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Inhibition of the replication of Venezuelan equine encephalitis virus by retinoic acid-inducible gene-I

Josh Q.H. Wu, Denny Huang, Erica Chan-Wong, and Damon Chau

Defence R&D Canada

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Abstract

Retinoic acid-inducible gene I (RIG-I) is a cytoplasmic protein that recognizes viral RNA molecules and subsequently elicits antiviral innate responses. This report describes a pilot study on the feasibility of developing RIG-I into a novel antiviral drug against alphaviruses. It was found that RIG-I, when expressed in cells from a plasmid vector, inhibits the replication of Venezuelan equine encephalitis virus (VEEV), which was used as a model pathogen of the alphaviruses. It appears that the inhibition is related to RIG-I's ability to induce the production of interferon alpha. The result from this study helps the further evaluation of RIG-I in a mouse challenge model of VEEV.

R□sum□

Le g□ne inductible I par un acide r□tino□que (RIG-I) est une prot□ine cytoplasmique qui reconna□t les mol□cules RNA virales et □licite par la suite des r□ponses antivirales inn□es. Ce rapport d□crit une □tude pilote sur la faisabilit□ de d□velopper RIG-I en une nouvelle drogue antivirale contre les arbovirus. On avait trouv□que, quand il est exprim□en cellules provenant d□un vecteur plasmide, RIG-I inhibe la r□plication du virus de l□enc□phalomy□lite □quine du Venezuela (VEEV) qui a □t□utilis□comme pathog□ne mod□le des arbovirus. Il semble que l□inhibition est li□e □la capacit□du RIC-I d□induire la production d□interf□ron alpha. Le r□sultat de cette □tude permet d□approfondir l□□valuation du RIG-I pour le VEEV chez un mod□le de souris.

Executive Summary

Inhibition of the replication of Venezuelan equine encephalitis virus by retinoic acid-inducible gene-I

Josh, Q.H. Wu, Denny Huang, Erica Chan-Wong, and Damon Chau; DRDC Suffield TM 2008-213; Defence R&D Canada – Suffield.

Introduction or background:

Venezuelan equine encephalitis virus (VEEV) is endemic to parts of Central and South America and causes encephalitis, a severe inflammation of the central nervous system, which is sometime fatal. The virus is normally transmitted by mosquito bites, but can also be transmitted by exposure to an aerosol of the virus. Currently, there are no commercial vaccines or antiviral drugs available for the prevention and treatment of the VEEV infection in humans, but efforts are underway at DRDC Suffield to develop medical countermeasures to this biothreat agent.

The alphaviruses, such as VEEV, use RNA for their genome. A mammalian cellular protein, RIG-I, has been shown to be play a critical role in the recognition and defence against a variety of RNA viruses, but little is known of its role against alphaviruses. If it is effective, then it might be possible to develop a RIG-I-based, broad-spectrum antiviral drug which would be effective against these viruses.

Results:

In order to know whether RIG-I is also effective against VEEV, the gene encoding RIG-I was first inserted into a DNA plasmid, which can produce RIG-I after being introduced into cells. We found that RIG-I was produced from cells containing the plasmid. We also found that cells containing the plasmid encoding RIG-I secreted a high level of interferon-alpha and blocked the replication of VEEV.

Significance:

Taken together, these results suggest that expression of RIG-I via a plasmid or viral vector could be a novel strategy for the development of an antiviral therapeutic for VEEV.

Future plans:

Future experiment will be focused on the determination of whether the expression of RIG-I provides protection against VEEV infection in an animal model.

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Inhibition of the replication of Venezuelan equine encephalitis virus by retinoic acid-inducible gene-I

Josh, Q.H. Wu, Denny Huang, Erica Chan-Wong, and Damon Chau; DRDC Suffield TM 2008-213; R & D pour la défense Canada – Suffield.

Introduction ou antécédents:

Le virus de l'encéphalomyélite équine du Venezuela (VEEV) est endémique à certaines régions de l'Amérique Centrale et du Sud. Il cause l'encéphalite, une inflammation grave du système nerveux central et peut quelques fois être fatal. Le virus se transmet normalement par la morsure de moustiques mais il peut aussi se transmettre par exposition au virus en aérosol. Il n'existe actuellement aucun vaccin commercial ou aucune drogue antivirale disponible pour la prévention et le traitement de l'infection du virus EEV chez les humains mais, à RDDC Suffield, on a entrepris de développer des contremesures médicales contre cet agent de menace biologique.

Les arbovirus, tels que le VEEV, utilise l'ARN pour leur génome. On a montré qu'une protéine cellulaire mammalienne, RIG-I, joue un rôle essentiel dans la reconnaissance et la défense contre une variété de virus ARN mais on connaît peu son rôle contre les arbovirus. Si elle est efficace, il se peut qu'il devienne possible de développer une drogue antivirale polyvalente à base de RIG-I pouvant être efficace contre ces virus.

