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Chapter 8

Use of RNA Domains in the Viral Genome as Innate Immunity Inducers for Antiviral Strategies and Vaccine Improvement

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/56099

1. Introduction

This chapter will focus on the role of innate immunity induction on antiviral responses with an emphasis on nucleic acids as type-I interferon (IFN) inducers and their use as antiviral compounds and vaccine adjuvants. A general and up-to-date view of the different mechanisms operating in the host cell for sensing viral genomes will be given, as well as viral strategies counteracting this response through immune evasion or specifically targeted antagonism. Our own recent data describing the ability to induce IFN and mediate protection against viral infection in vivo of synthetic RNA transcripts enclosing structural domains present in the 5′- and 3′-terminal regions of the foot-and-mouth disease virus (FMDV) genome will be summarized and discussed in this context. New vaccine formulations including innate immunity inducers are being developed for improvement of current vaccines. The potential of exogenous nucleic acids as modulators of immune response outcomes and vaccine adjuvants will be reviewed and discussed. A schematic summary of the interrelated topics addressed in this chapter is shown in Figure 1. Additionally, a glossary of all the acronyms and abbreviations used in the text and figures is shown in Table 1.

2. Innate immune response against viral infection

The mammalian immune system is composed of the innate and the adaptive arms which work in combination to battle against a large variety of pathogens such as bacteria, fungi, parasites

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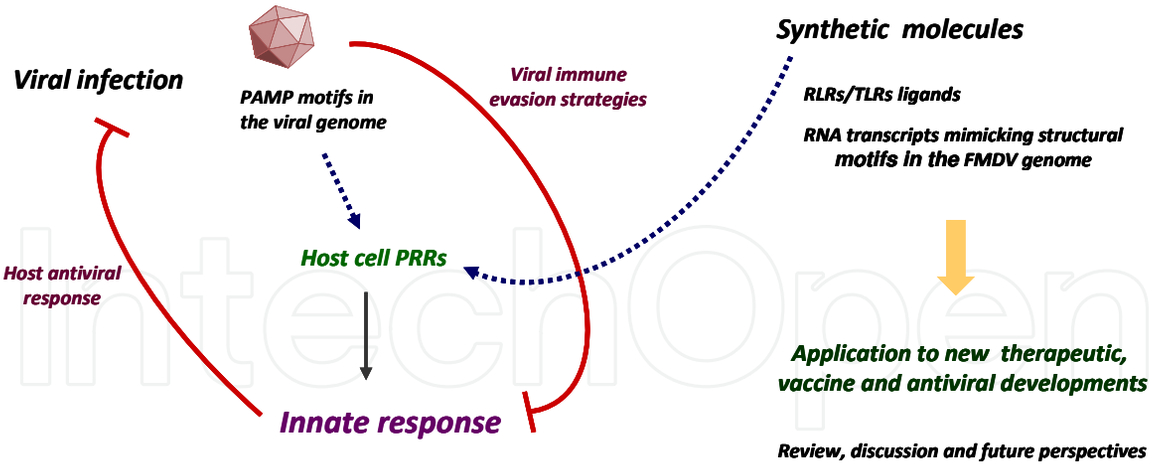

Figure 1. Schematic summary of the topics reviewed and discussed in this chapter

and viruses. Both systems have the molecular task to distinguish "self" from "non-self" components in the organisms in a sensitive and faithful manner. The innate immune system is hence the first line of defense against infection by pathogens. The existence of pattern-recognition receptors (PRRs) expressed in cells of the innate immune system that are capable to specifically sense pathogen-associated molecular patterns (PAMPs) was first proposed by Charles Janeway in 1989 [1]. PAMPs are chemical or structural features present in pathogens but not in host cells acting then as alert signals to the innate immune system of the host. Lipids, polysaccharides, nucleic acids and CpG DNA are among the basic PAMPs recognized by PRRs. Their recognition triggers cellular responses aimed to counteract the pathogen and initiate and promote other responses such as inflammation and adaptive immune responses.

In 1996, the gene/protein Toll, initially described as a transmembrane protein required for dorsal-ventral polarity in the Drosophila embryo, was found to play also a role in immunity against fungal infections [2]. One year later, their mammalian orthologs, the Toll-like receptors (TLRs), were reported to mediate recognition of pathogens by the innate immune system [3]. In 2004, a new and TLR-independent pathway was described for recognition of viral nucleic acids in the cytoplasm of the infected cells, the retinoic acid-inducible gene-1 (RIG-I) [4]. Four different families of PRRs have been found to date, including TLRs [5], RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs) [6] and nucleotide oligomerization domain (NOD)-like receptors, (NLRs) [7], as well as unidentified proteins that mediate sensing of cytosolic DNA or retroviral infection [8]. Among them, TLRs, RLRs and NLRs are involved in the recognition of viral nucleic acids [9]. NOD2, a member of the NLR family, typically involved in antimicrobial immune defenses, and highly expressed in antigen presenting cells (APCs) such as monocytes, macrophages and dendritic cells (DCs), has been shown to bind viral single-stranded (ss) RNA [10].

