Materials and Methods

Reagents

The synthetic virus dsRNA mimetic, polyinosinic/polycytidylic acid (poly(I:C); GE Healthcare), was used at a concentration of 10 or 100 μg/ml medium. Human IFNα and IFNA/D (universal IFN, Sigma-Aldrich) were used at a concentration of 250 U/ml medium. LPS (Sigma-Aldrich) was used at a concentration of 2.5 μg/ml medium. Unmethylated CpG DNA (human type C, M362 sequence) and the control CpG DNA (M363 sequence) (67) were prepared with a phosphorothioate backbone (MOBIX Laboratory, McMaster University, Hamilton, Ontario, Canada) and used at a concentration of 3.2 μg/ml medium. Plasmids containing DNA for TLR 1–9 were provided by Dr. B. Williams (Monash Institute of Medical Research, Victoria, Australia). Human TNF-α was harvested from supernatants of Vero cells infected with an adenovirus expressing human TNF-α (AdΔE1.E3huTNFα) (68) and dose-response assays were performed to delineate the optimal amount of supernatant required (data not shown). Cyclohexamide (Sigma-Aldrich) was used at a concentration of 50 μM to pretreat cells for 30 min at 37°C and was added to all medium when used.

Cell lines

HEL fibroblasts, BJ fibroblasts, and U2OS cells were purchased from the American Type Culture Collection (ATCC) and maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine (L-Gln), 100 U/ml penicillin, and 100 μg/ml streptomycin (pen/strep). Vero cells (ATCC) were maintained in DMEM supplemented with 5% FCS, L-Gln, and pen/strep. Beas-2B cells (obtained from S. Erzurum, Lerner Research Institute,

Table I. *Description of the cell lines used in this study*

Name	Organism	Cell Type	Age	Organ	Status
HEL	Human	Fibroblast	Embryonic	Lung	Normal[a]
BJ	Human	Fibroblast	Newborn	Skin	Normal[a]
Beas2B	Human	Epithelial	Adult	Lung	Immortalized
Vero	Monkey	Epithelial	Adult	Kidney	Immortalized
U2OS	Human	Epithelial	Adolescent	Bone	Transformed
MEFs	Mouse	Fibroblast	Embryonic		Normal
PBMCs	Human	Heterogeneous	Adult	Blood	Normal

[a] Nonimmortalized, Nontransformed.

Cleveland, OH) were cultured in flasks or dishes pretreated with coating medium (0.01 mg/ml fibronectin (Calbiochem), 0.03 mg/ml Vitrogen 100 (Cohesion), and 0.01 mg/ml BSA dissolved in LHC-9 medium (BioSource International)) for 10 min at 37°C followed by 10 min at room temperature. Beas-2B cells were maintained in LHC-9 medium supplemented with 10% FCS, L-Gln, and pen/strep. Human PBMCs were provided by Dr. D. Snider (McMaster University, Hamilton, Ontario, Canada) and maintained in RPMI 1640 medium supplemented with 10% FCS, L-Gln, pen/strep, 10 mM HEPES buffer, and 50 μM 2-ME. Murine embryonic fibroblasts (MEFs) were obtained from WT, TRIF$^{-/-}$ (69), MyD88$^{-/-}$ (70), TBK$^{-/-}$ (71), and IRF3$^{-/-}$ (5) mice, and maintained in α-MEM supplemented with 10% FCS, L-Gln, and pen/strep. The characteristics of the cell lines used in this study are outlined in Table I.

Viruses

HSV-1 (KOS strain) and VSV (Indiana strain) were grown on Vero cells whereas HCMV (AD169 strain) was propagated on HEL fibroblasts. Infections were performed with a multiplicity of infection (MOI) of 10 PFU/ml, unless otherwise stated. Infections with SeV (Cantell strain; Charles River Laboratories) were performed at 10 hemagglutinin units per 10^6 cells. All infections were performed at the indicated MOI for 1 h in serum-free medium at 37°C. Virus UV-inactivation was performed using a Stratalinker2400 (Stratagene) for the length of time required to prevent viral gene expression as determined by immunofluorescence microscopy.

VSV plaque reduction assay and antiviral assay

HEL fibroblasts were grown to 100% confluency in 6-well dishes overnight. Cells were subsequently left untreated or treated with increasing amounts of UV-inactivated virus for 1 h (MOI ranged from 1–100 PFU/ml for VSV-UV and 0.01–100 PFU/ml for HCMV-UV), rinsed three times with PBS to remove any unbound virus, and maintained in 5% DMEM overnight (primary (1°) infection). Half of the resulting medium was transferred to confluent 6-well dishes of HEL cells for 8 h (supernatant). Induction of an antiviral response was assessed by challenging monolayers with VSV-GFP, a lytic but IFN-sensitive virus (72) that expresses GFP under the control of a virus promoter (provided by B. Lichty, McMaster University). Levels of GFP fluorescence were visualized and quantified using a Typhoon scanner (GE Healthcare).