Résultats:

Pour savoir si RIC-I était aussi efficace contre le VEEV, le gène encodant RIG-I a d'abord été inséré dans un plasmide d'ADN pouvant produire RIG-I après avoir été introduit dans les cellules. On a trouvé que le RIG-I était produit à partir de cellules contenant le plasmide. On a aussi trouvé que les cellules contenant le plasmide encodant le RIG-I secrétaient un haut niveau d'interféron-alpha et bloquaient la réplication du VEEV.

Portée des résultats:

Ces résultats pris dans l'ensemble suggèrent que l'expression de RIG-I par vecteur plasmide ou viral pourrait devenir la nouvelle stratégie de développement d'une thérapeutique antivirale pour le VEEV.

Perspective d'avenir:

Les prochaines expériences auront pour but principal de déterminer si l'expression de RIG-I fournit une protection contre l'infection VEEV chez un modèle animal.

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Introduction

The alphaviruses, a group of arboviruses, that consist of a single-stranded, positive-sense, non-segmented RNA genome encased in an enveloped nucleocapsid. There are over 30 species of alphaviruses, which can be classified into eight antigenic complexes based on sequence analysis [1]. In addition, alphaviruses can be grouped into the Old World and New World alphaviruses according to geographic distribution.

The Old World alphaviruses can cause major outbreaks in humans with infections characterized by fever, rash and arthritis. As an example, about one-third of the population of R□union (an island in the south-western Indian Ocean with a total population of 770 000) was infected by Chikungunya virus during a recent outbreak [2]. The New World alphaviruses, which include Venezuelan equine encephalitis virus (VEEV), Eastern equine encephalitis virus (EEEV), and Western equine encephalitis virus (WEEV), are widely distributed throughout the Americas, causing encephalitis and sometimes death in humans and horses. VEEV, EEEV, and WEEV survive in nature through cycling between mosquitoes and small mammals or wild birds. Humans and equines are infected by the viruses through mosquito bites [3]. Natural outbreaks of the New World alphaviruses can have serious impact on both human and animal health. As another example of alphavirus epidemics, more than 100 000 human cases with more than 300 cases of fatal encephalitis were reported in a major VEEV outbreak in Venezuela and Colombia in 1995 [4]. About 350 000 horses were infected in both Canada and the United States during a WEEV outbreak in the 1930□s [5].

Besides natural transmission by mosquitoes, VEEV, EEEV, and WEEV can be easily spread to humans and animals through the aerosol route with high morbidity and mortality [6]. Currently, there is no commercial vaccine or antiviral drug available for the prevention or treatment of infections caused by VEEV, EEEV, and WEEV.

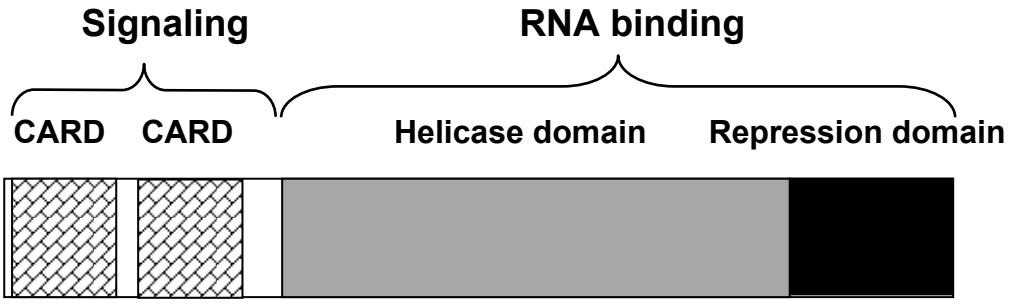

Figure 1. Schematic representation of retinoic acid-inducible gene I (RIG-I) protein. *CARD: caspase recruitment domain.*

Retinoic acid-inducible gene I (RIG-I) is a cytoplasmic protein that recognizes viral RNA molecules and subsequently elicits antiviral innate responses [7]. RIG-I contains two copies of caspase recruitment domains (CARD) at its N-terminus and an RNA helicase domain at its C-terminus [8] (Fig. 1). After the helicase domain binds to double-stranded RNA (dsRNA) [8] or single-stranded RNA (ssRNA) bearing 5′-triphosphate [9, 10], the CARD protein triggers downstream signal transduction pathways to produce alpha and beta interferon (IFN-α/β) [11]. These IFNs bind to cellular receptors to induce the synthesis of antiviral proteins that rapidly block viral replication [12].

