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Short Communication

Protection against Rift Valley fever virus infection in mice upon administration of interferon-inducing RNA transcripts from the FMDV genome

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ABSTRACT

In this work we have addressed the effect of synthetic, non-infectious, RNA transcripts, mimicking structural domains of the non-coding regions (NCRs) of the foot-and-mouth disease virus (FMDV) genome on the infection of mice with Rift Valley fever virus (RVFV). Groups of 5 mice were inoculated intraperitoneally (i.p.) with 200 μg of synthetic RNA resembling the 5′-terminal S region, the internal ribosome entry site (IRES) or the 3′-NCR of the FMDV genome. RNA inoculation was performed 24 h before (−24 h), 24 h after (+24 h) or simultaneously to the challenge with a lethal dose of RVFV. Administration of the IRES RNA afforded higher survival rates than administration of S or 3′NCR transcripts either at −24 h or +24 h after challenge. In contrast, when RNA inoculation and viral challenge were performed simultaneously, all mice survived in both IRES- and 3′NCR-inoculated groups, with an 80% survival in mice receiving the S RNA. Among survivors, a complete correlation between significant anti-RVFV circulating antibody titers and resistance to a second lethal challenge with the virus was observed, supporting a limited viral replication in the RNA-inoculated animals upon the first challenge. All three RNA transcripts were able to induce the production of systemic antiviral and pro-inflammatory cytokines. These data show that triggering of intracellular pathogen sensing pathways constitutes a promising approach towards development of novel RVF preventive or therapeutic strategies.

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Rift Valley fever virus is a negative strand RNA virus that belongs to the genus Phlebovirus, family Bunyaviridae. The virus is transmitted by many different mosquito species in Africa where recurrent outbreaks of the disease span for decades along the whole continent. More recently, the disease occurred in the Arabian Peninsula, Madagascar and other Indian Ocean islands, including the French island of Mayotte. The disease affects mainly young livestock, causing high abortion rates in pregnant ruminants. Humans usually become infected after epizootic outbreaks mainly due to close contact with infected animals, animal carcasses or by mosquito bites (Pepin et al., 2010). Previous studies have shown that RVFV is highly sensitive to the action of type-I interferon (IFN). Thus, the administration of poly I:C, a synthetic double-stranded RNA analog, known to be a potent IFN inducer, protected rodents from viral challenge (Peters et al., 1986). On the other hand, the administration of recombinant human interferon was able to protect rhesus macaques upon RVFV lethal challenge (Morrill et al., 1989), and transgenic mice lacking the IFN α/β receptor (IFNAR$^{-/-}$) were much more sensitive to the virus than wild type mice (Bouloy et al., 2001). Therefore, induction of IFN might be considered a potential therapeutic approach to combat RVF. Type I-IFN induction is triggered by the recognition of pathogen associated molecular patters (PAMPs) by a set of cellular pattern-recognition receptors (PRRs) (Berke et al., 2013; Thompson et al., 2011). Two main families of PRRs are involved in sensing viral RNA in infected cells, the Toll-like receptors (TLRs) (Kawai and Akira, 2011), type-I transmembrane proteins that traffic between the plasma membrane and endosomal vesicles, expressed in various immune cells, and the RIG-I like receptors (RLRs) (Goubau et al., 2013; Loo and Gale, 2011), a family of ubiquitous cytosolic RNA helicases. PAMP sensing by PPRs leads to initiation of a cascade of signaling events that result in the transcription of type-I IFN and pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-6. The use of PAMP motifs to induce innate immune responses is gaining attention as a powerful approach for the