TLRs are a family of type-I transmembrane proteins that traffic between the plasma membrane and endosomal vesicles, expressed on various immune cells, including dendritic cells, which recognize a wide range of PAMPs including double-stranded (ds) RNA (TLR3), ssRNA (TLR7 and TLR8) and DNA (TLR9). All TLRs signal as dimmers and share a common architecture of

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extracellular leucine-rich repeats and intracellular Toll/Interleukin-1 receptor (TIR) domain (Figure 2). Nucleic acid sensing-TLRs localize in intracellular vesicles, including endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes of dendritic cells and other innate immune cells. Intracellular localization enables TLRs to recognize nucleic acids delivered to intracellular compartments after the uptake of viruses or infected cells. Unc93b1 is a membrane protein which anchors TLRs to the ER and regulates their trafficking to the endosomal compartments. TLR signaling pathway depends on the recruitment of a TIR-domain containing adaptor, MyD88, for all TLRs (with the exception of TLR3) and culminates with NF-κB and MAP kinase activation and induction of inflammatory cytokines (Figure 2). TLR3 uses TRIF to activate NF-κB and IRF-3 through an alternative pathway and the induction of type-I IFN and inflammatory cytokines. Activation of the TLR signaling leads to maturation of DCs, contributing to the induction of adaptive immunity.

The RLRs are a family of ubiquitous cytosolic helicases consisting of the three members: RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology-2 (LGP2) (Figure 2). RIG-I and MDA5 have tandem caspase activation and recruitment domains (CARD) followed by a DExD/H box RNA helicase domain and a repressor domain. LGP2 lacks the N-terminal CARD domains and may function to regulate RIG-I and MDA5 as a repressor [11]. It has been reported that RIG-I recognizes ssRNA bearing a 5′-ppp and short dsRNA, while MDA5 senses long dsRNA [12-14]. When the inactive forms of RIG-I or MDA5 bind viral RNA, the helicases undergo a conformational change, multimerization and then, interaction with the adaptor molecule MAVS (also called IPS1, VISA or CARDIF), localised to the outer mitochondrial membrane via CARD-CARD interaction. Then, MAVS induces activation of IRF3/7 resulting in the transcription of type-I IFNs and also activates NF-κB (Figure 2). It has been recently shown that MAVS resides on peroxisomes also and can induce antiviral signaling from this organelle acting with mitochondrial MAVS sequentially to create an antiviral cellular state [15]. Upon viral infection, peroxisomal MAVS induces the rapid interferon-independent expression of defense factors for short-term protection, whereas mitochondrial MAVS activates an interferon-dependent signaling pathway with delayed kinetics, amplifying and stabilizing the antiviral response.

In addition to PRRs, which inhibit viral infections indirectly by activating signaling cascades that result in the transcription of IFN and other antiviral molecules, there are intrinsic antiviral factors which act blocking viral replication immediately and directly, often before the onset of IFN response, like PKR, MxA, TRIM5α or the IFIT and IFITM families [16]. Intrinsic innate factors preexist in certain cell types though they can be further induced by IFNs to amplify their antiviral activity.

Recent work supports the non-redundant functional requirement for TLRs and RLRs [17]. On the contrary, the cooperation and crosstalk between different PRRs mediates activation of an effective immune response and host defense against viral infections [8, 18]. Unique links between NLRs and RLRs signaling responses have also been identified [10, 19]. Polymicrobial infection involve complex host interactions that are likely to engage a variety of response pathways including different PRRs.

IFNs exert auto- and paracrine actions within a few hours in response to a viral infection. Their protective effect is dual: they induce an antiviral cellular state and promote the clearance of infected cells in synergy with other proapoptotic agents as tumor necrosis factor (TNF). Through the secretion of IFN, triggered by activation and translocation to the nucleus of NF-κB, IRF3 and IRF7, the antiviral response can be amplified and spread to surrounding uninfected cells by binding to the IFN-α/β receptor (IFNAR) in the cell surface. Binding of the cytokine triggers a Jak-STAT signaling pathway and subsequently activates hundreds of IFN-stimulated genes (ISGs), most of them encoding proteins with antiviral functions such as inhibition of viral gene expression or degradation of the viral genome [20].

In addition to its antiviral properties, IFNs exhibit potent immunomodulatory properties that contribute to their antiviral effect such as stimulation of the effector function of natural killer (NK) cells, cytotoxic T lymphocytes and macrophages, upregulation of MHC class I and II molecules, induction of immunoglobulin production by B cells and stimulation of proliferation of memory T cells [21]. This enables several ways to control viral replication by modulating of the innate and adaptive immune responses [22]. Type-I IFNs act through activation and maturation of dendritic cells leading to MHC upregulation. They can also regulate certain chemokines, chemokine receptors and costimulatory molecules, which, in turn, stimulate CD4- and CD8-positive T cell responses and promote Th1 differentiation, modulating T lymphocyte responses [23].