Yoneyama and colleagues [13] demonstrated that the CARD of RIG-I is enough to trigger the production of IFN-α/β. Because of its induction of IFN-α/β production, RIG-I plays a critical role in the recognition of and defense against RNA viruses, such as vesicular stomatitis virus [14, 15], measles virus [16], and flaviviruses [17].

IFN-α/β are required for early protection against alphaviral infection. For example, mice lacking the receptor for IFN-α/β are highly susceptible to infection by VEEV [18]. Injection of IFN-α protects animals from lethal challenge with WEEV [19] or VEEV [20]. Alphaviruses also produce proteins that can antagonize the function of IFN-α/β, further illustrated the importance of IFN-α/β for host's defense against these viruses [21-23]. Currently, little is known about the role of RIG-I in the IFN-α/β response against alphaviruses. Using VEEV as an example, we investigated whether alphavirus replication could be inhibited in cells expressing the CARD of RIG-I.

Materials and Methods

Cells and virus

Vero and HEK293 cells (ATCC) were grown in Dulbecco's modified Eagle media (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, antibiotics, and antimycotics. The TC-83 strain of VEEV [24] was provided by Dr. George Ludwig (the United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD). To prepare virus stocks, the original TC-83 viruses were passaged once in Vero cells at an MOI (multiplicity of infection) of less than 0.1. Supernatants from the infected cells were collected, aliquoted, and stored at −70 °C. The titers of the TC-83 stocks were determined by plaque assay in Vero cells.

Plasmid construction

pcD3-hCARD encoding the N- terminus (aa 1 to 292) of human RIG-I (hRIG-I) was made as follows. A 872 bp DNA fragment was isolated by PCR using pUNO-hRIG-I (InvivoGen, San Diego, CA) as a template, forward primer f-hRIG-I-N (5'-**CACC** ATG ACC ACC GAG CAG CGA CGC AGC- 3') and reverse primer r-hRIG-I-N (5'-CCC CTT TTG TCC TTG TGG GAA T- 3'). These two primers were designed based on the GenBank sequence of hRIG-I (accession no. NM_014314). Four nucleotides CACC (in bold) were added before the ATG start codon at the 5'-end of the forward primer f-hRIG-I-N for directional TOPO cloning of the PCR product. PCR was run by the ProofStart PCR kit (QIAGEN, Mississauga, ON, Canada) with 5 min of initial activation of the ProofStart DNA Polymerase at 95 °C, followed by 35 cycles of 30 sec of denaturing at 94 °C, 30 sec of annealing at 60 °C, and 1 min of extension at 72 °C. The PCR fragment was separated by 0.8% agarose gel, purified by QIAquick Gel Extraction kit (QIAGEN), and cloned into the pcDNA3.1D/V5-His-TOPO (Invitrogen, Burlington, ON, Canada) to yield pcD3-hCARD, containing a gene encoding the N-terminal 292 aa of hRIG-I fused with V5 epitope (GKPIPNPLLGLDST) [25]. The coding sequence for the fusion protein was verified by DNA sequencing using CEQ8000 Genetic Analysis System (Beckman Coulter INC., Fullerton, CA). pcD3-Empty, a control plasmid used for transient transfection experiments, was made by the digestion of pcD3-hCARD with *Hind*III and *EcoR*V, followed by filling-in the 5'-overhangs with T4 polymerase and self-ligating.

pcD3-mCARD encoding the N- terminus (aa 1 to 298) of mouse RIG-I (mRIG-I) was made in a similar method. A 894-bp DNA fragment was isolated by PCR using pUNO-mRIG-I (InvivoGen) as a template; and primers f-mRIG-I-N (5'-CACC ATG ACC GCG GCG CAG CGG CAG AAT C- 3') and r-mRIG-I-N (5'-AGC GAA GAA GAC CAC TTT CC- 3'). PCR was carried out by the ProofStart PCR kit with 5 min of ProofStart DNA Polymerase activation at 95 °C, followed by 35 cycles of 30 sec of denaturation at 94 °C, 30 sec of annealing at 65 °C, and 1 min of extension at 72 °C. The PCR fragment was cloned into pcDNA3.1D/V5-HIS-TOPO to generate pcD3-mCARD. The gene encoding the N-terminus of mRIG-I fused with V5 epitope was verified by DNA sequencing.

Cell transfection

Transfection of HEK293 cells with plasmids was done using Lipofectamine 2000 (Invitrogen) according to manufacturer□s instructions. Briefly, HEK293 cells were seeded at 10^6 cells per well in 6-well plates and grown overnight in DMEM containing 10% FBS and antibiotics. Next day, the media were replaced by DMEM containing 10% FBS only. Each well was transfected with 4 μg of DNA plasmids mixed with Lipofectamine 2000. The transfected cells were incubated at 37 □C in a 5% CO2 humidified atmosphere.

