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The Role of Innate Immunity in Regulating Rotavirus Replication, Pathogenesis, and Host Range Restriction and the Implications for Live Rotaviral Vaccine Development

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I. INTRODUCTION

Rotaviruses (RVs) remain one of the two most important viral causes of gastroenteritis despite the availability of several safe and effective live attenuated vaccines [1,2]. Rotavirus infection has its biggest health impact on children under the age of 3 years, in whom it still accounts for approximately 200,000 deaths annually, almost entirely in less-developed countries [2]. RVs can infect many cells of the nonimmune host, but the overwhelming bulk of viral replication occurs in the mature villus tip cells of the small intestine [3]. In this review, we focus on the regulation of rotavirus replication by the host innate immune system, the host-restricted

nature of the innate immune response to specific rotavirus strains, and the practical utility of these host range barriers in the development of safe and effective RV vaccines.

II. HOST INNATE IMMUNE SENSORS AND ROTAVIRUS INFECTION

A. Cytoplasmic Sensors

Infection with RV results in the immediate activation of a conserved cellular innate immune signaling pathway that involves multiple pattern recognition receptors (PRRs) recognizing

discrete RV-encoded pathogen-associated molecular patterns (PAMPs). A primary purpose of this diverse host-signaling system is to induce different types of interferons (IFNs) and a set of virus-induced stress genes (vISGs) through two principal transcriptional factors: nuclear factor- κ B (NF- κ B) and IFN regulatory factor 3 (IRF3) [4,5]. The induced IFNs and vISGs then function to restrict RV replication and pathogen-induced cell injury [6]. Of note, RVs, like virtually all other viral pathogens, have evolved a set of countermeasures to inhibit the host innate immune response, and these countermeasures are most pronounced during homologous RV infection (RV infection with a strain routinely isolated from that specific host species) [7]. Interestingly, RV strains that differ in their ability to regulate the secretion of IFNs similarly induce this early recognition pathway, as indicated by the transcriptional upregulation of IFNs and several vISGs [8]. Based on the collective evidence, initial RV transcription engages the two related PRRs RIG-I and MDA-5 (members of the family of RIG-I-like receptors, or RLRs) [8,9], which then trigger activation of the mitochondrial antiviral-signaling protein (MAVS). These receptors are likely to be stimulated by early RV transcriptional by-products such as exposed 5'-phosphate groups, incompletely methylated 5'-cap structures, and local dsRNA structures such as panhandle loops in viral transcripts [10]. In addition to inducing the secretion of different IFNs, RLR responses to RV are likely to orchestrate other host responses. Rotavirus activation of MDA-5 results in apoptosis, which occurs mostly in the pancreas of RV-infected mice, indicating that such PRR-dependent consequences can occur in a cell or organ type-specific fashion [11] (Chapter 6: Innate Immunity at Mucosal Surfaces).

In addition to RIG-I- and MDA-5-dependent host responses to RV RNA, other sensors are also recruited by the innate immune machinery to trigger early anti-RV responses. Among these is a third member of the RLR family: LGP2, which seems to exert a proviral effect on RV

replication [9] and whose activation during RV infection may represent a viral strategy to dampen this pathway. Yet another player in the innate recognition of RV is the dsRNA-dependent protein kinase PKR, which is essential for RV-infected cells to secrete IFN [8]. The molecular basis for PKR's role during RV infection is not well understood, but given the importance of PKR in antiviral signaling in general and its inhibition by a majority of viruses, PKR is likely to be important for RV pathogenicity.

B. Membrane-Associated Sensors

Distinct from the cytosolic receptors discussed above, RV recognition also involves the toll-like receptors (TLRs), a class of viral receptors that function in the context of cellular membranes, including surface and endosomal membranous components. This aspect of innate RV recognition possibly reflects the RV entry process that exploits endosomal vesicle transport to gain access into host cells. So far, TLR3, TLR7, TLR2, and TLR5 have all been implicated as potential players in the innate RV detection cascade [12–17]. The ability of TLR3 to recognize and regulate RV and thus perpetuate an antiviral effect has been tied to TLR3's age-dependent expression in the intestine [18]. Since RV typically causes severe disease and replicates in the intestine of infants and young children (in many mammalian species), coincidental with lower levels of TLR3 expression in infants [18], one possible implication is that age-restricted RV intestinal replication is partly due to enhanced TLR3 signaling with age in this mucosal compartment.