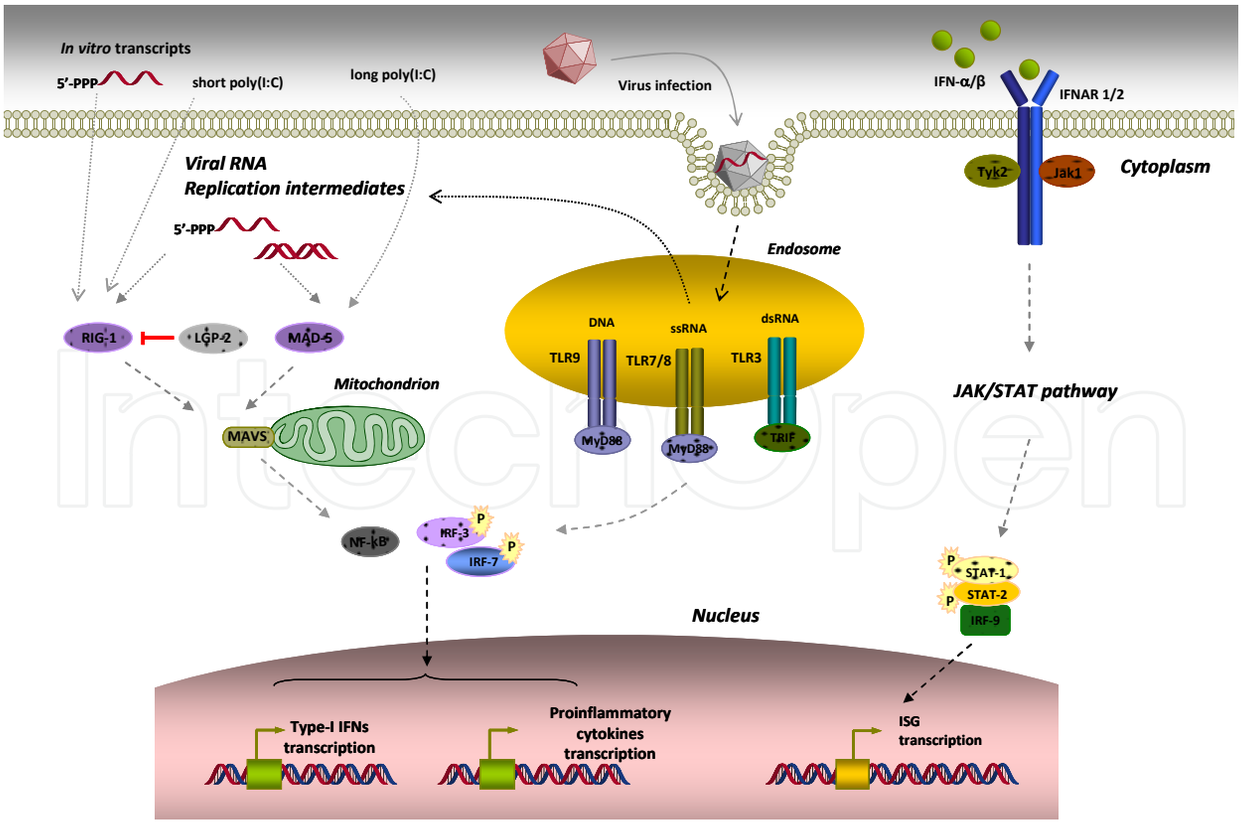

Figure 2. Schematic representation of viral RNA detection by TLRs and RLRs and signalling pathways

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Induction of innate immune signaling pathways through PRRs is a crucial step antagonised by many viruses [24, 25]. Over 170 different virus-encoded IFN antagonists from 93 different viruses had been reported by 2010 (reviewed in [26]), and the list keeps constantly growing, indicating that most viruses interfere with multiple stages of the IFN response. Four main mechanisms are used to circumvent host innate responses: general inhibition of gene expression, and sequestration, proteoltytic cleavage or proteasome degradation of key factors of the IFN circuit such as RLRs, MAVS, IRFs, Jak/STAT, PKR... Several IFN antagonists are conserved within different RNA virus families, while that seems not to be the case for DNA viruses [26]. This can be explained by the multi-functionality of RNA virus proteins, imposed by restriction in genome size, unlike large dsDNA viruses which might have a higher coding capacity for new viral proteins displaying a wider range of antagonistic activities.

The potential of IFN antagonists knockout viruses as promising candidates for live virus vaccines has been suggested based on studies with Influenza A/B viruses, Japanese encephalitis virus, human respiratory syncytial virus and coronaviruses [26-29]. These severely attenuated viruses are rapidly cleared in vivo by a potent IFN response, while inducing long-lasting immune memory due to their replication competent nature. Viral miRNAs may also function in evasion of the host antiviral response (reviewed in [30]). The contribution to viral evasion of type-I IFN response of small non-coding subgenomic flavivirus RNAs generated as degradation products by a host exoribonuclease, has been recently shown [31]. Hence, IFN antagonists are good targets for antiviral drugs development.