PCR analysis

Total RNA was isolated using the TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. RT-PCR was performed with 2 μg of RNA, 200 ng of random 6-mer primer, and 200 U of SuperScript II Reverse Transcriptase (Invitrogen Life Technologies), according to the manufacturer's instructions. PCR was subsequently performed using 1 μl of the RT-PCR or 1 ng of TLR plasmid DNA, 400 nM each primer set, and 0.5 U of *Taq*DNA polymerase (Invitrogen). Primers for TLRs 1–8 were provided by Dr. K. Rosenthal (McMaster University) and used with a 50°C annealing temperature for 30 cycles using the following human primers (5′–3′): TLR1 forward and reverse, CTT ATA AGT GTG ACT ACC CGG and CCA CAA TGC TCT TGC CAG G, respectively (382-bp product); TLR2 forward and reverse, GTT AAC AAT CCG GAG GCT GC and TTG GGA ATG CAG CCT GTT AC, respectively (438-bp product); TLR3 forward and reverse, CCC TTG CCT CAC TCC CC and CCT CTC CAT TCC TGG CCT, respectively (346-bp product); TLR4 forward and reverse, CTG GAC CTC TCT CAG TGT C and GGC AGA GCT GAA ATG GAG G, respectively (619-bp product); TLR5 forward and reverse, TGG GGG AAC TTT ACA GTT CG and CTG GGA TTC TCT GAA GGG G, respectively (379-bp product); TLR6 forward and reverse, GGG TTG AGA GTA TAG TGG TG and GTA GAT GCA GAG GGA GGT C, respectively (548-bp product); TLR7 forward and reverse, CCT CAG CCA CAA CCA ACT G and TTG TGT GCT CCT GGC CCC, respectively (348-bp product); and TLR8 forward and reverse, AAA CTT GAG CCA CAA CAA CAT TT and ATC TCC AAT GTC ACA GGT GC, respectively (580-bp product). TLR9 primers were reported by Droemann and colleagues (2005) and were used with a 48.8°C annealing temperature for 30 cycles with an expected product of 393 bp. Human RIG-I primers were previously reported by Cui et al. (73). IL-6 primers were 5′-GAC CAGAAGAAGGAATGCCC-3′ forward, and 5′-GAGAAGCTCTATCT CCCCTC-3′ reverse, with an expected product of 721 bp. ISG56 PCR was performed as previously described (51). PCR products were visualized on a 1.5% agarose gel containing 1.27 mM ethidium bromide (Invitrogen Life Technologies).

Immunofluorescence microscopy

HEL fibroblasts were seeded onto coverslips so they were 50% confluent after 24 h. Following treatment, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 2% goat serum. Coverslips were incubated with primary Ab (NF-κB (p65), 1/500 (Santa Cruz Biotechnology); HSV-1 infected cell protein 0, 1/1000 (Goodwin Institute); HCMV UL122/123, 1/1000 (Goodwin Institute); SeV 1/1000 (74); VSV glycoprotein G, 1/1000 (Roche)). Cy2- or FITC-conjugated secondary Ab (Jackson ImmunoResearch Laboratories) was used at a concentration of 1/500. All Ab dilutions were performed in 2% goat serum. Nuclei were stained with Hoechst stain (1/20000 dilution) before mounting onto slides. All images were taken using a Leica DM IRE2 microscope and analyzed using Openlab software (Improvision).

Results

TLR expression profile in human epithelial and fibroblast cells

The cellular distribution and expression patterns of TLRs suggest that certain cell types carry out specialized functional roles during

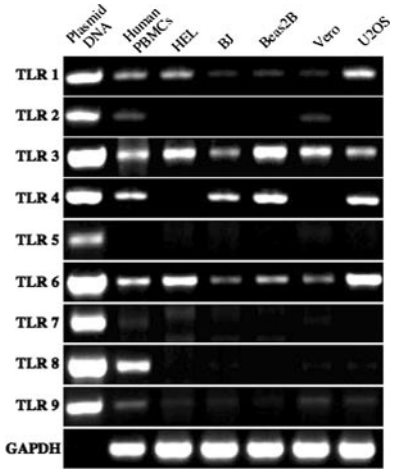

FIGURE 1. Expression of TLR message in various human cell lines. RNA was collected from untreated cells and subjected to RT-PCR analysis to assess the presence or absence of endogenous TLRs in nonimmune cells. GAPDH serves as an internal PCR control. Human PBMCs and TLR plasmid DNA served as positive controls for the recognition of endogenous TLRs and primer specificity, respectively.