Western blot detection

The expression of the N-terminus of hRIG-I from pcD3-hCARD and the N-terminus of mRIG-I from pcD3-mCARD was detected by a Western blot. HEK293 cells were transfected with pcD3-hCARD, pcD3-mCARD, or pcD3-Empty. Cell pellets were collected at different time points after transfection and lysed in 100 μl of NuPAGE LDS sample buffer (Invitrogen). The samples were boiled for 5 minutes, then loaded onto 10% Bis-Tris NuPAGE Novex gel (Invitrogen). The proteins in the samples were electrophoresed in MOPS SDS running buffer. The eletrophoresed proteins were transferred onto nitrocellulose membranes by an XCell II Blot Module (Invitrogen). The proteins were probed with a mouse monoclonal antibody (MAb) specific for V5 epitope (Invitrogen).

Measurement of human IFN-α

Enzyme-liked immunosorbent assay (ELISA) was used to measure the amount of human IFN-α (hIFN-α) produced from the cells transfected with pcD3-hCARD or pcD3-mCARD. HEK293 cells were transfected in duplicate with pcD3-hCARD, pcD3-mCARD, or pcD3-Empty. Supernatants from the cells were collected at different time points after transfection. hIFN-α was measured in triplicate by an ELISA kit purchased from PBL Biomedical Laboratories (Piscataway, NJ). The transfection experiment was done in duplicate.

Growth curve

Vero and HEK293 cells grown in T25 flasks were inoculated with TC-83 at an MOI of 0.1. Supernatants from infected Vero cells were collected at 1 h after the inoculation; and day 1, 2, 3 and 4 after inoculations. Supernatants from infected HEK293 cells were collected at 1 h, 8 h after the inoculations; and day 1, 2, 3, and 4 after inoculations. The yield of TC-83 in supernatants was determined by plaque assay in Vero cells.

Plaque Assay

Supernatants collected from infected cells were 10-fold serially diluted with DMEM containing antibiotics and antimycotics. Monolayers of Vero cells in 6-well plates were washed once with DMEM and each well was inoculated in duplicate with 250 μl of the diluted supernatants. The plates were incubated for 1 hour at 37 □C in a 5% CO_2 humidified atmosphere. The inoculated supernatants were removed and 2 ml of overlay containing 0.6% tragacanth gum (Acros Organics), 2% FBS, antibiotics and antimycotics was added to each well. At 48 h after incubation, cells were fixed and stained with Histochoice solution (Amersco) containing 0.3% crystal violet.

Virus inhibition assay

HEK293 cells were transfected in triplicate with pcD3-hCARD or pcD3-Empty. At 24 h after transfection, the cells were infected with TC-83 at an MOI of 0.1. Supernatants were collected from the infected cells at day 4 after infection and the number of TC-83 viruses in the supernatants was measure by plaque assay in Vero cells as described above. The transfection-infection experiments were done in triplicate.

Detection of serum mIFN-α in mice inoculated with plasmids

Female BALB/c mice (17 to 20 g) were obtained from the pathogen-free mouse breeding colony at the animal care facility of DRDC Suffield, with the original breeding pairs purchased from Charles River Canada. Animal experiment protocols were approved by the DRDC Animal Care Committee. The mouse experiments were carried out according to the guidelines set by the Canadian Council on Animal Care. Twelve mice were divided into four groups with three mice in each. Group I was injected with PBS; Group II with pcD3-Empty; Group III with pcD3-hCARD; and Group IV with pcD3-mCARD. Each mouse was injected with a total of 75 μg of plasmid DNA with about 37 μg in 50 μl of PBS injected into the rectus femoris muscle of each hind leg. Serum was collected from each mouse at days 1, 3, 7, and 9 after injection. Concentrations of mIFN-α in sera were measured in triplicate by an ELISA kit (PBL Biomedical Labs) with a detection limit of 12 pg/mL.

Statistical analysis

PRISM® 4 program (GraphPad Software Inc.) was used for statistical analysis. Two-tailed *t* test was used to determine statistically significant difference ($P < 0.05$) in the production of TC-83 from un-transfected cells or cells transfected with pcD3-Empty or pcD3-hCARD.

Results

Expression of RIG-I in cells transfected with pcD3-hCARD or pcD3-mCARD

Two eukaryotic expression plasmids were made to express the N-terminus of hRIG-I or mRIG-I in HEK293 cells. In both plasmids, a DNA fragment encoding the N-terminal RIG-I was placed between immediate early promoter of human cytomegalovirus and poly A of bovine growth hormone (Fig. 2). V5 epitope was fused with the C-terminus of RIG-I to help to detect the fusion protein by an MAb specific for the V5 epitope in Western blot.