3. RNA motifs in the viral genome can trigger innate responses

Accurate discrimination of self from non-self is critical to avoid immune triggering against self that leads to autoimmunity [32]. In that sense, it has been proposed for hepatitis C virus (HCV) that a combinatorial non-self signature in the viral genome for PRR binding may lead to accurate PAMP discrimination [33].

TLRs involved in recognition of the viral genomes are TLR3, TLR7/8 and TLR9, all of them localized to the endosomal compartment [34]. TLR3 is widely expressed in innate immune cells with the exception of neutrophils and pDCs and responds to dsRNA, a common viral PAMP, and its synthetic analog polyriboinosinic-polyribocytidylic acid (poly I:C) [35]. TLR7 and TLR8 are closely related receptors that recognize nearly any long ssRNA with some differences between them. Short ssRNA containing certain motifs preferentially activate TLR7, and activation with synthetic agonists specific to TLR7 or TLR8 trigger different cytokine profiles [36]. TLR9 is highly expressed in pDCs and responds to the unmethylated deoxycytidylate-phosphate-deoxyguanylate (CpG) motifs in viral and bacterial DNA [37].

Different features have been defined for RIG-I recognition as RNA PAMPs, including the presence of a free 5′-triphosphate, absent from eukaryotic cytoplasm due to RNA metabolism in the nucleus, length (longer than 19 nt), secondary structure characteristics (a base-pairing region of 10-20 nt near the 5′-ppp) [38] and nucleotide sequence motifs (such as a 3′-poly U/UC tract in the HCV genome) [33]. Panhandle structures adopted by Sendai virus DI-

genomes or self-complementary influenza virus genome have been described as potent PAMPs sensed by RIG-I [39]. Data on MDA5 ligands are scarce. MDA5 seems to sense dsRNA analog poly I:C in mice [40] and higher-order RNA structures present in infected cells have been found to activate MDA5 [41]. A recent report shows the direct interaction of MDA5 with dsRNA replicative intermediate forms of positive strand RNA viruses [14]. RLRs have evolved to sense the presence of largely different sets of viruses but not always acting in a mutually exclusive way [13, 42].

In addition to the direct antiviral function of RNase L degrading ssRNA, RNase L can generate viral or host-derived small RNAs that amplify the IFN response by generating PAMPs that activate the RLR pathway. RNase L mediated cleavage of HCV RNA generates svRNA that activates RIG-I, thus propagating innate immune signaling to the IFN-β gene [43, 44].

Given the ability of RLRs to sense viral RNAs and activate IFN signaling cascades that eliminate viral infections, many viruses have developed immune evasion strategies to overcome detection by RLRs. This is carried out through RNA modification of viral RNA genomes to prevent host detection [24]. For example, some viruses engage cap snatching (e.g. influenza virus), modification of 5′-ppp to monophosphate through virus encoded enzymes (e.g. Borna disease virus, Lassa virus), 2′O-methylation of viral mRNA cap structure by virus encoded methyltransferases, exploiting nucleotide modifications found at higher frequency in eukaryotic versus prokaryotic/viral RNA, and the use of proteins to protect the 5′ends (picornavirus have a virus encoded protein, VPg, covalently linked to the 5′end of their genome) or overhangs (e.g. arenavirus) [24].

In 2008, Saito et al. showed that the 3′ non-coding region (NCR) of HCV (a flavivirus) encoded PAMP motifs triggering innate immune signaling in the host cell. Thus, the 100 nt-polyuridine motif (poly U/UC) within the 3′ NCR was identified as a potent PAMP, substrate of RIG-I recognition and immune triggering in human and murine cells [45]. In contrast, the structured 3′-terminal X region failed to trigger signaling. The entire HCV 5′NCR, containing four major stem-loop structures including the internal ribosome entry site (IRES), was a weak inducer of IFN promoter signaling. However, prior treatment of cells with IFN-β to increase RIG-I levels rendered them responsive to signaling induced by the 5′NCR or the X region [45], suggesting that dsRNA regions of the HCV genome are not potent PAMPs but may confer signaling during the IFN response. Some studies on the 5′- and 3′-NCRs of other flaviviruses show remarkable differences in their IFN-inducing capacity. The 5′and 3′NCRs of dengue virus (DEN) elicited low but measurable stimulation of innate immune signaling, while the smaller highly structurally conserved 3′-terminal stem-loop RNAs of DEN, West Nile virus (WNV) and yellow fever viruses were minimally active [46]. Additionally, the base-pairing extent of the 5′-triphosphate of the RNAs may have an enhancing effect on RLR recognition and signaling [38, 47]. Therefore, the ability of different RNAs as IFN inducers must be tested independently, being difficult to predict their behaviour/potency by their sequence, secondary structure or homology with analog molecules. In this sense, we have recently shown that FMDV (a picornavirus) full-length transcripts with the 3′NCR deleted induce lower levels of IFN-β than complete RNA transcripts in cell culture [48]. These results are equivalent to those reported for HCV transcripts lacking the PAMP motif poly-U/UC. In this case, it has been