Other TLRs play specialized roles in discrete types of cells during RV infection. The RV-encoded enterotoxin NSP4 may function as a PAMP and in macrophages triggers inflammatory cytokine secretion by a TLR2-dependent pathway [14]. During RV infection in human enteroid cultures [19] and in different species of mammals [20–25], different types of IFNs are

secreted, and as will be discussed below, antiviral actions of these IFNs are actively countered in a host-range-specific manner by pathogenic RVs. Of the IFNs, type I IFN is mostly expressed in the intestinal hematopoietic cell compartment rather than in the epithelium where RV primarily replicates [26]. Studies to date have implicated TLR-dependent signaling in dendritic cells in the type I IFN secretion process during RV infection [16,27,28]. Infection of plasmacytoid dendritic cells with RV, which are a major source of type I IFN secretion during viral infections, leads to endosome-dependent (and possibly TLR7-mediated) type I IFN secretion that is triggered by viral genomic dsRNA (or, potentially, a RV structural protein) [13,16,27,28]. A central role for TLR-dependent defense against RV is also indicated by the finding that the absence of MyD88, an essential convergent adaptor in signaling from the different TLRs, results in increased RV infectivity, severity of diarrhea, and impaired humoral immunity [12]. In addition, RV is susceptible to the antiviral effects of TLR5, since activation of this receptor by bacterial flagellin prevents or cures RV infection by a process that involves the secretion of IL 22 [17,29,30].

C. Other Sensors

Inflammasomes are cytosolic multiprotein complexes that remain quiescent at resting state [31]. Upon activation, apoptosis-associated speck-like protein containing CARD protein, named ASC (encoded by *PYCARD*) and caspase-1 (encoded by *CASP1*), oligomerize and mediate the proteolytic processing of proinflammatory cytokines such as pro-IL-1 β and pro-IL-18 and the pore-forming protein gasdermin D, ultimately leading to a lytic form of cell death known as pyroptosis [32]. These events not only contribute to the host defense against bacterial and other microbial infections, but also regulate the homeostasis of the immune system and the development of various inflammatory diseases and cancer [33].

Although it is known that AIM2 and IFI16 inflammasomes recognize DNA viruses and that NLRP3 inflammasome responds to general stress and breach of plasma/endosomal membrane integrity [34,35], how inflammasomes control RNA virus replication is less well understood. In addition, whether noncanonical inflammasomes operate in cell types other than myeloid cells is largely unknown. Recently, we found that oral infection of suckling mice with murine RV-induced robust activation of CASP1 in the small intestinal tissue, indicating a potential role for inflammasomes in RV pathogenesis [36]. Of note, in contrast to other NOD-like receptors, including NLRP3, NLRP6, NLRC4, and NAIPs, targeted deletion of NLRP9b in intestinal epithelial cells (IECs) of suckling mice led to an increase in diarrhea, RV shedding in the feces, and intraintestinal viral replication compared to wild-type pups, highlighting a crucial role of NLRP9b in controlling RV replication. Mechanistically, we found that during RV infection, DExH-box helicase 9 (DHX9) binds to viral RNA PAMP and interacts with NLRP9 to activate the downstream signaling pathway (Fig. 40.1). Furthermore, primary mouse intestinal enteroids generated from DHX9- or NLRP9-deficient mice produced less IL-18 and underwent less pyroptosis compared to wild-type enteroids upon RV infection, confirming a role for DHX9 in the activation of the NLRP9b inflammasome during RV infection [36].

Identification of the DHX9-NLRP9b-ASC-CASP1 cascade as a novel RV-sensing pathway opened up new research directions. Are there other inflammasome sensors of RV or other enteric viruses? How do different RNA-binding proteins (DHX9, RIG-I, MDA-5, etc.) coordinate in the cytoplasm? What is the physiological relevance of these sensors in the human intestine? Addressing these fundamental issues will provide new insights into the biological functions of host innate immune recognition during acute RV infection and, more generally, in overall human enteric health

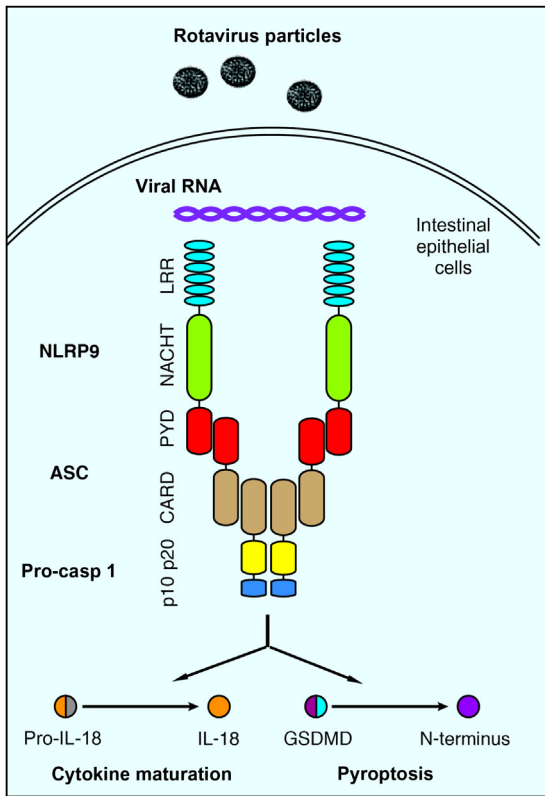


FIGURE 40.1 Rotavirus sensing by the NLRP9b inflammasome. Within the IECs, RV RNA is sensed by DHX9 and the NLRP9 inflammasome and activates the downstream ASC-CASP1 complex, which activates IL-18 secretion, GSDMD cleavage, and pyroptosis.

and disease. In addition, answering these basic questions is likely to inform more practical considerations, such as the development of better therapeutics and preventive strategies for enteric infectious diseases.