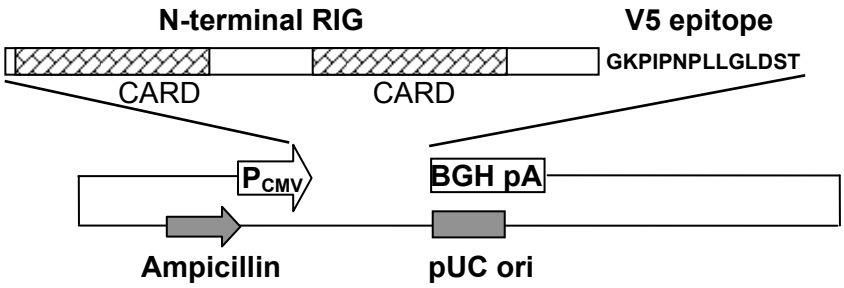

Figure 2. Schematic diagram of the DNA plasmid expressing the tandem CARD of human or mouse RIG-I. *The eukaryotic expression plasmid containing the gene encoding N-terminal human or mouse RIG-I fused with V5 epitope. PCMV: cytomegalovirus immediate-early promoter. BGH pA: bovine growth hormone polyadenylation signal.*

As shown in Fig. 3, the MAb detected a protein band with a molecular mass of about 40 kDa in the cell lysates prepared from cells transfected with pcD3-mCARD or pcD3-hCARD transfected cells. The 40-kDa protein band detected by the MAb is consistent with the calculated molecular mass of the N-terminus-RIG-I-V5 fusion protein. The protein band was detected as early as day 1 after transfection. No such protein band could be detected in cells transfected with control plasmid pcD3-Empty, indicating that the protein detected by the anti-V5 MAb is specific for cells transfected with pcD3-mCARD or pcD3-hCARD.

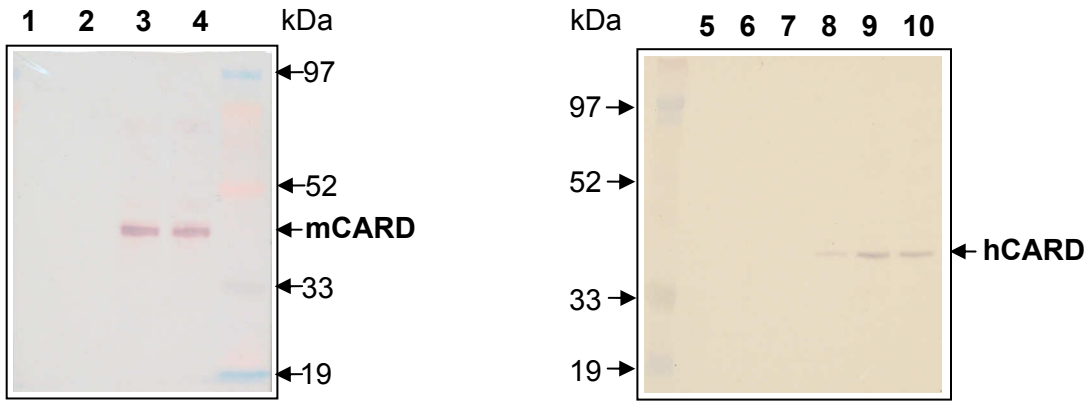

Figure 3. Detection of the CARD domain of the mouse and human RIG-I by Western blot. *Proteins in cell lysates collected at different time points after transfection with pcD3-hCARD or pcD3-mCARD were separated by 10% SDS-PAGE and probed with rabbit polyclonal antibody against V5. Cell lysates collected from pcD3-Empty-transfected HEK293 cells were included as controls. Lanes 1and 2: cell lysates collected at day 1and day 2 after transfection with pcD3-Empty; Lanes 3 and 4: cell lysates collected at day 1 and day 2 after transfection with pcD3-mCARD. Lanes 5, 6, and 7: cell lysates collected at day 1, 2, and 3 after transfection with pcD3-Empty; Lanes 8, 9 and 10: cell lysates collected at day 1, 2, and 3 after transfection with pcD3-hCARD.*

Induction of IFN-α by pcD3-hCARD or pcD3-mCARD

Having confirmed the expression of the N-terminal hRIG-I or mRIG-I protein, we next determined whether the protein could induce the production of IFN-α. HEK293 cells were transfected with pcD3-hCARD or pcD3-mCARD. hIFN-α in supernatants of transfected cells was measured by ELISA. By day 3 after transfection, hIFN-α was present at a high concentration in supernatants from pcD3-hCARD or pcD3-mCARD transfected cells (Fig. 4). A small amount of hIFN-α was also induced from cells transfected with control plasmid pcD3-Empty by day 3 after transfection. No or very few hIFN-α was produced in mock- or lipofectamine-treated cells.