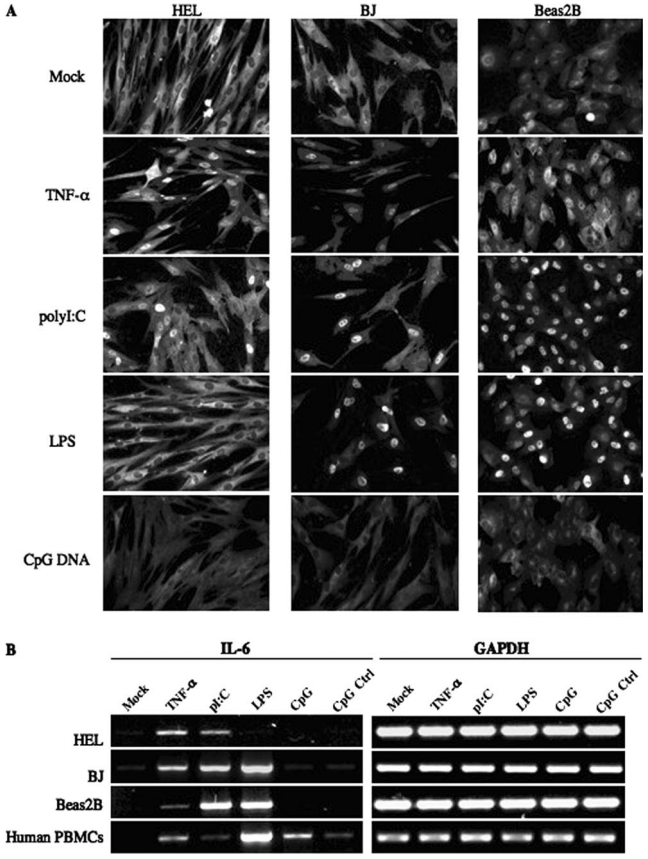

FIGURE 2. The subcellular localization of NF-κB and the induction of IL-6 following TLR ligand treatment. *A*, Cells were subcultured onto coverslips in 12-well dishes for 24 h and treated with poly(I:C) (10 μg/ml), LPS (2.5 μg/ml), CpG DNA (3.2 μg/ml), and a CpG control oligonucleotide (3.2 μg/ml) for 2 h. NF-κB localization was detected using an NF-κB- (p65 subunit) specific primary Ab and an Alexa Fluor-conjugated secondary Ab. *B*, Cells were subcultured into 6-well dishes for 24 h and treated with poly(I:C) (10 μg/ml), LPS (2.5 μg/ml), CpG DNA (3.2 μg/ml), and a CpG control oligonucleotide (3.2 μg/ml) for 8 h. RNA was collected using the TRIzol method and the presence of IL-6 was examined by RT-PCR analysis. GAPDH served as an internal PCR control. Human TNF-α was used as a positive control for the nuclear localization of NF-κB and induction of IL-6. Human PBMCs were used as a positive control for the induction of IL-6 in response to all TLR ligands tested. Ctrl, Control.

innate antiviral responses. Because much of this work has been conducted in immune cells such as PBMCs and DCs, we aimed to determine the presence or absence of TLRs in nonimmune epithelial and fibroblast cells. Specifically, HEL and BJ fibroblasts were used because they comprise nontransformed, nonimmortalized cells that have been used to characterize IFN-independent antiviral responses. Beas-2B, in contrast, is an immortalized epithelial cell line in which TLR characterization has been previously performed (75). RNA was isolated and analyzed by RT-PCR using primers for human TLR1–9. Human TLR plasmid DNA served as a positive control (Fig. 1). As previously demonstrated, human PBMCs express the majority of TLRs (76). In epithelial and fibroblast cells, TLR1, TLR3, and TLR6 expression was ubiquitous, TLR2, TLR4, and TLR9 expression was restricted, and TLR5, TLR7, and TLR8 expression was not detected.

