Antiviral defense: interferons and beyond

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Mice lacking the adaptor protein that initiates an antiviral response downstream of the RNA helicases retinoic acid–inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) have recently been described. These studies highlight the essential and nonredundant role of nucleic acid recognition in the induction of type I interferon production and raise important questions regarding the nature of cell-autonomous virus detection in coordinating the antiviral response.

Viruses are the most abundant pathogens on earth, and the survival of many organisms depends on their ability to sense and restrict viral infection. Detection of nucleic acids is a fundamental means of viral recognition in both prokaryotes and eukaryotes, although the types of receptors that recognize nucleic acids and the responses induced by these receptors vary greatly across phyla. In jawed vertebrates, nucleic acid–sensing receptors induce the production of type I interferons (IFNs). These cytokines, originally identified as a soluble activity that blocked virus spread among cultured cells (1, 2), have several essential functions in the antiviral response. Type I IFNs act in both an autocrine and paracrine fashion by inducing the expression of hundreds of genes that together establish an "antiviral state," which restricts the spread of virus among neighboring cells. In addition, type I IFNs enhance the function of natural killer (NK) cells and the differentiation of virus-specific cytotoxic T lymphocytes (CTLs), both of which recognize and eliminate virus-infected cells.

Despite the attention that type I IFNs have received since their identification, the receptors that trigger their production remained mysterious until quite recently. Two types of receptors are now known to link pathogen detection to the type I IFN response. The first type is Toll-like receptors (TLRs), which are expressed primarily by macrophages and dendritic cells (DCs) (for review see reference 3). Several features of TLR-mediated nucleic acid recognition indicate that these receptors are only part of the overall antiviral response. First, TLRs specific for viral nucleic acids are expressed by only a small fraction of specialized cells, whereas almost all cells produce type I IFNs when infected with virus or transfected with nucleic acids. Second, the TLRs that sense nucleic acids are localized in the endosomes of sentinel cells that are generally not infected with virus. These cells sample endocytosed material but do not detect the presence of intracellular infection. Third, mice lacking the adaptor protein MyD88, which are unable to activate most TLR signaling pathways, are remarkably resistant to infection with several viruses (4). Thus, although TLRs can detect viral infection, a second, more widely expressed receptor system must signal the presence of virus from within all cells.

The missing link

Two years ago, Fujita and colleagues published a landmark study that sparked the current advances in our understanding of antiviral immune responses (5). The group screened for cDNAs that could enhance the activation of an IFN regulatory factor 3 (IRF3) reporter after transfection with polyinosinic–polycytidylic acid (poly I:C)—a synthetic double-stranded (ds)RNA polymer that potently induces the production of type I IFNs. Their screen turned up the caspase-recruitment domain (CARD)–containing cytosolic RNA helicase RIG-I and the closely related MDA5 (5). Shortly afterward, Balachandran et al. demonstrated that the signaling proteins Fas-associated via death domain (FADD) and receptor-interacting protein 1 (RIP1) were essential for the production of type I IFNs in response to infection with vesicular stomatitis virus (VSV) and transfection with poly I:C (6). Another signaling protein, TNF receptor–associated factor 3 (TRAF3), was also shown to be involved (7). But the link between the RNA helicases and these signaling molecules remained undefined.

Less than a year ago, four groups independently identified this missing link as a CARD-containing adaptor protein, naming it IFN-β promoter stimulator-1 (IPS-1) (8), mitochondrial antiviral signaling protein (MAVS) (9), virus-induced signaling adaptor (VISA) (10), and CARD adaptor–inducing IFN-β (CARDIF) (11). Here we refer to this protein as IPS-1. Overexpression of IPS-1 activated IRF3 and NF-κB, and knockdown of IPS-1 expression blocked signaling in response to both RNA viruses and poly I:C. Interestingly, one group found that IPS-1 is localized to the outer membrane of mitochondria (9), and another group showed that it is cleaved and inactivated by the hepatitis C virus (HCV) protease NS3/4A (11), which had previously been shown to intercept dsRNA-activated signaling to IRF3 and IFN-β (12, 13).

Studies of mice lacking either RIG-I or MDA5 showed that the two helicases are essential for type I IFN production in response to distinct classes of RNA virus. RIG-I responds to in vitro–transcribed dsRNA and is required for the in vivo response to VSV, Newcastle disease virus (NDV), Sendai

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virus, and influenza virus (14, 15). MDA5, by contrast, is the principal receptor for poly I:C and is essential for the antiviral response to the picornavirus encephalomyocarditis virus (EMCV) (15, 16).