III. HOST INNATE RESPONSES TO ROTAVIRUS AND THEIR EFFECTS ON VIRAL REPLICATION

The concept of host range restriction (HRR) is central to many aspects of RV replication and disease, including the development of several

of the currently available safe and effective RV vaccines [37]. RVs are distinguished by being highly pathogenic and infectious to their homologous host species (i.e., the species of host normally infected by the particular RV strain and the species in which that RV strain spreads efficiently) [38]. RVs are also subject to very severe species-specific restriction of replication and transmission in heterologous host species [39]. These fundamental properties of RVs are not only of great importance for viral pathogenicity. They also form the basis for several licensed live attenuated orally administered RV vaccines (which are attenuated by virtue of their HRR).

In traditional continuous cell line culture systems, most RV strains efficiently block the induction of type I IFN and have evolved to target several different host factors that regulate the IFN pathway [40]. This multipronged subversion of the IFN response is accomplished primarily by the versatile RV nonstructural NSP1 protein, the product of RV gene 5 (see below). Most IFN-sensitive RV strains encode forms of NSP1 that exhibit defective IFN inhibition and therefore elicit enhanced IFN secretion [40–48]. Although such strains are still viable infectious agents, their ability to replicate is substantially hampered. In addition, IFN sensitivity of RVs encoding full-length “functional” NSP1 proteins also occurs in specific cell lines, possibly reflecting NSP1’s inability to target host innate factors across different species [49].

Enteric infection of suckling (i.e., 3- to 5-day-old) mice with a homologous murine RV compared to a heterologous simian RV strain reveals a substantial (~4–5 log) host restriction of the simian RV replication in the intestine [7,26,50,51]. This host restriction phenotype is substantially reduced (down to 1 log or less) in mice lacking and three IFN receptors (IFNRs) or STAT1, a key transcription factor relaying antiviral signals from different IFNRs [7,26,50,51]. The replication phenotype strongly cosegregates with the genetic origin of the

murine or simian RV NSP1 encoding gene 5 segments. The suckling mouse thus presents a highly tractable model in which IFN-specific effects on RV replication can be studied within the biologically relevant framework of intestinal RV replication in a natural host and in a host-range-restricted manner.

Ectopic parenteral injection of purified IFN types I, II, or III in many species, including mice, results in the activation of the key downstream transcription factor STAT1 in small IECs (the predominant site of RV replication) [52]. Multiple lines of evidence indicate important roles for IFN types I, II, and III in restricting RV replication in the gut and in cell culture [7,26,50,51,53]. In mouse embryonic fibroblasts lacking both types I and II IFNRs, the replication of several nonmurine RV strains is substantially enhanced (by four to five orders of magnitude). In suckling mice lacking the types I, II, and III IFNRs (either singly or in combination) significant enhancement of simian RV intestinal replication occurs demonstrating the sensitivity of heterologous nonmurine RVs to different IFN types in the mouse gut [7,26,50,51]. In contrast, replication of the homologous murine RV is quite resistant to these IFNs (~1 log or less replication gain). The collective evidence thus highlights the important role of different types of IFNs (and their inhibition by the RV NSP1 protein) in RV pathogenicity and attenuation [7,26,50,51,53]. Deciphering the mechanisms underlying these interactions is key for rational RV strain attenuation and designing improved third-generation live attenuated RV vaccines.

IV. REGULATION OF THE INTERFERON INDUCTION PATHWAY BY ROTAVIRUS

In a manner analogous to that of other RNA viruses, RV-induced IFN activation is dependent on an intact RNA sensing pathway [8].

Postrecognition of viral RNA by the cytoplasmic sensors RIG-I and/or MDA-5, epithelial cells activate the MAVS, a mitochondria-resident adaptor protein that is alternatively known as IFN- β promoter stimulator 1 (IPS-1), CARD adapter inducing IFN beta (Cardif), or virus-induced signaling adapter (VISA) [54–57]. MAVS serves as a central hub in the IFN induction pathway by activating further downstream kinases including TANK-binding kinase 1 (TBK1) and inhibitor of kappa light polypeptide gene enhancer in B cells, kinase epsilon (IKK- ϵ) that phosphorylate IRF3 and NF- κ B, respectively [58]. These molecules translocate into the nucleus upon phosphorylation and function as transcription factors, which ultimately leads to the expression of different IFNs and the activation of IFN-stimulated response elements (ISREs). In addition to IRF3, IRF7 has been characterized as an important transcription factor for type I IFN induction in immune cells, in particular dendritic cells [59]. Similar to IRF3, IRF7 undergoes phosphorylation and subsequent translocation into the nucleus in response to RV infection and activates IFN expression by functioning as transcription factors. To block such an important pathway, the RV-encoded NSP1 protein efficiently degrades both IRF3 and IRF7 in a virus-strain-dependent manner [42,60]. This process takes place first through the recognition of IRF3 using an ELLIS motif localized at the C-terminal end of NSP1 present on the NSP1 molecule derived from simian, murine, and some other nonhuman RV strains [61]. The NSP1–IRF3 interaction subsequently results in a rapid and efficient degradation of IRF3 at the proteasome and suppression of IFN production in RV-infected cells (Fig. 40.2).