Due to the lack of a suitable panel of Abs for TLR detection via immunoblotting, we confirmed our RT-PCR data via two functional assays: by examining the nuclear localization (activation) of NF-κB by immunofluorescence microscopy and by examining the expression of IL-6 by RT-PCR using the rationale that all TLR

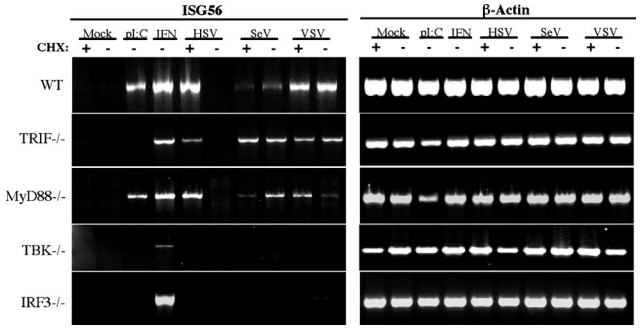

FIGURE 3. TRIF and MyD88 are not required for the induction of ISG56 in response to enveloped virus particles but TBK and IRF3 are essential. The presence of absence of ISG56 mRNA was assessed by RT-PCR in WT, TRIF$^{-/-}$, MyD88$^{-/-}$, TBK$^{-/-}$, and IRF3$^{-/-}$ MEFs following 6 h of treatment. β-Actin served as an internal PCR control. CHX, Cycloheximide.

subtypes activate NF-κB to induce the expression of proinflammatory cytokines such as IL-6 (23, 24). We focused on TLR2, TLR3, TLR4, and TLR9 using poly(I:C) (TLR3), LPS (TLR2 and TLR4), and CpG DNA (TLR9), because TLR2 and TLR9 have been implicated in the recognition of viruses, including herpesviruses, and TLR3 and TLR4 signal upstream of IRF3. TNF-α was used as a positive control for the nuclear localization of NF-κB (Fig. 2*A*) and induction of IL-6 expression (Fig. 2*B*). In untreated HEL and BJ fibroblasts, NF-κB expression was detected uniformly throughout the cytoplasm whereas in untreated Beas-2B cells, NF-κB expression was less intense within the cytoplasm and appeared perinuclear in a proportion of cells. Upon treatment with various TLR ligands, NF-κB nuclear localization was detected in all three cell lines following treatment with poly(I:C) but only in BJ and Beas-2B cells following LPS treatment. Treatment with CpG DNA did not result in NF-κB activation in any of the cell lines examined. In all cases, the extent of IL-6 mRNA accumulation correlated with the intensity of NF-κB nuclear localization. In PBMCs, IL-6 up-regulation was observed following treatment with all TLR ligands examined. Detection of IL-6 message in response to CpG control sequence has been reported with PBMCs (67). A similar induction pattern to IL-6 was observed for the NF-κB-responsive cytokine RANTES (data not shown). In summary, fibroblast and epithelial cells have a more restricted TLR expression profile than human PBMCs.

IRF3 and TBK, but neither TRIF nor MyD88, are essential for ISG induction in response to enveloped virus particle entry

The restricted TLR expression profile in fibroblasts suggested that TLRs do not play a role during the innate antiviral response to virus particle entry previously observed in these cells. To confirm this hypothesis, we examined the presence or absence of ISG56 in WT, TRIF$^{-/-}$, MyD88$^{-/-}$, TBK$^{-/-}$, and IRF3$^{-/-}$ MEFs. In this experiment, we used cyclohexamide to prevent de novo protein expression (viral and cellular), as we have found that low-passage primary MEFs are particularly sensitive to viral stimuli and as such produce small amounts of IFNβ in response to virus particle entry (66). In WT MEFs, ISG56 expression was detected following all treatments except replicating HSV-1, as previously demonstrated (51) (Fig. 3). A similar profile was observed in TRIF$^{-/-}$ and MyD88$^{-/-}$ MEFs. However, in TBK$^{-/-}$ and IRF3$^{-/-}$ MEFs, the expression of ISG56 was detected in IFN-treated samples only, agreeing with our previous study demonstrating the essential role of IRF3 in

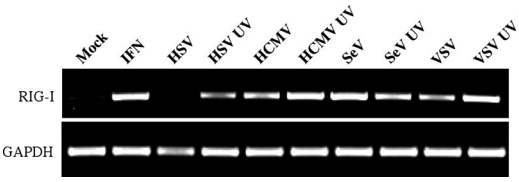

FIGURE 4. The presence of endogenous RIG-I was absent in untreated HEL cells but induced following virus treatment. RNA was collected from untreated or virus-exposed HEL fibroblasts following 8 h of treatment and subjected to RT-PCR analysis. GAPDH served as an internal PCR control.

response to particle entry (55). Collectively, these results suggest that in fibroblasts, TBK and IRF3, but not components of the TLR pathway, are essential for ISG induction in response to virus particle entry.