Two recent studies, one in the July issue of the *JEM* and another in a recent issue of *Immunity*, reported that the phenotype of IPS-1–deficient mice is essentially the sum of the phenotypes of RIG-I and MDA5 single knockout mice (17, 18). In cells from IPS-1–deficient mice, RNA virus– and dsRNA-induced signaling and type I IFN production were profoundly impaired. The only exception to this rule was in plasmacytoid (p)DCs, which exclusively use TLRs to link RNA recognition to type I IFN production (14, 18). IPS-1–deficient cells were unable to activate IRF3 and NF-κB, failed to produce type I IFNs and did not activate IFN-inducible genes downstream of RIG-I and MDA5 (17, 18). In vivo, *Ips-1*$^{-/-}$ mice were highly susceptible to infection with VSV (17, 18), despite normal serum levels of type I IFNs (probably induced by pDCs through TLR-dependent mechanisms) (18). Interestingly, *Ips-1* was highly haploinsufficient in vivo: heterozygous (*Ips-1*$^{+/-}$) and homozygous (*Ips-1*$^{-/-}$) mice were equally susceptible to infection with VSV (18). Furthermore, as with MDA5 deficiency (15, 16), *Ips-1*$^{-/-}$ mice were more susceptible to lethal infection with EMCV (17). Unlike VSV infection, however, EMCV infection failed to induce the early production of type I IFNs in the serum of IPS-1–deficient mice, suggesting that this nonenveloped picornavirus might evade TLR-dependent detection by pDCs.

These studies show that IPS-1 is essential for antiviral responses to RNA viruses and synthetic dsRNA. In contrast, IPS-1 was not required for type I IFN production in response to transfected DNA (17, 18), or to infection with the intracellular bacterium *Listeria monocytogenes* (18) or the Vaccinia poxvirus (17). These data, together with recent reports demonstrating a TLR-independent response to intracellular DNA (19–21), suggest that cytosolic RNA and DNA recognition activate the production of type I IFNs via distinct signaling pathways that converge on IRF3.

The characterization of IPS-1–deficient cells and mice is an important advance in our understanding of how cells respond to infection with RNA viruses. But these studies also raise interesting questions regarding the nature of nucleic acid recognition and the different ways in which infected cells coordinate antiviral immunity.

Are nucleic acids the sole initiators of antiviral type I IFN production?

The phenotype of IPS-1–deficient cells formally demonstrates that RIG-I and MDA5 are the primary, nonredundant sensors that link cytosolic dsRNA detection to the type I IFN response. Before the characterization of RIG-I and MDA5, the IFN-inducible protein kinase R (PKR) had been thought to be important for the type I IFN response to dsRNA, because PKR-deficient cells and animals show reduced type I IFN production and are susceptible to infection with VSV (22). However, in light of the identification of the RNA helicase pathway of viral recognition, it now appears that PKR does not induce the type I IFN response to cytosolic dsRNA. Instead, PKR appears to have a separate, but still important, role in cell-autonomous control of viral infection. The function of PKR most likely involves its ability to inhibit translation of host cell mRNA by phosphorylating the translation initiation factor eIF2α—the only known substrate of PKR (22). Perhaps the reduced type I IFN production by PKR-deficient cells reflects the possibility that type I IFNs are more efficiently translated under conditions where eIF2α is limiting, as would occur during viral infection.

Not only do these findings clarify the relative contribution of PKR to the antiviral response, they also imply a more general principle with fundamental implications for antiviral responses and autoimmunity: nucleic acid recognition might be the only way for most cells to induce type I IFN production. In models of infection with various classes of RNA virus, IPS-1–deficient cells are completely unable to mount a type I IFN response. This suggests that no component of these viruses other than the RNA, including envelope glycoproteins and capsid proteins, can induce the production of type I IFNs. Moreover, it indicates that stress responses, which are activated in cells supporting rapid viral replication, are also insufficient to induce the production of type I IFNs. In this regard, it would be interesting to examine mitogen-activated protein kinase activity during viral infection in IPS-1–deficient cells, as it is likely that intact stress response pathways are activating the expression of genes other than type I IFNs.