Besides the IRF family members, NF- κ B has been shown to be another key arm of the host innate immune response downstream of MAVS in many virus-infected cells [56]. NF- κ B signaling is robustly activated by virus infection as well as proinflammatory cytokines, including

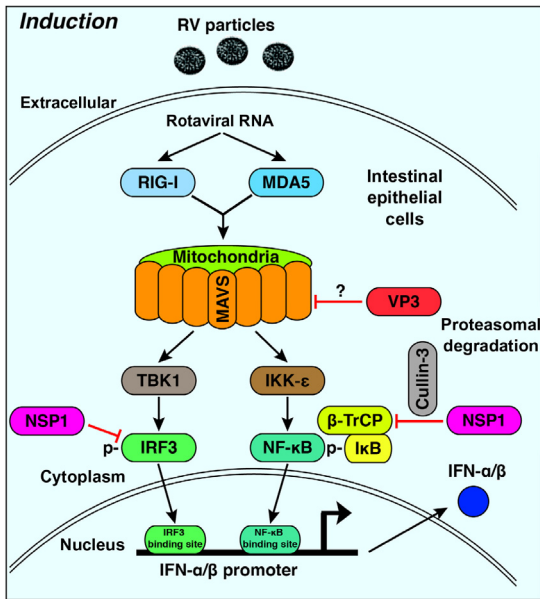


FIGURE 40.2 Rotavirus regulation of the IFN induction pathway. RV RNA PAMP activates the RNA sensing pathway, which is antagonized by RV-encoded proteins at multiple steps. In unpublished studies, VP3 has been shown to directly induce the proteasomal degradation of MAVS. In addition, NSP1 from different RV strains degrades either IRF3 or β -TrCP, the latter of which is mediated by the host cullin 3–E3 ligase complex. Together, these RV proteins work in concert to dampen IFN induction in RV-infected cells.

IL-1 β and TNF- α , the latter of which has recently been shown to be directly antiviral against RV [62]. For RV infection of HT-29 cells, the secretion of IL-8 is dependent on the NF- κ B activation [63]. In a suckling mouse model, other chemokines such as CCL3, CCL5, CXCL10, and GM-CSF were also upregulated following RV infection [64], although whether these canonical NF- κ B cytokines are activated through MAVS or TLRs remains unknown. Similar to IRF3, β -TrCP, a critical protein essential for degrading cellular NF- κ B inhibitor I κ B, is also targeted by NSP1 for proteasomal degradation [45]. In the case of β -TrCP, the binding domain within NSP1 maps to a C-terminal DSGIS motif in human and porcine RV strains [65]. Importantly, this is the same region as the

ELLIS motif responsible for IRF3 binding mentioned above. This interesting dichotomy of NSP1–substrate interaction may stem from the distinct contribution of IRF3 versus β -TrCP in IFN induction in different human and animal RV species [45]. In contrast to the previous speculation of NSP1 as a viral E3 ligase due to the presence of an N-terminal RING finger domain, we recently discovered an interesting codestruction mechanism, in which NSP1 localizes to the Golgi apparatus and hijacks the host cullin 3–RING box protein 1 E3 ligase complex to induce the proteasomal degradation of both β -TrCP and NSP1 itself [44]. Chemical blockade or siRNA knockdown of cullin-3 components impaired NSP1’s ability to degrade β -TrCP, leading to a significant increase in the levels of β -TrCP and reduced RV replication (Fig. 40.2). Interestingly, the cullin complex did not appear to be required for NSP1-mediated IRF3 degradation, suggesting an alternative mechanism of action at work.

More recent unpublished data from our lab indicate that in addition to IRFs and β -TrCP, MAVS itself is also subject to RV inhibition. MAVS levels were significantly reduced during RV infection, and this process is mediated, surprisingly, by the RNA methyl- and guanylyl-transferase VP3 protein (Fig. 40.2). By localizing to the mitochondria and binding to MAVS through an N-terminal domain, VP3 induced efficiently proteasomal degradation of MAVS in a host-species- and virus-strain-specific manner. MAVS inhibition by VP3 is another striking manifestation of RV’s ability to subvert host innate immune signaling. This is the first example of MAVS degradation by an enteric virus, and it would be of interest to further test other enteric RNA viruses such as norovirus.