To find whether IFN-α could be induced in mice inoculated with plasmids expressing the N-terminus of human or mouse RIG-I, we injected mice intramuscularly with a single dose of pcD3-hCARD or pcD3-mCARD. Serum was collected at days 1, 3, 7, and 9 after injection and concentrations of mIFN-α were measured by ELISA. We did not find any measurable mIFN-α in the serum.

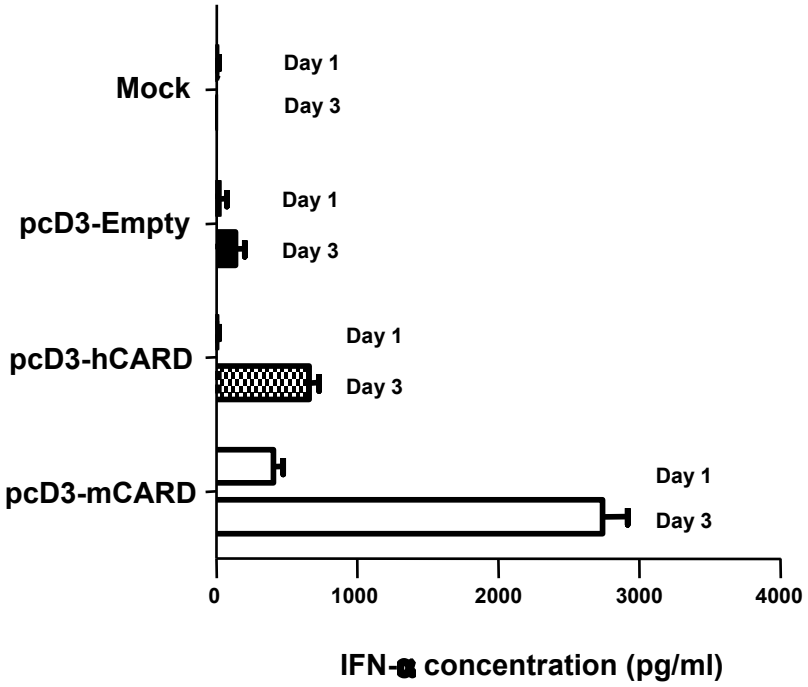

Figure 4. Induction of IFN-α in cells transfected with pcD3-hCARD or pcD3-mCARD. *HEK293 cells were transfected in duplicate with 4 µg of the DNA plasmid. At day 1 and day 3 after the transfection, supernatants from the transfected cells were collected and the concentration of hIFN-α was measured in triplicate by ELISA. Controls are supernatants from mock-transfected, lipofectamine-treated, and pcD3-Empty-transfected HEK 293 cells. Error bars are the standard deviations of IFN-α concentrations.*

Inhibition of VEEV Replication in cells transfected with pcD3-hCARD

We chose HEK293 cells for VEEV inhibition study because these cells have better transfection efficiency than Vero cells. We first determined if HEK293 cells are permissive for the TC-83 strain of VEEV. Fig. 5 shows that by day 1 after infection, virus yield in the supernatant from infected HEK293 cells is similar to that in the supernatant from infected Vero cells, suggesting that HEK293 cells are permissive for TC-83 replication. It appears that the production of the virus maintained at a steady level in HEK293 cells for at least 4 days. However, no CPE could be seen in infected HEK293 cells during 4-day observation period whereas CPE was found in Vero cells by day 2 after infection. By day 4 after infection, the Vero cell monolayer was completely destroyed, which could be related to low virus production at the late stage of infection.

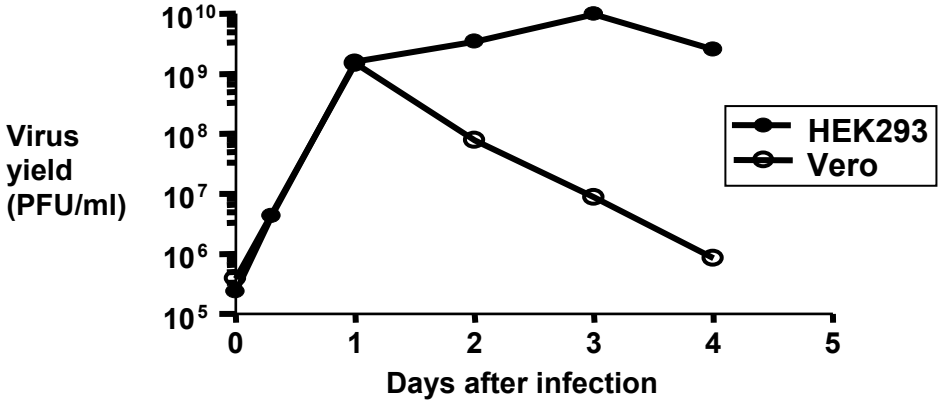

Figure 5. Replication of VEEV in HEK293 cells. *Vero and HEK293 cells were infected with TC-83 at an MOI of 0.1. Supernatants from infected cells were collected at different time points and assayed for the yield of TC-83 using plaque assay on Vero cells.*