If nucleic acid recognition is the only way for most cells to induce the production of type I IFNs in response to infection with RNA viruses, does a similar principle apply to recognition of DNA viruses? Until recently, dsRNA was thought to be the main signature of viral replication inside cells, produced by replicating RNA viruses and bidirectional transcription of DNA viruses. However, the finding that IPS-1–deficient cells mount a normal antiviral response to transfection with DNA, and to infection with *L. monocytogenes* or a poxvirus (17, 18), indicates that cytosolic dsRNA is not the only trigger of the type I IFN response. This raises the question of why two signaling pathways are needed to link nucleic acid detection to the antiviral response. The simplest explanation is that RNA and DNA viruses activate different cell-intrinsic effector responses that are tailored to combating the particular type of virus. Importantly, neither type I IFNs nor generic IFN-inducible genes account for the specificity of this response, as transfection with DNA and poly I:C activates a similar profile of IFN-inducible genes (21). Instead, the two signaling pathways might drive the transcription of distinct IFN-independent genes and might also activate different transcription-independent antiviral mechanisms. If so, this would have important implications for the design of antiviral therapeutics, which would need to target the IFN-independent response appropriate for the type of virus infection.

Another implication of these studies relates to autoimmune disorders, which are often associated with the production of type I IFNs (23). Most cells tested respond to RNA viruses and dsRNA in an IPS-1–dependent manner, and a similarly ubiquitous response exists to cytosolic DNA (20, 21). Thus, an inappropriate response of these sensor proteins to self-derived, intracellular nucleic acids might be a critical contributor to autoimmunity. This scenario implies a cell-autonomous initiation of autoimmunity, which is distinct from several current models in which defective clearance of apoptotic cells results in the accumulation of extracellular nucleic acids (24). TLRs might have a key role in the detection of these extracellular self-nucleic acids (25), particularly chromatin and RNA–protein complexes (26–30), whereas the cytosolic RNA and DNA sensors might be responsible for a distinct set of autoimmune disorders that may only somtimes be associated with the failed clearance of apoptotic cells (19). Breeding IPS-1–deficient mice with autoimmune-prone mouse strains would be a simple way to begin to address this hypothesis.

Cell-autonomous defense: beyond type I IFNs?

The observation that IPS-1–deficient mice are highly susceptible to infection with VSV despite generating a normal systemic type I IFN response (18) is reminiscent of the phenotype of FADD-deficient fibroblasts, which are unable to control VSV replication even after pretreatment with type I IFNs (6). It is possible that local concentrations of type I IFNs are too low to control viral replication in peripheral tissues in the absence of IPS-1. But there is another possible explanation that would suggest a role for IPS-1 signaling beyond the induction of type I IFNs: detection of cytosolic nucleic acids might trigger cell-intrinsic apoptosis and/or flag the infected cells—but not their uninfected neighbors—for elimination by NK cells and CTLs.

One of the key functions of type I IFNs is to provide a paracrine alarm signal and induce an antiviral state in uninfected neighboring cells. For example, RIG-I and MDA5 are induced by type I IFNs, which then increase the sensitivity of nucleic acid detection in neighboring cells. Another key role of type I IFNs is to enhance the cytotoxic functions of NK cells and $CD8^+$ T cells, which are important for inducing non–cell-autonomous apoptosis in infected cells. The recognition and elimination of infected cells must be carefully regulated such that only the infected cells—and not their immediate neighbors—are targeted. The IPS-1–dependent production of type I IFNs cannot supply the dominant "kill signal" for cytotoxic cells; this signal must be cell bound and it must be unique to infected cells (Fig. 1). Because RIG-I and MDA5 (and the currently unidentified DNA sensor(s)) detect viral nucleic acids within infected cells, they are perfectly suited to induce surface expression of the ligands that uniquely flag infected cells for elimination. This induction must be IFN independent and could even be IRF3 independent (Fig. 1).

What might these ligands be? Promising candidates are ligands for activating and inhibitory receptors that are expressed by NK cells and cytotoxic $CD8^+$ T cells (31). Coordinated down-regulation of inhibitory ligands and up-regulation of activating ligands can determine the susceptibility of target cells to NK cell–mediated killing (31), but the mechanisms and signaling pathways responsible for altering the expression of these ligands remain largely unknown. These mechanisms must be cell autonomous, however, and could conceivably be controlled by IPS-1–dependent signals in infected cells. One activating receptor expressed by NK cells and some effector $CD8^+$ T cells is NKG2D, which recognizes cell surface ligands induced by various stresses, including viral infection (32). One prediction