A. Regulation of the Interferon Signaling Pathway by Rotavirus

The antiviral IFN response to RV infection follows a biphasic pattern consisting of an

initial IFN induction phase followed by a ligand-mediated (and IFN receptor-related) amplification phase [7,8]. As was discussed above, RVs are adept at inhibiting IFN induction and the RV NSP1 protein functions to degrade the essential factors β -TrCP and/or IRF3 during IFN induction in a RV strain-specific manner [66]. Interestingly, in spite of viral antagonism of IFN induction, infection with RV leads to the transcriptional induction and secretion of different IFN types in both cell culture and in vivo [8,19,26,49,52,67]. At least two factors contribute to the failure of RV to completely suppress the induction of IFN secretion. First, synthesis of the IFN antagonist NSP1 occurs only after viral entry, uncoating of the virion, RV transcription, and translation. During this initial infection process, several by-products of viral transcription are generated that act as potent triggers of the IFN induction pathway [8,10]. Second, RV entry into different types of cells may not always result in productive infection. For example, RV exposure to primary human pDCs results in two distinct populations of cells that differ in their level of viral infectivity [16]. Dendritic cells that are not productively infected nevertheless exhibit robust activation of the IFN induction response [16,27]. Given the remarkable efficiency of IFN secretion in this cell type, they are a likely source of substantial IFN secretion from a non-epithelial cellular compartment where RV does not replicate efficiently. In suckling mice, infection with RV leads to significant induction of different types of IFNs, of which the type I IFNs are induced primarily in intestinal immune cells rather than being derived from IECs, where viral infectivity is maximal [26]. The secretion of IFNs from different cell types poses a unique challenge to successful viral propagation and spread to uninfected bystander IECs. This is because IFN binding to its cognate cell surface receptor activates a positive feedback loop that amplifies the expression of IFNs as well as more than 300 different

IFN-stimulated genes [68]. This IFN release then efficiently amplifies the expression of antiviral proteins targeting a variety of viral replication steps in uninfected bystander cells. Each of the three major IFN types (I, II, and III) that are found to be induced in the intestine following RV infection is capable of mediating phosphorylation of the key convergent transcription factor STAT1 (at Y701, an event that is critical for unlocking the transcriptional program resulting in an antiviral state) (Fig. 40.3). Each of these IFN types is biologically relevant in the context of modulating RV infection and spread [7,52].

Several lines of evidence indicate that RV employs potent countermeasures to subvert the antiviral state mediated by secreted IFNs during initial infection [7,19,26,52,67]. In cell culture, addition of purified exogenous IFNs after RV adsorption does not significantly hamper viral replication; instead, IFN treatment of cells prior to RV infection is required to achieve efficient RV replication restriction [69]. In the RV suckling mouse model, infection with a homologous murine RV and infection with a heterologous simian RV strain result in comparable levels of induction of different IFNs from the intestine [7,26]. However, as was noted above, the presence of IFNs during infection has a negligible effect on murine RV replication (\sim 1-log restriction in titer) but has a potent effect on heterologous simian RV replication (4- to 5-log restriction in titer) [26,50]. These observations suggest that in order to replicate successfully, homologous RVs have evolved strategies to induce resistance to the actions of different secreted IFN types in cells prior to their actual infection (bystander cells). Classical reassortment genetic studies of the IFN-mediated replication phenotype of murine and simian RVs implicated a constellation of RV genes (encoding the VP3, NSP1, NSP2, and NSP3 viral proteins) in determining the resistance to IFN signaling [51]. Of these, NSP1 is a necessary and the major determinant of efficient intestinal RV replication in an IFN-dependent fashion.