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production of type-I IFN with both therapeutic and prophylactic purposes (Olive, 2012; Schmidt et al., 2012). In this context, RNA transcripts mimicking structural domains in the non-coding regions of the foot and mouth disease virus (FMDV) genome (NCR-RNAs) have been recently found to be potent inducers of innate immune signaling. Thus, synthetic transcripts corresponding to the internal ribosome entry site (IRES), S region and the 3′ non-coding region (3′NCR) of FMDV were able to trigger type-I IFN induction when transfected into porcine cultured cells (Rodriguez-Pulido et al., 2011a). Furthermore, after intraperitoneal (i.p.) administration, these RNAs were able to protect mice upon virulent FMDV or West Nile virus challenge in suckling and adult mice respectively (Rodriguez-Pulido et al., 2012, 2011b). To test whether this strategy of protection by endogenous IFN induction was also valid in the context of a RVFV infection, RNA transcripts corresponding to the 3′NCR, the S fragment and the IRES of the FMDV genome were generated, as described previously (Rodriguez-Pulido et al., 2011b). Then, groups ($n = 5$) of BALB/c mice (Harlan) were inoculated with a lethal dose (10^3 pfu intraperitoneally) of the virulent RVFV strain 56/74 from a virus stock grown in BHK-21 cells. This lethal dose usually kills 80–100% of challenged BALB/c mice, as described previously (Lopez-Gil et al., 2013). Mice experiments were handled in accordance with the guidelines of the EU directive 2010/63/EU for animal experiments, and protocols approved by the Committees on Biosafety and Ethics of Animal Experiments of INIA (permit codes CEEA 2012/014 and CBS 2012/017). At different times relative to viral challenge, 200 μg of each type of transcript delivered in a lipofectamine-complexed mixture, as described previously (Rodriguez-Pulido et al., 2011a) were also administered i.p. Thus, 24 h before the viral challenge, three groups of mice were treated with the RNAs (namely pre-challenge groups), another three groups of mice were inoculated with RNA simultaneously to the viral challenge (co-challenge groups) and a third set of three groups was inoculated with RNA 24 h post viral challenge (post challenge groups). As a negative control, a group of five mice was mock-inoculated only with a PBS – lipofectamine mixture. Statistical differences were analyzed using the Graph-Pad software package v 5.0. Survival curves comparison for each RNA treatment and time were subjected to the Mantel–Cox (log-rank) test. When side by side comparisons between two survival curves were performed, the significance level was adjusted according to a Bonferroni's corrected threshold.

Protection of mice against lethal RVFV infection could be achieved by means of the effect triggered by some of the RNAs and, in all cases regardless the transcript or the time of inoculation, all RNA-treated groups showed higher survival rates than the control group (Fig. 1).

Best protection results were achieved when RNA was administered simultaneously to the viral challenge, rendering statistically significant levels of protection (log-rank test $p = 0.0058$), ranging between 80% and 100% (Fig. 1B and D). Notably, this protection was observed in the absence of detectable clinical signs in the challenged mice. Particularly, both IRES and the 3′NCR rendered 100% protection, while the S RNA was able to protect 4 out of 5 mice (Fig. 1D). Administration of RNA 24 h before or after the viral infection led also to protection, though with lower levels than those observed for the simultaneous RNA and virus inoculation (Fig. 1A and C). Among mice receiving RNA before viral infection, those inoculated with IRES and 3′NCR RNA transcripts showed higher protection rates, with 60% of the animals surviving challenge, whereas only 40% of mice that received the S RNA survived viral challenge. In the group of mice receiving IRES RNA the average survival was slightly increased compared to the other groups (Fig. 1A). Finally, in the groups receiving the RNA 24 h after viral infection the survival rates decreased to 80% for IRES, 60% for the S fragment and 40% for the 3′NCR (Fig. 1C and D). In spite of the observed survival trend, comparison of −24 h and +24 h survival curves did not reach enough statistical significance (Mantel–Cox log-rank test) in part due to the reduced power of this test with