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proposed that, as the virus must maintain this motif in the 3′NCR for its viability, the host takes advantage of this and targets this region as a discriminator of PAMP RNA through RIG-I interaction [45]. Thus, HCV infection seems to be regulated by hepatic immune defenses triggered by the cellular RIG-I helicase. For FMDV, we also found that RNA transcripts corresponding to structural domains predicted to enclose stable dsRNA regions in the 5′and 3′NCRs of the viral genome were able to trigger an IFN-α/β response in epithelial porcine kidney cultured cells and induce an antiviral state [48] (Figure 3). A direct link between antiviral activity induced by FMDV NCR transcripts and IFN could be established in cultured cells, as treatment with monoclonal antibodies against IFN-α/β effectively blocked the antiviral activity induced by the RNAs [48]. Different levels of IFN-β mRNA induction were observed for the different RNAs assayed, being the one mimicking the complete 3′NCR, enclosing two predicted stem-loop structures, the best inducer. The in vitro RNA transcripts corresponding to the complete 5′ NCR, the IRES and the S hairpin (Figure 3), were also able to induce IFN-β transcription, though at lower levels than the 3′NCR transcript. The removal of the poly A tail within the 3′NCR RNA had a detrimental effect on IFN-β induction, but milder than removal of the 5′-ppp by treatment with alkaline phosphatase, which strongly reduced but did not completely abolish induction. However, deletion of any of the 3′NCR stem-loop (SL) structures rendered RNAs minimally active for IFN-β signaling, suggesting a relevant role for RNA structure in this region for its recognition as a PAMP. Unlike the FMDV NCR transcripts, the 5′-end of the viral genome is linked to the viral protein VPg lacking a 5′-ppp, making difficult to draw conclusions on the putative role of these structural regions in viral pathogenesis.

Encouraged by the results of IFN-β induction in swine cultured cells transfected with the NCR transcripts, we aimed to address the potential of such molecules as type-I IFN inducers in vivo. For that, the FMDV NCRs were inoculated intraperitoneally into Swiss suckling mice and the levels of IFN-α/β proteins and antiviral activity in sera were measured [48]. Newborn mice are a suitable model for innate immune responses while their adaptive immunity is still immature. All the FMDV NCRs were able to induce a peak of IFN-α/β in sera of the inoculated animals at 8 h after injection, remarkably higher than those observed for poly I:C-transfected mice. This peak was maintained up to 24 h in the case of the S RNA. The presence of antiviral activity in sera from NCR-transfected mice was also detected and measured, and a good correlation with IFN-α/β levels tested by ELISA was found. Interestingly, even those transcripts showing a lower capacity for IFN-β induction in porcine cultured cells were able to induce an innate immune response in mice. On one hand, this suggests that the effect of low level-inductions of type-I IFN observed in cultured cells can be magnified in vivo. On the other hand, the action of other viral sensors in vivo, mainly TLRs, may account for the enhancing effect observed. Thus, the specific immunostimulatory activity of each NCR RNA may be different depending on the host cell context assayed. This was the case for the IRES: despite of its complex structure, it was a poor inducer in cultured cells. However, the IRES acted as a strong IFN inducer in suckling mice. We further showed that the innate immune responses triggered by the NCRs in suckling mice resulted in a reduced susceptibility to FMDV infection in all cases, being remarkable the antiviral effect of inoculation with the IRES RNA [48, 49].

The antiviral effect exerted in vivo by these small synthetic non-infectious RNA molecules was analyzed extensively, using a wide range of viral doses and different serotype isolates [49]. The time course of resistance to FMDV of the RNA-transfected mice was also studied. Inoculation with all RNAs remarkably increased the 50% lethal dose (LD_{50}) of the virus, determined for the control group. Mice inoculated with IRES or S transcripts 24 h before challenge became at least 10000-fold less susceptible to the virus than PBS-inoculated mice. Interestingly, 90% of the IRES-transfected mice survived after infection with a viral dose of 7×10^6 plaque forming units (PFU) (undiluted viral stock), showing the outstanding protective effect of these RNA molecules. The level of protection against viral infection was dose-dependent. Complete or very high protection was achieved when IRES RNA was inoculated 8 or 24 h prior to FMDV infection with 7×10^4 PFU, with 100 and 86% survival, respectively. Inoculation of the transcripts at longer times pre-infection strongly decreased their protective effect against viral infection. Co-inoculation of S or IRES transcripts and the virus induced high levels of protection (about 90%), and the IRES RNA had a higher protective effect inoculated at 8 h than at 4 h before infection, suggesting that a fine balance between the routes activating the innate immune response by the RNAs and the viral replication kinetics or antagonistic mechanisms triggered by the virus, might determine either the outcome of disease or the viral clearance. Additionally, high survival percentages were observed for those groups inoculated with the RNAs at short times after infection (89 and 87% of mice inoculated with the IRES at 4 h and 8 h post-infection survived, respectively), and complete protection (100% survival) was achieved when mice were inoculated with the S transcripts at 4 or 8 h post-infection [49]. No protective effect was observed for mice inoculated with the RNAs 24 h after viral infection. These results suggest that the antiviral response induced by the RNAs is rapidly established and effective to counteract the viral replication if administered shortly after infection, while 24 h later it was too late to restrain the progress of infection. Our data support the potential use of this RNAs as both prophylactic as well as therapeutic molecules in a certain time window. These small non-infectious RNAs could be useful to induce a rapid antiviral state in combination with effective FMD vaccines to overcome the problem of the susceptibility window until protective levels of antibodies are produced by vaccinated animals. These results provide, as well, a new insight into broad-spectrum antiviral development strategies (Figure 3).