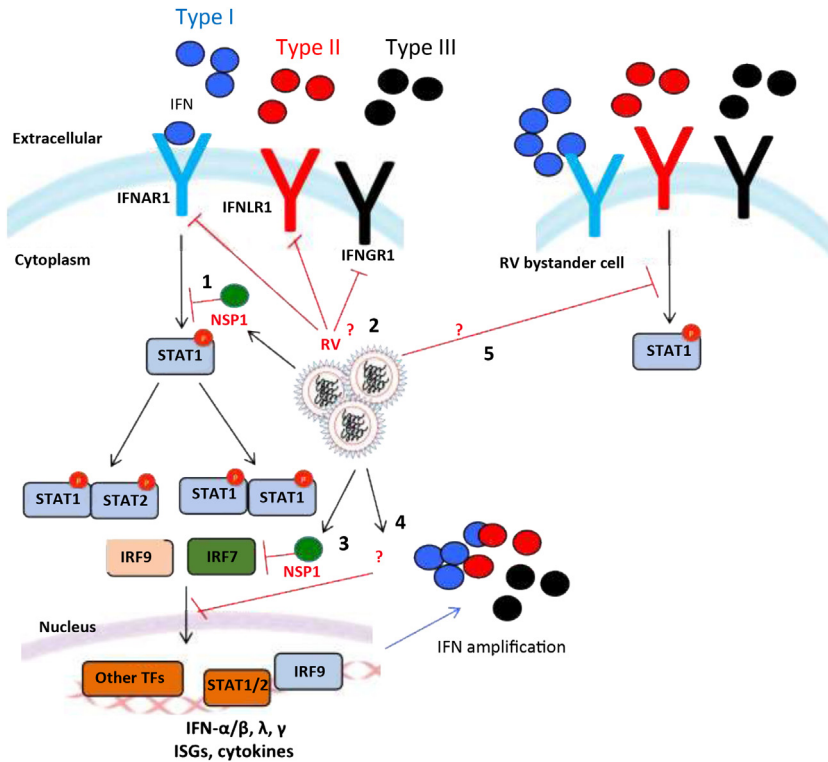


FIGURE 40.3 RV regulation of the IFN amplification pathway. Following infection, RV NSP1 efficiently blocks exogenous IFN-directed phosphorylation of STAT1 at Y701, shown here for the IFNAR1 (1). In the virus-infected cell, RV also mediates the lysosomal degradation of receptors for types I, II, and III IFN by an unknown process (2). Along with IRF3 and IRF5, the viral NSP1 protein also proteasomally degrades IRF9 and IRF7 in the infected cells, which are required for optimal IFN amplification responses (3). In addition, RV can inhibit the nuclear translocation of STAT1-pY701 by an unknown mechanism (4). Remarkably, in addition to these viral effects in infected cells, RV also potently inhibits STAT1 phosphorylation in uninfected bystander cells in response to different types of IFNs (5). The viral and host factors underlying this bystander inhibition of IFNs are unknown.

B. Regulation of STAT1 by Rotavirus

Direct evidence for RV subversion of the antiviral state mediated by exogenous IFNs comes from the finding that RV-infected HT-29 cells (a human IEC colonic cancer cell derived line) are able to efficiently block STAT1-Y701 phosphorylation in response to exogenously added purified IFNs I and II [52,67]. Using single-cell analytic techniques, IFN-mediated STAT1 inhibition is found to occur within RV-infected cells. Remarkably, STAT1 responses to exogenous IFN ligand are also potently

inhibited in RV uninfected bystander cells, which do not express any detectable viral antigen [67] (Fig. 40.3). Although initially described for a porcine RV strain SB1-A, this bystander inhibitory effect has now been observed in vitro with several other RV strains, albeit with lower efficiency (Sen and Greenberg, unpublished observations). The ability of RV to block IFN-dependent signaling has also been observed in vivo. Suckling mice infected with murine RV are able to significantly suppress IEC STAT1-Y701 phosphorylation and subsequent transcription that occurs in response to parenterally

administered purified IFNs I or III [52]. Although IFNA4, IFNA5, and IFNA5 transcripts are induced in the intestines of murine RV-infected mice, transcriptional analysis of isolated mature villous enterocytes revealed that within this compartment (where RV replication predominantly occurs), both infected and bystander cells fail to amplify the type I IFN genes [26]. In the villous epithelium, RV bystander cells also do not express elevated levels of transcripts encoding IRF7, which is upregulated in response to stimulation of cells with secreted IFNs and is critical for the optimal expression of several antiviral genes.

C. Degradation of Different Types of Interferon Receptors

The effectors in RV-infected cells that mediate STAT1 inhibition in bystander cells and the rotaviral factors responsible have not been identified. In contrast, RV inhibition of IFN-directed STAT1 activation in RV-infected cells is well characterized [26,52,67]. Recent findings demonstrate that at the single-cell level, RV infection results in the efficient depletion of type I, II, and III IFNRs within RV (VP6 antigen⁺) infected cells [52]. Such RV-mediated IFNR degradation is unlikely to be directed by secreted IFNs (i.e., by a ligand-dependent pathway) and despite prolonged infection of cells is restricted exclusively to the subset of cells expressing VP6. The depletion of IFNRs in RV-infected cells occurs from 6 to 8 hours post-infection (hpi) onwards by a lysosomal–proteasomal pathway of protein degradation and is not observed in the RV (VP 6 antigen) uninfected bystander cells, which are nevertheless highly refractory to IFN-directed STAT1 activation (Fig. 40.3). The relevance of RV-mediated IFNR degradation is shown *in vivo* by the significant decrease in intestinal type I and II IFNR protein expression in the small intestine following murine RV infection [52].

Degradation of different types of IFNRs by RV represents an ingenious strategy to ensure that any autocrine IFN antiviral amplification is inhibited, thus allowing viral replication and cell to cell spread to proceed efficiently [52]. Interestingly, these findings indicate the likelihood that RV targets a common host-signaling pathway that is responsible for the expression of all three IFNRs. Continuing to unravel the mechanisms by which RV also inhibits the response to different IFNs in bystander cells is important for several reasons. First, since the level of RV replication substantially determines host pathogenicity, such information will enable more rational attenuation of homologous and heterologous RV strains and their use as candidate third-generation live vaccines. Second, for several diseases (including sepsis and systemic lupus erythematosus), an excessive IFN response is undesirable and implicated as a causative and/or exacerbation trigger of disease. In these situations, discovering novel therapeutic modalities that can dampen IFN signaling is potentially valuable.

D. STAT1 Sequestration in the Cytoplasm

Other RV strategies have also been identified that are directed at disrupting STAT1 signaling during infection. The ability of RV to perturb STAT1 signaling was first reported by Holloway and colleagues [70,71], who observed that as early as 6 hpi, several RV strains inhibited the nuclear translocation of phosphorylated STAT1-Y701 in response to exogenous IFN stimulation (Fig. 40.3). Although viral factors responsible for this inhibitory effect downstream of STAT1 activation have not yet been identified, it is possible that redundancy in RV inhibition of the STAT1 pathway exists, perhaps indicative of the vital role of inhibiting IFN signaling in enabling RV replication.