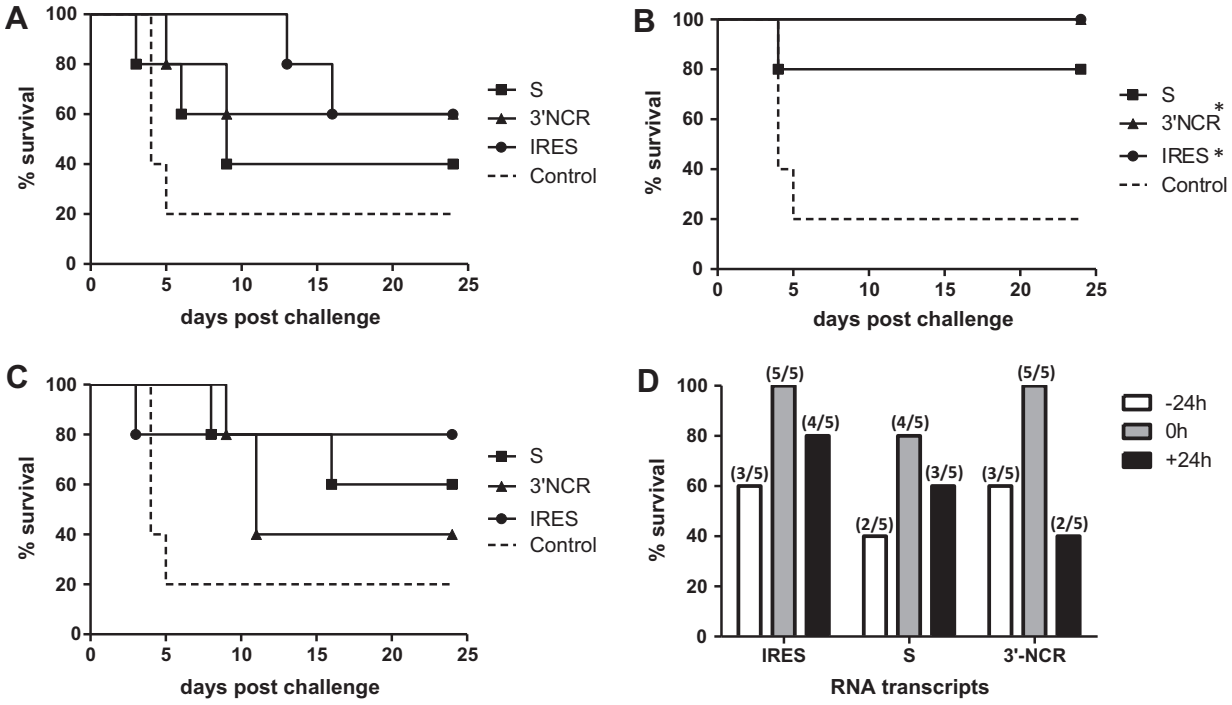

Fig. 1. Protective effect of NCR-RNAs inoculation at different times upon lethal RVFV challenge. Survival (Kaplan–Meier plots) of mice receiving different FMDV NCR-RNA transcripts 24 h before (A), simultaneously (B), or 24 h after (C) viral challenge. In all graphs the plots are compared to a same sized group of mice ($n = 5$) receiving identical dose of challenge virus but without RNA administration (control group, dotted line). End point percent survival of the mice (survivors vs inoculated mice in parenthesis) is represented in D. Asterisk (*) denote statistical significance differences between the corresponding RNA-inoculated and control groups ($p < 0.05$).

the sample size used in the experiments. However, it cannot be ruled out that the RNA treatment at these time points, or perhaps closer to the time of viral challenge (i.e. −8 h or +8 h) might have any effect in the mortality rates. Further experiments are warranted to test this hypothesis, increasing the number of mice, to assess whether these differences are biologically meaningful.