RIG-I expression is not detected in untreated fibroblasts

Recently, the RNA helicase, RIG-I, has been shown to respond to dsRNA produced during viral infection in an IRF3-dependent, TLR-independent manner (22). To assess the expression and role of endogenous RIG-I in our model, RNA was collected from HEL fibroblasts that were either untreated or treated with a diverse group of replicating and nonreplicating viruses (Fig. 4). As a positive control for the induction of RIG-I, HEL cells were treated with IFN (22). RIG-I mRNA was not detected in mock-treated fibroblasts, yet displayed typical ISG-induction kinetics following exposure to replicating and nonreplicating virus particles, with the exception of replicating HSV-1, as previously reported (51, 59, 66). Similar patterns of expression were detected upon protein analysis via Western blotting, thereby confirming our RT-PCR data (data not shown). Because we failed to detect RIG-I expression in mock-treated cells, and de novo protein synthesis is not required for ISG induction in our model, RIG-I likely does not play a critical role during the innate antiviral response to virus particle entry in these cells.

Virus particle entry fails to activate the NF-κB signaling pathway

Although virus infection has been shown to activate NF-κB (77), the lack of IFN-α or -β and proinflammatory cytokine production in response to virus particle entry suggests that entry alone is insufficient to activate this signaling cascade. To test this hypothesis,

Table II. *Nuclear localization of NF-κB in response to replicating and nonreplicating (UV) virus*[a]

Treatment	Duration of Treatment	
	2 h	6 h
TNF	+++++	+++++
Mock	−	−
HSV-1	−	+++++
HSV-1 UV	−	−
HCMV[b]	−	−
HCMV UV[b]	−	−
HCMV[c]	++	++
HCMV UV[c]	++	−
SeV	++	+++
SeV UV	−	−
VSV	+	++
VSV UV	−	−

[a] −, <1%; +, 1–5%; ++, 6–10%; +++, 11–20%; ++++, 20–80%; +++++, >80%.
[b] MOI of 0.1.
[c] MOI of 10.

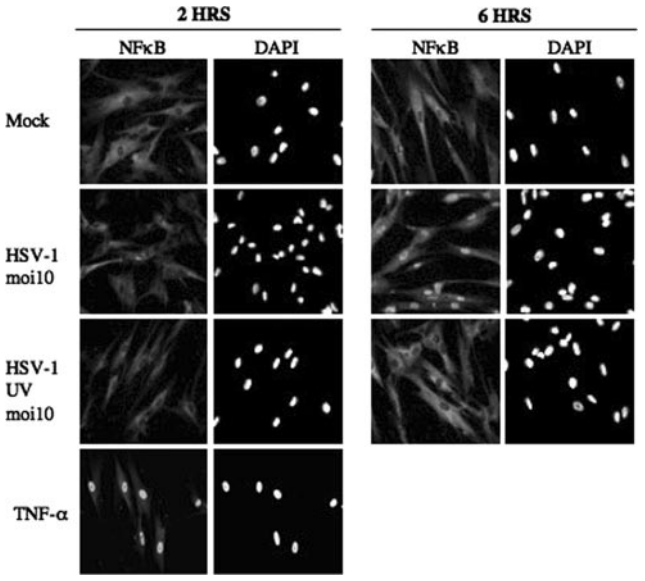

FIGURE 5. The NF-κB-signaling pathway is not activated in response to nonreplicating HSV-1 particles. HEL fibroblasts were subcultured onto coverslips in 12-well dishes for 24 h and treated with replicating and UV-inactivated HSV-1 (MOI 10) for 2 and 6 h. NF-κB localization was detected using an NF-κB- (p65 subunit) specific primary Ab and an Alexa Fluor-conjugated secondary Ab. Viral protein synthesis was detected in samples treated with replicating virus but absent in samples exposed to UV-inactivated virus (data not shown). Treatment of cells with human TNF-α (30 min) served as a positive control for the nuclear localization of NF-κB.

we examined the subcellular localization of NF-κB by immunofluorescence microscopy in HEL cells following treatment with replicating and nonreplicating virus (Table II and Fig. 5). Viral protein expression was examined in parallel to ensure complete UV-inactivation whereas IFN production (or lack thereof) was tested via plaque-reduction assays (data not shown).

Following HSV-1 infection, NF-κB nuclear localization was observed 6 h postinfection with replicating virus but remained cytoplasmic in the absence of replication, consistent with previous reports (78–80, and see Fig. 5). To determine whether the inability to activate NF-κB is a general phenomenon for nonreplicating enveloped viruses, we performed this same experiment with HCMV, SeV, and VSV. Cytoplasmic localization of NF-κB was observed at all times following treatment with nonreplicating SeV and VSV whereas treatment with replicating SeV and VSV resulted in the nuclear localization of NF-κB at 2 and 6 h (Table II). Treatments with HCMV were performed at low (MOI 0.1) and high (MOI 10) MOI, because we have previously observed differential cellular responses to HCMV entry based on the MOI (66). Following low MOI with replicating and nonreplicating HCMV, NF-κB was observed within the cytoplasm at all time points. In contrast, high MOI with replicating HCMV resulted in the nuclear localization of NF-κB at 2 and 6 h. With high multiplicity-nonreplicating HCMV infection, nuclear detection of NF-κB was only observed at 2 h postinfection. This two-tiered activation of NF-κB following HCMV infection agrees with previously published studies (81, 82). In all cases, the activation of NF-κB correlated with IFN production (data not shown). Taken together, these results demonstrate that NF-κB is not routinely activated upon virus particle entry, which accounts for the lack of IFN production during this antiviral response.