E. Regulation of IRF7 and IRF9

The ability of the RV NSP1 protein to target IRFs for proteasomal degradation extends to IRF7 and IRF9, two additional factors that are critical for the optimal amplification of IFN-dependent antiviral responses [40,42]. Whereas early induction of different IFNs and antiviral transcripts is mediated primarily by IRF3, IFN-mediated signaling results in an increase in IRF7 expression, which subsequently orchestrates the amplification of IFNs and of ISGs. The IFN-mediated effects on transcription of several genes (including IRF7) require the assembly of a transcriptional complex ISGF3, which includes STAT1 and IRF9. The role of IRF7 and IRF9 degradation in IFN-dependent responses during RV infection has not yet been well studied. Nevertheless, the degradation of IRF7 and IRF9 by NSP1 is likely to be an additional weapon in the RV arsenal that can be used to halt an efficient IFN amplification response (Fig. 40.3).

F. Rotavirus Regulation of Other Effector Antiviral Factors

In addition to the IFN induction and amplification pathway, RV is equipped with the ability to block further downstream effector antiviral proteins. One such example is ribonuclease L (RNaseL), a key enzyme in the IFN-inducible 2'-5'-oligoadenylate synthetase (OAS)-RNaseL pathway responsible for potent RNA degradation, including both host and viral RNA molecules [72]. The RV RNA capping enzyme VP3 encodes a C-terminal phosphodiesterase (PDE) domain that was recently shown to induce the degradation of 2',5'-oligoadenylate, the second messenger responsible for RNaseL activation and dimerization [73]. The RV VP3 PDE domain functionally replaced the comparable domain in the murine coronavirus ns2 protein and inhibited RNaseL activity. A more recent study suggests that another VP3-independent,

yet-to-be identified, mechanism also exists and contributes to RV inhibition of RNaseL [74]. However, the actual physiological roles of RNaseL in modulating RV replication and of VP3 in antagonizing RNaseL in vivo remain to be demonstrated.

V. TAKING ADVANTAGE OF ROTAVIRUS HOST RANGE RESTRICTION TO RELIABLY ATTENUATE LIVE ROTAVIRUS VACCINE CANDIDATES

There are currently two time-honored and demonstrably successful approaches to developing safe and effective human viral vaccines [75]. In the first case, a wide variety of current viral vaccines rely on the parenteral administration of replication-incompetent inactivated whole virus, the parenteral administration of a viral protein(s) component, or the administration of a molecularly produced virus-like particle (VLP). All of these entities are selected because immunity to the individual protein, inactivated whole virus, or VLP induces protective immunity to the host and, at the same time, is both safe and well tolerated. There are numerous highly successful examples of this category of viral vaccine (e.g., the inactivated polio vaccine, the various preparations of inactivated influenza hemagglutinin-based vaccines, the human papilloma VLP vaccine, the hepatitis B virus vaccine, and the recently licensed herpes zoster gE protein-based vaccine). In all cases, these vaccines are administered parenterally. Several are administered with adjuvants of one kind or another to enhance immune responses, and in all cases, they appear to function primarily by stimulating systemic immunity, with the primary effector function generally mediated by systemic antibodies. Of note, none of these types of vaccines are directed at a predominantly enteric pathogen, although an investigation of a potential parenterally administered

norovirus VLP vaccine is currently under way [76], and there are plans to study the utility of a heat-inactivated RV virion-based vaccine in humans [77]. The greatest advantages of the inactivated/recombinant protein-based vaccines are their general safety and the fact that they can be produced even when the actual pathogen cannot be readily propagated. The general disadvantage of such vaccines is that they are almost always less effective at stimulating T-cell-based immune responses, they are not very efficient at stimulating mucosal immune responses, that highly effective mucosal immune adjuvants are not yet readily available, and in some cases, immune responses to these inactivated vaccine preparations tend to diminish over time more rapidly than do responses to several live viral vaccines. Other potential disadvantages of inactivated vaccines become apparent when the antigen or antigens required to induce protective immunity are difficult to synthesize artificially or when immunity is most potent when it is directed at multiple antigens that are correctly folded only on the actual or recombinant multiprotein viral particle. Of note, RVs have at least two separate proteins (VP4 and VP7) that are targets of protective antibodies, and VP4 is cleaved by enteric trypsin into two separate protein components: VP8* and VP5* [78]. Both VP8* and VP5* are individually targeted by protective antibody responses. VP7 is correctly folded into its proper antigenic trimeric form only within the context of the RV virion, and a similar issue likely is true for VP5*. On the other hand, the RV VP8* protein can be relatively simply and accurately synthesized in several prokaryote and eukaryote systems, and because of this feature, it is currently being examined as a potential inactivated vaccine to be administered parenterally [79] (Chapter 41: Development of Oral Rotavirus & Norovirus Vaccines).