To test whether the protective effect was due to a total blockade of virus replication upon challenge or to limited virus replication levels that could have elicited adaptive responses, a second challenge of the remaining mice (survivors from the first challenge) was performed 24 days after the first challenge with an equivalent lethal dose of RVFV. All the surviving mice from the co-challenge and post challenge groups survived the second challenge, whereas lower protection levels (50–66%) were observed in the group that had received the RNA 24 h before virus infection (Fig. 2B). Survival always correlated with the presence of high titers of neutralizing antibodies (Fig. 2A) before challenge, indicative of virus replication and in agreement with activation of a strong immune response. The antibody titers were similar to those of control mice that survived spontaneously the challenge (not shown). Accordingly, the three mice that did not survive to the second challenge had developed barely detectable levels of neutralizing antibodies. Since all three mice had received the RNA 24 h before the challenge, it could be hypothesized that earlier administration of these RNA molecules was able to induce an antiviral state strong enough to reduce viral replication to levels lower than those required for protective antibody development.

To look for a correlation between the observed levels of protection and the induction of an IFN response, the antiviral activity in the serum of RNA-inoculated mice was determined by a vesicular stomatitis virus (VSV) infection-inhibition assay on L929 cells. Briefly, groups of three BALB/c mice were inoculated with 200 μg of each FMDV NCR-RNA transcript and serum samples were taken at 4, 8 and 24 h post inoculation (hpi). Then, monolayers of L-929 cells in 96-well microtiter plates were incubated with serial 2-fold dilutions of the corresponding group pooled sera for 24 h. Then, media were removed and replaced with 100 μl of fresh media containing 10^2 50% tissue culture infectious doses ($TCID_{50}$) of VSV. Cytopathic effect was monitored at 72 h post infection by microscopy examination. A peak of antiviral activity was observed at 8 hpi in RNA-treated but not in mock-treated mice; at this time a peak of IFN-α induction was also detected by ELISA of pooled sera (Fig. 3A). Similarly, the presence of other pro-inflammatory cytokines such as TNF-α, IL-6 or IL-1β in mice sera was also measured by ELISA (PBL Interferon Source). Only IL-6 was readily detected, in all RNA-treated mice, whereas neither IL-1β nor TNF-α could be detected above the assay sensitivity threshold. The highest levels of IL-6 were detected at 8 hpi except for IRES-inoculated animals showing highest levels even earlier at 4 hpi (Fig. 3B). In none of the mock-treated groups of mice (PBS – lipofectamine only) cytokine expression levels above the established sensitivity threshold could be detected (not shown).

The results shown here demonstrate that the administration of the NCR-RNAs results in a fast, specific and measurable induction of innate responses that may prevent the spread of the infection in a time window covering at least 24 h. As previously found in other viral infection models (Rodriguez-Pulido et al., 2011b) the transcripts corresponding to the IRES sequence rendered the best results, providing survival rates of 60%, 100% and 80% at the three time points analyzed, respectively. This higher individual protection coverage correlates with the induction of high levels of IFN-α in RNA inoculated mice (Fig 3A), as well as a very early production of IL-6 (Fig 3B), a polyfunctional pro-inflammatory cytokine playing a role in bridging innate and adaptive immune