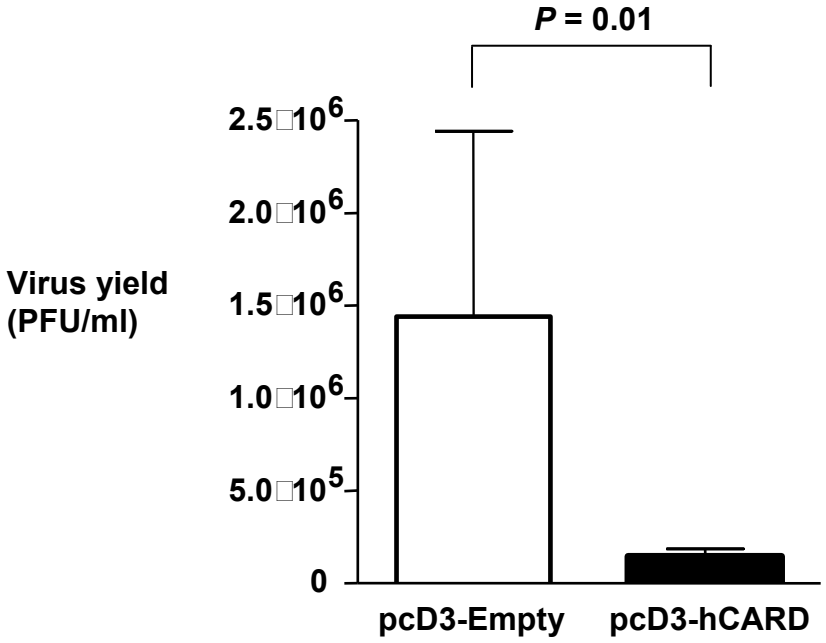

Figure 6. Inhibition of the replication of the TC-83 strain of VEEV in cells transfected with pcD3-hCARD. *HEK293 cells were transfected in triplicate with 4 μg of pcD3-hCARD or 4 μg of pcD3-Empty. At 24 h after transfection, cells were infected with TC-83 at an MOI of 0.1. The yield of the virus in cell culture media at day 4 after TC-83 infection was determined by plaque assay on Vero cells. Error bars are the standard deviation of virus concentration.*

Having demonstrated that 293 cells support TC-83 replication, we next investigated whether the cells transfected with pcD3-hCARD would block the replication of TC-83. HEK293 cells were first transfected with pcD3-hCARD. At day 2 after transfection, the cells were infected with TC-83 at an MOI of 0.1 and the yield of virus in supernatants at day 4 after infection was measured by plaque assay on Vero cells. Figs. 6 shows that TC-83 yield in cells transfected with pcD3-hCARD was 10-fold less than that in cells transfected pcD3-Empty ($p<0.05$). In addition, virus yield in mock-cells was higher than that in cells transfected with pcD3-hCARD or pcD3-Empty (Fig. 7). These results demonstrate that the tandem CARD of RIG-I inhibits the replication of VEEV.

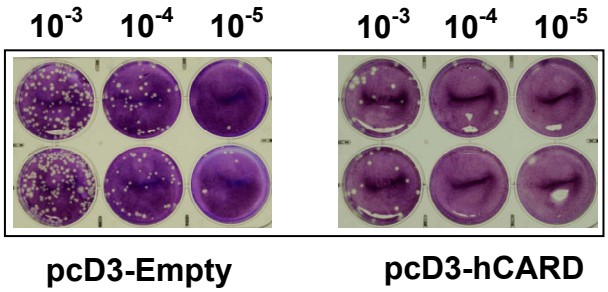

Figure 7. Plaque assay result of the supernatants collected from VEEV-infected cells that were pre-transfected with pcD3-hCARD or pcD3-Empty. *Vero cells were inoculated with 10-fold serially diluted culture media. At 48 h after incubation, cells were stained with 0.3% crystal violet staining solution.*

Discussion

In this study, we used the TC-83 strain of VEEV as an example for the alphaviruses to demonstrate that cells expressing the tandem CARD blocked alphavirus replication. Similar to a previous study [13], we found that IFN-α was produced in cells transiently expressing the CARD of either human or mouse RIG-I (Fig. 4). The production of IFN-α in the cells was correlated to the inhibition of TC-83 replication (Figs. 6 and 7).