4. Exploiting innate responses for antiviral, therapeutic and adjuvation strategies

The example described above illustrates the potential of RNA regions in the viral genome, known to elicit innate responses, for antiviral and therapeutic applications.

Viral pattern recognition system may offer unique translational implications in medical approaches, taking advantage of the innate immune function of PRRs to trigger cell autonomous responses in tumour cells along with cytotoxic T-cell responses that target tumour cells. Tumour cells are much more sensitive to cytotoxic effects after RLR ligation than are untransformed cells, allowing for tumour-specific effects despite systemic application of the ligands in mouse models [50, 51]. The concept of using targeted application of PAMPs to mimic a situation of viral infection for clinical application like immunotherapy is being extensively explored [52]. In addition to being interesting targets for the immunotherapy of cancer, RLRs

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have been found to play a role in other disease conditions like type-I diabetes and other autoimmune diseases like psoriasis or selective IgA-deficiency [53-55]. RLRs ligands have been shown to have a therapeutic effect for autoimmune inflammatory disease of the central nervous system in mice [56].

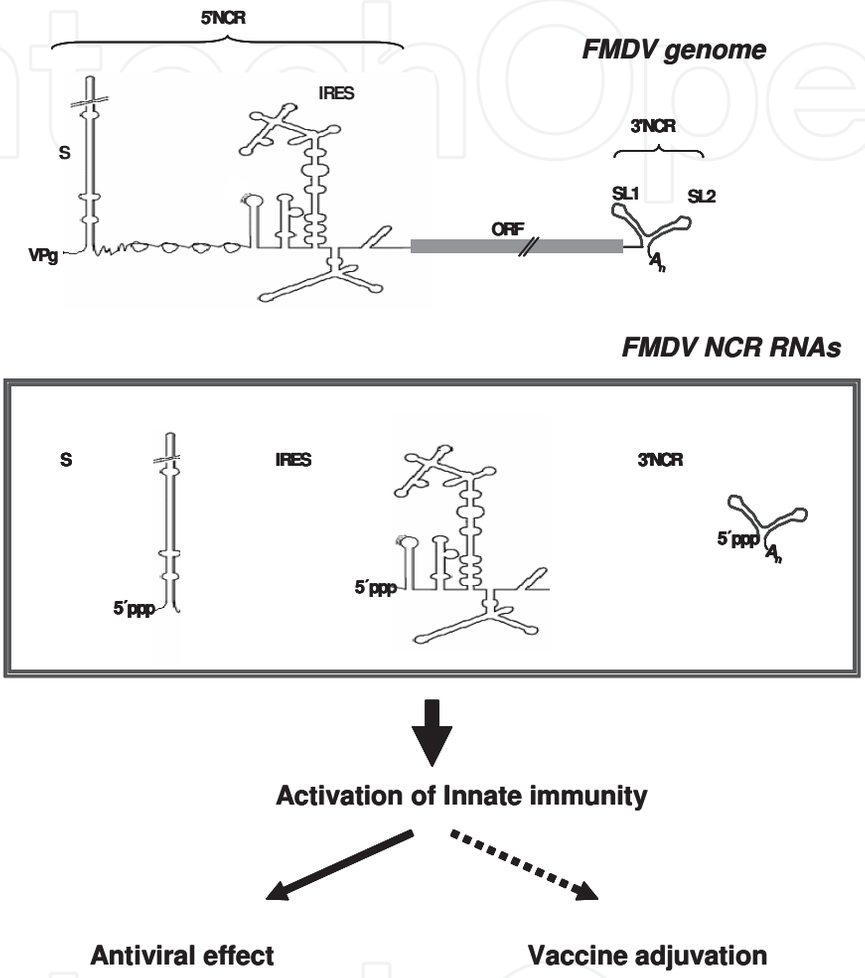

Figure 3. Synthetic RNA transcripts derived from structural domains present in the 5' and 3'NCRs of the FMDV genome as innate immunity elicitors

Other than its role in driving innate immune defenses, IFN plays a major role in modulating the adaptive immune response [57]. IFN is required to promote T cell survival and clonal expansion after antigen presentation. IFN also induces the cytolytic activity of NK cells and cytotoxic lymphocytes and promotes B cell differentiation and antibody production, as well as expression of MHC class I molecules [58, 59]. The specific role of RLR signaling in regulating IFN production and its regulation of the adaptive immune response is less clear and appears to vary from virus to virus [60].