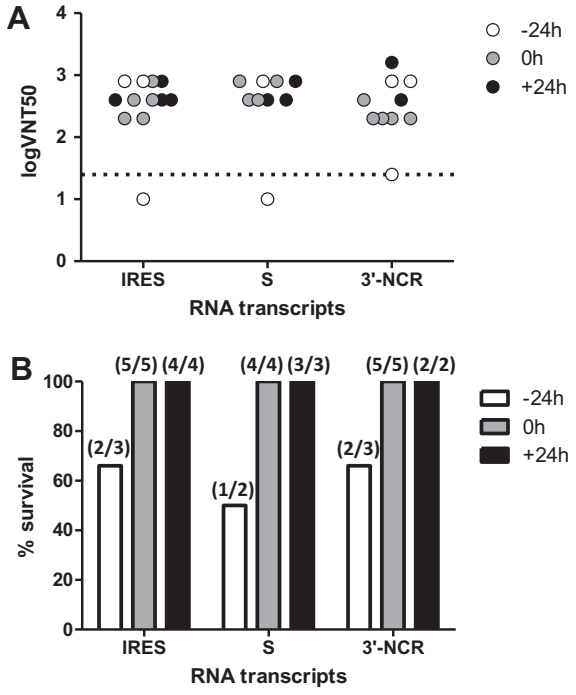

Fig. 2. Survival of convalescent mice after a second challenge correlates with detection of serum neutralizing antibodies. Serum samples from surviving (convalescent) mice were taken at day 24 post viral challenge and tested for the presence of neutralizing antibodies. (A) Scatter data plot of log_{10} seroneutralization values. The RNA inoculated mice that survived the first virus challenge were re-inoculated i.p. with an equivalent dose of RVFV 56/74 and monitored daily up to 7 days. Dotted line indicates sensitivity of the assay. (B) Proportions of mice from each group that survived a second challenge performed 24 days after the first challenge.

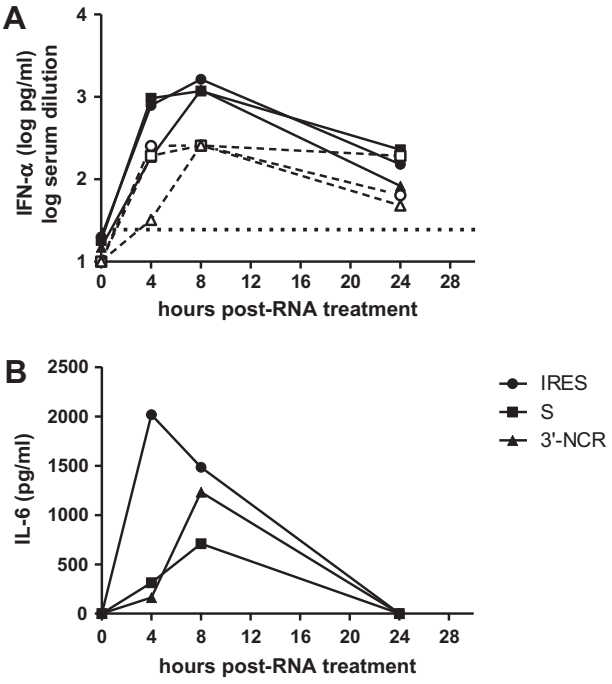

Fig. 3. Antiviral activity and cytokine detection in sera from RNA-inoculated mice. Mice were inoculated with 200 μg of each NCR-RNA and serum samples were collected at 4, 8 and 24 hpi. (A) Antiviral activity (discontinuous lines and open symbols) was expressed as log transformed values of the reciprocal of the highest serum dilution protecting cells from cytotoxicity in 50% of the wells. Serum IFN-α levels (solid lines and symbols) were also analyzed by ELISA. Dotted line indicates sensitivity of the ELISA assay (Symbols in A correspond to the legend displayed in figure B). (B) Detection of IL-6 levels in sera by ELISA.

responses and promoting immunoglobulin production by B-cells (Rincon, 2012). The fate of these RNA molecules after delivery with respect to their cell or tissue tropism upon intraperitoneal administration has not yet been elucidated. It could be assumed that the RNA will enter quickly the blood stream and reach the liver through the portal vein system. Thus, hepatic cells could be one of the first RNA targets. The fact that the RVFV replicates extensively in the liver could explain the efficacy of the RNA treatment.

Interestingly, the IRES RNA has been shown to act as vaccine adjuvant, enhancing the adaptive immune responses of an inactivated FMDV vaccine in Swiss ICR-CD1 mice (Borrego et al., 2013). Thus, the combination of an early antiviral response together with an improvement in the development of the adaptive immunity induced by the RNA treatment could represent an efficient antiviral strategy in the case of suspected RVFV outbreak. These NCR-RNAs are non-infectious and easy to produce molecules with a proven capacity to stimulate innate immune responses, suggesting their potential for vaccine adjuvancy and antiviral strategies against a wide range of viral pathogens. Additional work is warranted to elucidate how the induction of innate immunity by these NCR-RNAs modulates subsequent protective immune responses.

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