The second highly successful approach to human viral vaccine development has been the production of live attenuated viral vaccines

that actually infect susceptible people but are attenuated to such a degree that their level of reactogenicity and pathogenicity is acceptable, while they reliably generate protective immunity. As with inactivated vaccines, a number of highly effective replication-competent viral vaccines are currently available (e.g., oral Sabin polio vaccine, measles vaccine, rubella vaccine, yellow fever vaccine, smallpox vaccine, live attenuated influenza vaccine, and, of course, several licensed RV vaccines, such as RotaTaq, Rotarix, and Rotavac) [75]. Most but not all of the live attenuated viral vaccines are administered by parenteral injection; however, oral polio, rotavirus, and live attenuated influenza vaccine are all administered to a mucosal surface (the GI tract and the respiratory tract, respectively). The general thinking is that live attenuated viral vaccines more closely mimic the type and level of immunity induced by natural infection than parenterally administered inactivated vaccines do. If natural infection is an effective preventative of severe secondary infection, then reproducing it without undue reactogenicity can be desirable. This feature is present when natural immunity is operative primarily at a mucosal surface, as is the case for RVs. Natural RV infection efficiently protects against severe reinfection, and for this reason, a variety of experimental and licensed live attenuated RV vaccines have been developed or proposed.

The key issue to overcome in developing a live viral vaccine is to discover a method that reliably attenuates viral pathogenicity while retaining the ability of the viral infection to induce protective immunity. Traditionally, such modification has been accomplished by multiple passage of virulent viral strains in cell culture with the hope that such multiple passages will lead to the acquisition of sufficient mutations in the viral genome (acquired to enhance cell culture replication) and that these cell culture adaptations will attenuate viral pathogenicity in the target host. This strategy is

time honored and has been used to develop multiple vaccines (e.g., live polio, measles, mumps, rubella, yellow fever, and some RV vaccines). While this approach is frequently successful, there is no way to determine how many passages are needed to eliminate residual virulence while retaining immunogenicity, so it is often tedious and always an inexact approach. In addition, concerns about reversion to virulence are always present. That said, this approach was used successful to develop a highly effective human RV vaccine (R1, Rotarix), which consist of a single human RV strain that was repeated passed in several cell culture systems and, over the passage history, became attenuated in people [80]. While the genomic sequence of both the virulent wild-type parental Rotarix strain and the eventual vaccine strain are known, the exact genomic mutations responsible for attenuation are unclear. What has been established, however, is that, given the very substantial and decade-long safety record, this human RV vaccine strain has sufficient attenuating mutations to ensure a high degree of genetic stability in humans. Of note, the R1 Rotarix vaccine represents a single G and [P] serotype yet reliably induces protective immunity to virtually all frequently circulating human RV strains. This finding strongly supports the conclusion that serotype-specific immunity is not a major determinant of immunity to severe RV disease in humans [81].

The other strategy that has proven highly successful for the reproducible attenuation of the RVs used in live attenuated RV vaccines has been to take advantage of the HRR (see above) of heterologous (nonhuman origin) RVs as vaccine candidates for humans [82]. Several currently licensed RV vaccines (e.g., the R5 vaccines RotaTeq and Rotasil and the R1 vaccine Rotavac) consist of either natural or experimentally selected reassortants between animal RVs (in these cases, all bovine RVs) and human RVs. Both R5 vaccines are pentavalent constructs derived experimentally on the basis of

bovine RV genomes but containing individual VP7- or VP4s-encoding genes derived from various human serotypes. RotaTeq is broadly licensed around the world, while Rotasil is currently licensed only in India [83]. Rotavac is, interestingly, a naturally occurring reassortant RV derived from a human RV and a bovine RV. It contains only a bovine VP4, with all other ten RV gene segments derived from a human RV strain. This virus was originally isolated from a neonatal nursery where asymptomatic RV infection was endemic [84]. Finally, of relevance to this review, an entirely lamb origin RV strain is currently licensed for RV prevention in China. This vaccine is also highly attenuated, presumably because of the HRR of a lamb origin RV in humans. While this vaccine seems safe, the data on its efficacy are limited [85]. The key point here is that all these animal virus origin based RV vaccines are highly attenuated in humans, and this attenuation, as was discussed above, is most likely based on their host range replication restriction in humans. This HRR is primarily due to the inability of heterologous RV to efficiently suppress the human intestinal innate immune response, primarily the human IFN response, owing to the presence of heterologous NSP1s in the vaccine candidates. The active human IFN response to these heterologous RV vaccines suppresses their replication sufficiently to restrict pathogenicity and reactogenicity but not so much that the generation of effective RV immunity is suppressed. However, in the case of the Indian Rotavac vaccine, attenuation might be based on the heterologous bovine origin VP4, which might be expected to reduce RV binding to human IECs.

The big question for the future is whether, with the advent of a tractable reverse genetic system and our improved understanding of the genetic determinants of HRR, we will be able to better fine-tune the replication competence and immunogenicity potential of RV vaccine candidates to further enhance their efficacy.

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