Luke et al. showed that the potency of a DNA vaccine against influenza virus could be augmented by the incorporation of a RIG-I activating immunostimulatory RNA into the vector

backbone [61]. Mice receiving the vaccine exhibited increased virus-specific serum antibody response as compared to those receiving the DNA vaccine alone. These results suggest that RLR signaling can enhance antibody development after vaccination by activation of innate immunity and improved adaptive immune responses. However, in a previous study Koyama et al. found a defect in antigen-specific B and T cell activation in MyD88-defficient mice, unlike MAVS-deficient mice, suggesting that adaptive immune responses against influenza A virus are governed by the TLR pathway [62]. On the contrary, MAVS-deficient mice infected with WNV displayed uncontrolled inflammation including elevated systemic IFN, proinflammatory cytokine and chemokine levels, and enhanced humoral responses marked by complete loss of virus neutralization activity with a failure to protect against WNV infection [63]. This work defined an innate/adaptive immune interface mediated through MAVS-dependent RLR signaling that regulates the quantity, quality and balance of the immune response to WNV. Using MDA5-defficient mice, Anz et al. showed that the loss of T regulatory cell function on infection with encephalomyocarditis virus (EMCV) is strictly dependent on RLR signaling [64]. In a different study, a normal and effective adaptive immune response against respiratory syncytial virus (RSV) infection was reported in the absence of both MAVS and MyD88, RLR- and TLR-adaptor molecules, respectively [65]. All together, the results suggest that, in the case of flavivirus and picornavirus infections, RLR signaling is important in the control of the quantity, quality and balance of the adaptive immune response, with specific and differential mechanisms of regulation operating depending on the specific virus infections. The understanding of the molecular features underlying these processes could offer new strategies for immune and antiviral therapy targeting the RLR pathway for therapeutic control of the viral infection and enhancement of the induced immune response.

The vaccine development field is evolving from traditional whole cell vaccines to more defined and safer subunit vaccines. In the idea of exploiting the innate responses in vaccine adjuvant design, a growing demand for the use of immunopotentiators in poorly immunogenic subunit vaccines is arising with the development of a new generation of vaccine adjuvants. New vaccine adjuvants are designed to improve the recruitment and activation of dendritic cells, then enabling transition from the innate to adaptive immune system for priming of B- and T-cell responses. Endogenous or therapeutically induced early type-I IFN responses may confer protection until adaptive immunity is activated to an extent that the pathogen can be eliminated. In that context, PRRs come into sight as targets of new vaccine adjuvants beside their role as sentinels in innate immunity.

Evidence is now emerging that many empiric vaccines and adjuvants inherently stimulate PRRs, like the yellow fever vaccine 17D, one of the most effective vaccines available, shown to activate multiple DC subsets through stimulation of several TLRs (including TLR-7, -8 and -9) [66], highlighting the potential of vaccination strategies that use combinations of different PRRs ligands to stimulate polyvalent immune responses.

The current vaccine adjuvants licensed for use in human vaccines are limited [67], but other PRR agonists in clinical stages of development are emerging as potential vaccine adjuvant candidates [68], such as the TLR3 and MDA5 agonist poly I:C, a promising mucosal adjuvant for intranasal H5N1 influenza vaccination [69]. Clearly, TLR agonists are the most clinically

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advanced in adjuvant development, but require additional considerations, as variation in TLR expression and influence of age in responsiveness or the risk of autoimmunity by induction of excessive inflammatory responses [68]. Other TLR agonists currently in clinical trials of a malaria vaccine are the TLR-9 agonists CpG oligodeoxynucleotides (ODNs).

In addition to recognition of viral ssRNA like that derived from HIV (Human immunodeficiency virus) or influrenza viruses, TLR7/8 can also be activated by certain synthetic agonists such as the imidazoquinoline derivatives imiquimod and resiquimod (R-848), as well as the guanine nucleotide analog loxoribine. TLR7/8 imidazoquinolines can activate appropriate immune cells and modulate cellular and humoral immunity and have been found to be excellent vaccine adjuvants [70]. Most TLR agonists induce antibody and Th1 responses, although some can induce Th2 and possibly Th17 responses. Knowledge of the response outcomes in terms of cytokines, chemokines and T-cell subtypes generated by activation of combination of PRRs would help in the design of vaccine formulations including the appropriate combination of adjuvants in the future that can contribute to develop new vaccines against infectious diseases.