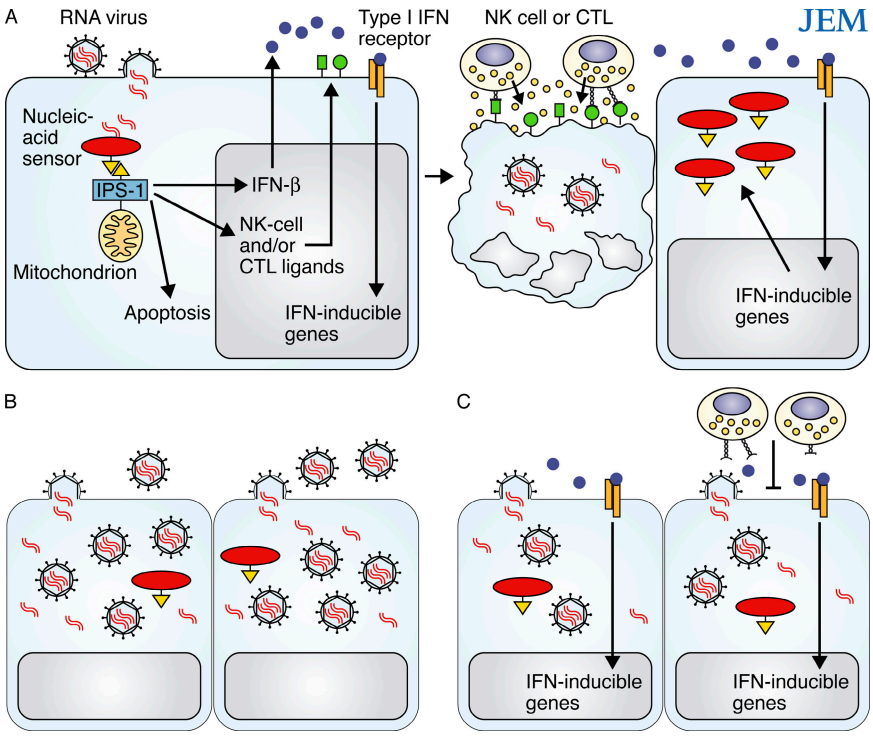

Figure 1. Control of antiviral defense by IPS-1. (A) In normal cells infected with an RNA virus, IPS-1–dependent signaling activates type I IFNs, which turn on IFN-inducible genes in both infected cells and neighboring, uninfected cells. IPS-1 might also control cell-intrinsic apoptosis and the induction of ligands for NK cells and CTLs, but only in infected cells. (B) In IPS-1–deficient cells, all four functions depicted in part A are lost and antiviral defenses are fully compromised. (C) Treatment of IPS-1–deficient cells with type I IFNs (either TLR-activated systemic IFNs or exogenous type I IFNs) only restores the IPS-1–independent response mediated by IFN-inducible genes.

of this model is that IPS-1–deficient mice would initiate adaptive immune responses normally after infection with RNA viruses but that effector $CD8^+$ T cells and NK cells would be unable to kill infected cells. Another clinically relevant prediction is that treatment of virus-infected individuals with recombinant type I IFNs would only restore paracrine IPS-1–dependent immune responses and not the cell-autonomous effector functions downstream of IPS-1 (Fig. 1). For viruses such as HCV, which encodes the IPS-1–cleaving protease NS3/4A, pharmacological inhibitors that block NS3/4A would restore all functions of IPS-1 and would thus be far more effective than treatment with type I IFNs alone (33).

IPS-1 might also control induction of cell-intrinsic apoptosis in an IFN-independent manner (Fig. 1). Because IPS-1 is a transmembrane protein localized to mitochondria, it would be interesting to see if it interacts with or alters the function of the BCL2 family of proteins, which control mitochondria-dependent apoptosis. However, infection with viruses other than VSV would be required to firmly establish this, as VSV-infected cells can be killed by the strong cytopathic effect of this virus, especially in the absence of type I IFN-induced signaling. Importantly, anchoring IPS-1 to other membranes in the cell impairs signaling, suggesting that mitochondrial localization, and not membrane attachment itself, is essential for its function (9). This observation, coupled with the fact that IPS-1 is not IFN inducible and is strongly haploinsufficient in vivo, implies that IPS-1 is coordinately regulated with the numbers of mitochondria and might be most abundant (and most sensitive to nucleic acid detection) in cells with the highest metabolic activity.

Conclusions and future directions

The description of IPS-1–deficient mice marks an important milestone in the rapidly advancing field of nucleic acid recognition. In just two years, the nonredundant cytosolic RNA sensors and several of the key signaling molecules that link RNA recognition to the antiviral response have been identified and characterized in vitro and in knockout mice. Along the way, we have realized that an entirely separate system exists for the detection of intracellular DNA that is likely to be important for host responses to DNA viruses and intracellular bacteria. Together with the current knowledge of TLR-mediated nucleic acid recognition, we are now in a position to dissect the relative contribution of all of these systems to immunity and immunopathology.

We thank members of our lab for discussions.

D.B. Stetson is a postdoctoral fellow of the Cancer Research Institute. R. Medzhitov is an investigator of the Howard Hughes Medical Institute.

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