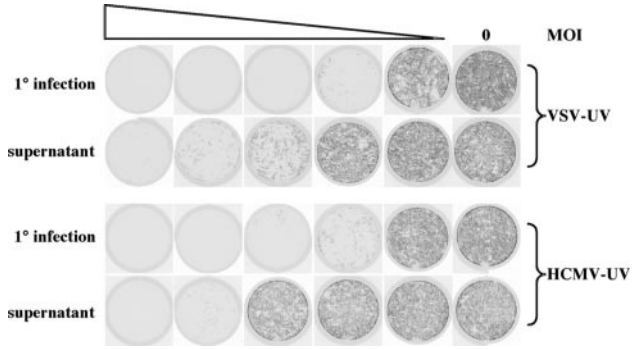

FIGURE 6. The IFN-independent antiviral response against enveloped virus particles is MOI-dependent. HEL fibroblasts were treated with increasing multiplicities of UV-inactivated virus for ~12 h (1° infection). The resulting medium was subsequently transferred to untreated HEL cells for 8 h (supernatant). Both sets of cells were infected with replicating recombinant VSV expressing GFP to assess the presence of biologically active IFN resulting from the 1° infection. GFP expression (virus replication) was visualized using a Typhoon scanner and is depicted in this figure. The MOI ranged from 1 to 100 PFU/ml for VSV-UV and 0.01–100 PFU/ml for HCMV-UV.

The IFN-independent nature of the antiviral response to virus particle entry is multiplicity dependent

We have previously demonstrated that IRF3 is differentially modified in response to low and high MOI exposure to enveloped virus particles (51). In this study, our observations with HCMV (Table II) suggested that MOI also influences the activation of NF-κB, resulting in the production of IFN. To test this hypothesis, we treated HEL fibroblasts with increasing amounts of nonreplicating virus (1° infection) and subsequently transferred supernatants to naive HEL fibroblasts to assay for the presence of secreted IFN (supernatant; Fig. 6). Induction of an antiviral response was measured by assaying the ability of VSV-GFP to replicate within the cultures. In response to both nonreplicating VSV and HCMV, low MOI protected the monolayers in the absence of IFN production, as indicated by the lack of protection following transfer of the supernatants to naive monolayers. However, as the multiplicity increased, naive monolayers became protected upon supernatant transfer, suggesting the production and secretion of IFN during the primary infection. Viral protein expression was undetected at all MOIs (data not shown). Similar results were found with additional enveloped RNA and DNA virus particles (data not shown). These results suggest that virus replication per se is not required for the activation of NF-κB and subsequent IFN production but can be stimulated upon entry of high numbers of virus particles.

Discussion

Our laboratory has previously demonstrated that the entry of enveloped virus particles leads to a cellular antiviral state characterized by the induction of a subset of ISGs in an IFN-independent but IRF3-dependent manner (51, 52). In this study, we found that TLRs and RIG-I are not responsible for the activation of TBK/IRF3 observed in our model. Recent studies support the existence of TLR- and RIG-I-independent antiviral mechanisms that result in the activation of the TBK/IRF3 pathway (48–50). These responses were observed following the recognition of cytoplasmic bacterial DNA (48, 49) and viral DNA (31), although the cellular mediator(s) responsible for initiating these responses remain elusive. Interestingly, nucleic acid is not required to stimulate the IFN-independent response to virus particle entry. Therefore, although there are similarities between the cellular responses to cytoplasmic DNA and virus particle entry, the cellular mediator(s) associated with these responses are likely to differ and remain to be identified.