In the case of FMDV, many efforts are being invested on development of new vaccine formulations aimed to improve currently used vaccines [71]. Although FMD vaccines are available since the early 1900s, the disease still affects millions of animals around the globe and remains the main sanitary barrier to the commerce of animals and animal products. Among the limitations of the currently available inactivated antigen vaccines are the short duration of immunity and the lack of serotype-cross protection. Administration of this vaccine or an experimental vaccine based on a replication-defective human adenovirus (Ad5) vector that delivers the FMDV capsid and 3C proteins requires ~7 days to induce protective immunity in animals [72, 73]. New approaches aimed to shorten this susceptibility window and induce a more robust and long-lasting adaptive immune response are being developed, such as expression of type-I,-II and –III IFNs with Ad5 vectors with good but differential results depending on the host species assayed [74, 75]. Treatment with IFN has proved, so far, to be the best biotherapeutic approach tested against FMDV. Recent data show that poly I:C stabilized with poly-L-lysine and carboxymethyl cellulose (poly ICLC), a TLR-3 and MDA5 agonist, is a potent stimulator of IFN and ISGs in swine and at an adequate dose is sufficient to induce complete protection against FMD [77]. A different study shows that the combined application of recombinant adenoviruses expressing IFN-α or siRNA and other antiviral agents such as ribavirin may enhance their inhibitory effect on FMDV [77]. Our own data, discussed above, support the use of the FMDV NCR RNAs, mimicking structural domains in the viral genome acting as potent type-I IFN inducers, as promising non-infectious and synthetic molecules in future antiviral and vaccine developments against FMDV and likely other viral infections.

5. Conclusions

Understanding how the innate immune system senses the infection of different viruses with a variety of genome structures and signals and the crosstalk between different PRRs will help to understand the complex regulation of immunity to infection. The increasing knowledge on

the nature of PAMPs and sensor specificity will surely contribute to the development of safer and more effective vaccines for infectious diseases. PRR agonists arise as promising molecules due to their synergistic effects on cytokine production and contributing to effective immune responses. The success of rationally designed vaccine formulations in the near future will likely correlate with the advances on understanding cell signalling mechanisms as well as PRR adjuvanticity and response outcomes. Targeted immunomodulatory strategies will require knowledge of the virus-specific aspects of the pathway. Viral proteins with IFN antagonistic activity are potential drug targets for antiviral strategies. Moreover, small, synthetic and non-infectious RNAs mimicking viral PAMPs can act as potent IFN inducers and exert an antiviral effect in vivo, providing new insight into broad-spectrum antiviral development strategies.

Acronyms and abbreviations

Ad5	replication-defective human adenovirus vector
APC	Antigen presenting cell
CARD	Caspase activation and recruitment domain
CLR	C-type lectin receptor
CpG	Unmethylated deoxycytidylate-phosphate-deoxyguanylate
DC	Dendritic cell
DEN	Dengue virus
DI genomes	Defective-interfering genomes
ds	Double-stranded
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
FMDV	Foot-and-mouth disease virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
IFITM	Interferon inducible transmembrane protein
IFN	Interferon
IFNAR	IFN-α/β receptor
IRES	Internal ribosome entry site
IRF	Interferon regulatory factor
ISG	IFN-stimulated gene
JAK/STAT	Janus kinase/signal transducers and activators of transcription
LD_{50}	50% lethal dose

http://dx.doi.org/10.5772/56099

LGP2	Laboratory of genetics and physiology-2
MAP Kinase	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral signaling
MDA5	Melanoma differentiation-associated gene 5
MHC	Major histocompatibility complex
miRNA	Micro RNA
NCR	Non-coding region
NK	Natural killer
NLR	Nucleotide oligomerization domain (NOD-like receptor)
ODNs	Oligodeoxynucleotides
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cell
PFU	Plaque forming units
PKR	Protein kinase R
Poly I:C	Polyriboinosinic-polyribocytidylic acid
Poly ICLC	poly-L-lysine and carboxymethyl cellulose
PRR	Pattern-recognition receptor
RIG-I	Retinoic acid-inducible gene-1
RLR	RIG-I-like receptor
RSV	Respiratory syncytial virus
siRNA	Small interfering RNA
SL	Stem-loop structure
ss	Single-stranded
svRNA	Small viral RNA
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRIF	TIR-domain-containing adaptor inducing interferon
TRIM	Tripartite motif protein
VPg	Virus encoded protein
WNV	West Nile virus

Table 1. List of the acronyms and abbreviations used in this chapter

Acknowledgements

The work on FMDV discussed in this chapter was supported by grants BIO2008-04487-C03-01, AGL2011-24509 and BIO2011-24351 and by the ICTS program from the Spanish Ministry of Science and Innovation. We also thank Francisco Mateos, Nuria de la Losa and Lourdes Peña for their assistance in mice experiments. We are also grateful to Encarna Martínez-Salas for reagents and helpful discussion.

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