The reason that we chose to express the CARD instead of full-length RIG-I is that CARD is able to constitutively stimulate the production of IFN-α/β [13]. Saito and coworkers demonstrate that in normal cells, the CARD activity is suppressed by an internal repressor domain at the C-terminus [26]. Upon virus infection, RIG-I changes its conformation through binding to viral RNA, which allows CARD to activate the downstream signal transduction pathway for IFN-α/β production. Therefore, CARD alone is a strong inducer for IFN-α/β. Indeed, we found that HEK293 cells, which are human embryonic kidney cells [27], secreted close to 3000 pg/ml of IFN-α at day 3 after transfection with pcD3-mCARD (Fig. 4). Similarly, more than 500 pg/ml of IFN-α was produced in HEK293 cells at day 3 after transfection with pcD3-hCARD. These results also indicate that there is no specie specificity for mouse CARD.

At this time, we cannot explain why the production of IFN-α is much higher in cells transfected with pcD3-mCARD than in cells transfected with pcD3-hCARD. We speculate that the amount of CARD proteins expressed from pcD3-mCARD may be higher than that expressed from pcD3-hCARD or the induction of IFN-α by mCARD in HEK293 cells is more efficient than that by hCARD.

The expression of hCARD in HEK293 cells inhibited TC-83 replication (Fig. 6). However, we also found that the TC-83 replication was inhibited in cells transfected with the control plasmid pcD3-Empty (data not shown), although the degree of inhibition is less dramatic than that in cells transfected with pcD3-hCARD. A previous study showed that DNA plasmids can trigger Toll-like receptor 9 to induce IFN-α/β production [28]. In this study, we also found that low levels of IFN-α were detected in cells transfected with pcD3-Empty (Fig. 3). Thus, the partial inhibition of TC-83 replication by pcD3-Empty could be due to the ability of pcD3-Empty to stimulate IFN-α production.

Although the transfection of cells with pcD3-hCARD or pcD3-mCARD induced IFN-α production (Fig. 4), we could not detect serum IFN-α after intramuscular injection of mice with these plasmids. This could be due to the intramuscular injection route not being ideal for targeting CARD-expressing plasmids to IFN-α/β-producing cells, such as plasmacytoid dendritic cells [29]. Alternatively, the amount of CARD expressed in mice after a single inoculation with the plasmids may not be high enough to induce IFN-α production. Future experiments will be carried out to test whether the administration of pcD3-hCARD or pcD3-mCARD through other routes such as intradermal or intravenous injection would induce IFN-α/β production. In addition, the expression of CARD might be improved by the delivery of the gene encoding CARD through viral vectors.

Conclusion

This study demonstrated for the first time that overexpression of RIG-I in cells inhibited the replication of VEEV. Future experiments will be conducted to address the following questions: 1) whether overexpression of RIG-I could also inhibit the replication of other alphaviruses such as EEEV and WEEV; 2) whether delivery and expression of RIG-I gene would provide protection against VEEV in animals; and 3) whether the delivery of RIG-I via a viral vector would improve the antiviral activity of RIG-I. Obtaining answers for these questions will pave the way for the possible development of a RIG-I-based, broad-spectrum antiviral drug which could be used to prevent or treat infection caused by the alphaviruses.

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List of symbols/abbreviations/acronyms/initialisms

ATCC	American type culture collection
CARD	Caspase recruitment domains
DMEM	Dulbecco's modified Eagle media
EEEV	Eastern equine encephalitis virus
ELISA	Enzyme-linked immunosorbant assay
FBS	Fetal bovine serum
IFN-α	Interferon-alpha
MAb	Monoclonal antibody
MOI	Multiplicity of infection
PCR	Polymerase chain reaction
PFU	Plaque forming units
RIG-I	Retinoic acid-inducible gene-I
VEEV	Venezuelan equine encephalitis virus
WEEV	Western equine encephalitis virus

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Retinoic acid-inducible gene I (RIG-I) is a cytoplasmic protein that recognizes viral RNA molecules and subsequently elicits antiviral innate responses. This report describes a pilot study on the feasibility of developing RIG-I into a novel antiviral drug against alphaviruses. It was found that RIG-I, when expressed in cells from a plasmid vector, inhibits the replication of Venezuelan equine encephalitis virus (VEEV), which was used as a model pathogen of the alphaviruses. It appears that the inhibition is related to RIG-I's ability to induce the production of interferon alpha. The result from this study helps the further evaluation of RIG-I in a mouse challenge model of VEEV.

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Retinoic acid-inducible gene-I, Venezuelan equine encephalitis virus

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