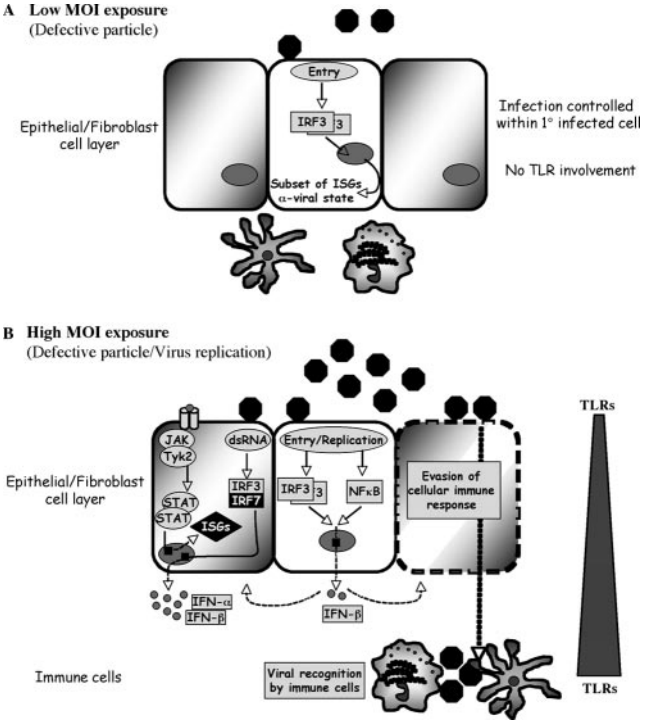

FIGURE 7. Model depicting the varying levels of innate antiviral defense based on the extent of virus exposure. *A*, During a low MOI infection, the initial entry of enveloped virus into epithelial/fibroblast cells triggers an IFN-independent antiviral response that contains the viral inspread to the primary infected cell. This response does not require the involvement of TLRs, RIG-I, or NF-κB but the participation of IRF3 is essential. *B*, A breach of the primary line of defense initiates a secondary line of cellular defense involving the activation of IRF3 and NF-κB, resulting in production of IFN and protection of neighboring cells. In the event that a virus infection evades the cellular immune response at the epithelial/fibroblast layer, immune cells such as monocytes/macrophages and DCs participate in another level of cellular defense, which involves IFN, cytokines, and initiation of an adaptive immune response. The involvement of TLRs is cell-type dependent and increases as the complexity of the immune response increases.

Distinct cellular responses can be elicited against viruses depending on the nature of the infection, the cell type being infected, and the anatomical location of these cells. For many infections, epithelia and fibroblasts are the first to encounter a virus and thus provide an initial barrier of antiviral defense. Given the likelihood that these cells are exposed to low levels of virus particles on a routine basis, we propose the following model (Fig. 7). Epithelial and fibroblast cells are primed to respond to low levels of virus infection by activating IRF3 and induce an intracellular antiviral response that is limited to the production of a small subset of ISGs. Such a response would occur before virus replication and in the absence of IFN and proinflammatory cytokine production. If successful, virus replication is blocked within individual infected cells without causing cellular damage due to cytokine secretion and immune cell infiltration. However, if a threshold of virus particle entry is surpassed, both IRF3 and NF-κB are activated, leading to the production and secretion of IFN and proinflammatory cytokines. This second level of antiviral defense warns surrounding cells to induce an antiviral response and attracts immune cells to the site of infection. Under conditions where this second level innate antiviral response is insufficient to block virus replication,

the production and subsequent release of stimulatory viral proteins and genomic material would serve to activate infiltrating immune cells.

We propose that the first line of innate antiviral defense occurs in response to virus particle entry, before virus replication (Fig. 7*A*). This response is characterized by the induction of an antiviral state following the expression of a subset of ISGs in an IFN-independent, IRF3-dependent manner (51, 52). In turn, the cell is able to control infection within the primary infected cell without activating an elaborate immune response. This provides an important advantage to the host as it prevents the recruitment of inflammatory cells and unnecessary cellular damage. In support of this view, we have previously demonstrated a lack of IFN and proinflammatory cytokine production in response to nonreplicating viruses (51, 52, 66). In this study, we provide an explanation for these observations as we observed a differential activation of NF-κB based on the number of virus particles that correlated with the production of IFN, as high but not low multiplicities of nonreplicating-virus particle entry resulted in the activation of NF-κB and the expression of IFN. Therefore, the nature of the IFN-independent antiviral response is specific to low numbers of virus particles. Recently, TLR3, TLR4, and RIG-I have been shown to mediate antiviral responses via the activation of IRF3 (22, 25–28). Our data, however, do not support a role for either of these antiviral systems during the primary response to nonreplicating virus particles. First, epithelial and fibroblast cells express a restricted TLR profile and can induce ISGs in the absence of TRIF or MyD88. Second, the activation of NF-κB, which is a result of TLR and RIG-I stimulation, was not observed in response to low multiplicity virus entry. Third, although RIG-I and the related protein melanoma differentiation-associated gene 5 serve as an intracellular sensors for viral dsRNA and function in a TLR-independent manner (22, 83), we failed to detect basal RIG-I expression in untreated cells. The lack of TLR and RIG-I involvement in our model supports previous observations in which virus replication, and thus the production of stimulatory viral dsRNA, DNA, and proteins, was not required to trigger this primary innate response (12, 54, 55).

During the secondary line of antiviral defense, cells respond to virus infection via the production and secretion of IFN (Fig. 7*B*). During this response, exposure to viral stimuli exceeds a cellular threshold that results in the activation of both IRF3 and NF-κB, which promotes the production and secretion of IFNβ. In turn, IFNβ interacts with type I IFN receptors on neighboring cells, inducing ISGs via the JAK/STAT pathway and amplifying the IFN response. Although the induction of IFN in response to viral replication has been well established, our data suggests that IFN is also generated in response to high multiplicities of nonreplicating virus, which physiologically may occur following an initial round of infection with viruses that produce large amounts of defective particles. The production of IFN is essential during this secondary response as it communicates with surrounding cells in an attempt to inhibit further virus replication and limit viral spread. The production of dsRNA during virus replication provides a significant target that allows cells to recognize and respond to infection. In addition, the cellular damage resulting from infection may also provide a means by which dsRNA is released into interstitial spaces, thereby exposing nearby cells. As a result, TLR and RIG-I would serve an important role during the IFN-dependent antiviral response in epithelial and fibroblast cell layers. For instance, TLR3 has been shown to combat infection with ssRNA viruses such as influenza and rhinovirus in lung epithelial cells (84–86). Similarly, TLR3 has been suggested to mediate protection against viral infection in vaginal (87, 88) as well as uterine epithelial cells (89, 90). Our data agree with a functional role for TLR3 in epithelial and fibroblast cells as this receptor was ubiquitously detected in all nonimmune cells examined in our study. In addition to TLRs, RIG-I has been shown to mediate the expression of IFN in lung MEFs following infection with RNA viruses (91). Interestingly, Kato et al. failed to detect RIG-I expression in untreated MEFs (91), however, the expression of RIG-I was induced following treatment with poly(I:C). In combination with our data, a role for this RNA helicase during IFN-dependent but not IFN-independent responses is strongly suggested. Therefore, in contrast to the primary antiviral response, the secondary line of defense requires IFN production as well as TLR3- and RIG-I-mediated antiviral responses to combat an increased viral assault.

In the event that an infection breaches the immune response within the epithelial and fibroblast cell layer, infiltrating immune cells such as macrophages and DCs provide another layer of antiviral protection. Under these conditions, the level of infectious viruses has increased, and the resulting cellular damage in the epithelial and fibroblast layer will promote the presence of stimulatory viral components such as dsRNA, CpG DNA, and/or glycoproteins. Immune cells are well equipped to combat infection because they can activate several innate antiviral mechanisms, as well as initiate the adaptive arm of the immune system. In contrast to epithelial and fibroblast cells, immune cells express most, if not all, TLRs involved in antiviral responses (76, 92). Activation of TLRs has been shown to play a role in the expression of proinflammatory cytokines and IFNα following virus infection, particularly in plasmacytoid DCs (33, 41, 93). These cell types are also capable of initiating antiviral responses via TLR-independent mechanisms. For instance, RIG-I has been shown to induce IFN in conventional DCs following RNA virus infection (91). In contrast, plasmacytoid dendritic cells were found to predominantly use the TLR system to induce IFN in response to virus infection, thereby demonstrating cell type-specific mechanisms for initiating antiviral responses (91). Other studies have shown that HSV infection can induce the expression of proinflammatory cytokines and IFN in a TLR9-dependent manner in DCs (41, 94). In addition to their role during innate antiviral immunity, macrophages and DCs are also capable of presenting viral Ags and secreting a wide variety of cytokines, thereby initiating and shaping an adaptive immune response. Ultimately, this line of antiviral defense employs all levels of the immune system to control virus spread as well as facilitates the clearance of viruses during the later stages of infection. In addition, an infected individual will also experience and display symptoms of clinical illness during this phase of the antiviral response. It can be appreciated that this level of immune activation is not required to control low-level virus exposure.

The results presented herein contribute to our ongoing characterization of the innate antiviral response to virus particle entry. Our studies confirm the existence of unidentified receptor(s) and ligand(s) that are capable of activating the TBK/IRF3 pathway because TLRs and RIG-I are not critical during this response. Moreover, we have demonstrated that this response is initiated upon low multiplicity exposure to virus particles in a first line of antiviral defense. We propose that the biological role of this response is to manage an initial encounter with either low multiplicity WT infection or exposure to higher levels of defective virus particles without alarming the immune system, thereby preventing any unnecessary cellular damage as well as the clinical symptoms of infection.

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Disclosures

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