A novel attenuated enterovirus A71 mutant with VP1-V238A,K244R exhibits reduced efficiency of cell entry/exit and augmented binding affinity to sulfated glycans

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Running title: Virulence Attenuation of EV-A71 mutant VP1-V238A,K244R

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Abstract

Enterovirus A71 (EV-A71) is one of the major etiological agents of hand, foot, and mouth disease, and its infection occasionally leads to fatal neurological complications in children. However, only inactivated whole virus vaccines against EV-A71 are commercially available in Mainland China. Furthermore, the mechanisms underlying the infectivity and pathogenesis of EV-A71 remain to be better understood. By adaptation of an EV-A71 B5 strain in monkey Vero cells in the presence of brilliant black BN (E151), an anti-EV-A71 agent, a double mutant with VP1-V238A,K244R emerged to be E151 enhanced infection. The growth of reverse genetics (RG) mutant RG/B5-VP1-V238A,K244R (RG/B5-AR) was promoted by E151 in Vero cells, but inhibited in other human and murine cells; while its parental wild type RG/B5-wt was strongly prevented by E151 from infection in all tested cells. In the absence of E151, RG/B5-AR exhibited defective cell entry/exit, resulting in reduced viral transmission and growth in vitro. It had augmented binding affinity to sulfated glycans, cells and tissue/organs, which probably functioned as decoys to restrict viral dissemination and infection. RG/B5-AR was also attenuated with 355 times higher 50% lethal dose and shorter timing of virus clearance than RG/B5-wt in suckling AG129 mice. It, however, remained highly immunogenic in adult AG129 mice to protect their suckling mice from lethal EV-A71 challenges through maternal neutralizing antibodies. Overall, discovery of the attenuated mutant RG/B5-AR contributes to better understanding of virulence determinants of EV-A71 and further development of novel vaccines against EV-A71.
Importance

Enterovirus A71 (EV-A71) is highly contagious in children and has been responsible for thousands of deaths in Asia-Pacific region since 1990s. Unfortunately, the virulence determinants and pathogenesis of EV-A71 are not fully clear. We discovered that a novel EV-A71 mutant VP1-V238A,K244R showed growth attenuation with reduced efficiency of cell entry/exit. In Vero cell line which has been approved for manufacturing EV-A71 vaccines, the growth defects of the mutant were compensated by a food dye brilliant black BN. The mutant was also with augmented binding affinity to sulfated glycans and other cellular components which probably restricted the viral infection and dissemination. Therefore, it was virulence attenuated in a mouse model but still retained its immunogenicity. Our findings suggest the mutant as a promising vaccine candidate against EV-A71 infection.

Introduction

Enterovirus A71 (EV-A71) is classified into enterovirus A species in the Enterovirus genus of the Picornaviridae family, which contains many human pathogens. EV-A71 is a non-enveloped, icosahedral particle with a positive-sense, single-stranded RNA genome. Its capsid is about 30 nm in diameter and consists of 60 copies of protomers, each of which has four structural proteins, VP1, VP2, VP3 and VP4 (1-2). VP1 is the most immunogenic protein to elicit neutralizing antibodies and is involved in cell entry. Based on the nucleotide sequence of VP1, EV-A71 strains are divided into seven distinct genogroups, A, B, C, D, E, F and G. Moreover, genogroup B and C are further divided
into subgenogroups B1 to B5 and C1 to C5, respectively (3-5). Although EV-A71 infection usually causes mild symptoms, including hand, foot and mouth disease (HFMD), it occasionally leads to neurological complications which could be lethal (6-10). Outbreaks of EV-A71 infection amongst children in the Asia-Pacific region have resulted in several thousand deaths during the last 30 years (11).

Three types of formalin inactivated whole virus EV-A71 vaccines have been commercially available in Mainland China since 2016 (12), but no EV-A71 vaccine has approved in other regions yet. All these vaccines use EV-A71 subgenogroup C4 wild type strains as virus seeds which grow in cultured African green monkey kidney Vero or human diploid KMB-17 cells (13-15). Therefore, their safety control is a challenge because specialized vaccine manufacturing equipment with biosafety level 2+ is required and incomplete inactivation may lead to vaccine-derived infections. Although the inactivated EV-A71 vaccines have an advantage over live attenuated vaccines for safety reasons, they require at least two doses to elicit a solid humoral immunity against EV-A71 infection and the immunity wanes after the first 6 months of vaccination (16). On the other hand, live attenuated EV-A71 vaccines could elicit both humoral and cell-mediated immunities to provide strong and durable protection based on the success of attenuated poliovirus vaccines, but this strategy needs to overcome safety issues about virulence determinants and pathogenesis of EV-A71. Identification of attenuated EV-A71 mutants not only unveils the mechanisms underlying viral infection and pathogenesis but also benefits development of attenuated EV-A71 vaccines. For example, a temperature sensitive mutant of EV-A71 BrCr strain exhibits attenuation in cynomolgus monkeys, induces high levels of neutralizing antibodies with cross-reactivity to other EV-A71
genogroups and protects the monkeys against the lethal challenge of EV-A71 (17, 18). A high-fidelity double mutant of EV-A71 with L123F and G64R in the viral polymerase 3D is also attenuated in immunodeficient AG129 mice (19). Moreover, combination of different attenuation mutations in one strain could be synergic and reduces virulence reversion (20, 21).

Efficient infection of EV-A71 relies on the success of every stage of its life cycle. At first, EV-A71 attaches onto surface of host cells by interacting with viral attachment factors, which includes scavenger receptor B2 (SCARB2) (22), P-selectin glycoprotein ligand-1 (PSGL1) (23) and heparan sulfate (HS), a type of sulfated glycosaminoglycans (GAGs) (24). After viral internalization through endocytosis, EV-A71 undergoes uncoating, which is induced by low pH and uncoating factors SCARB2 and cyclophilin A (CypA) (25), and finally releases viral genome into the cytosol. Biosynthesis of viral proteins and genomes happens in the cytosol and is followed by virus assembly and progeny release.

Previous studies indicate that strong binding affinity of viruses, including EV-A71, to sulfated GAGs enhances viral attachment and infection in vitro, but restricts viral spread/dissemination and results in virulence attenuation in vivo (26-27). The positively charged vertex of 5-fold axis of EV-A71, mainly determined by amino acids at VP1-98, VP1-145, VP1-242 and VP1-244, is involved in the viral attachment to HS (28, 29) and affects the viral pathogenesis (26-27, 30, 31) and immunogenicity (32, 33).

Brilliant black BN, a food azo dye with E number E151, has been identified previously as a promising drug candidate against almost all EV-A71 circulating strains (34). By adaptation of an EV-A71 B5 strain in the presence of E151 in a vaccine production Vero cell line, the infection of the 14th passaged mutant with was found to be partially
dependent on E151. In contrast to parental wild type RG/B5-wt which was inhibited by E151, RG mutant RG/B5-AR with VP1-V238A.K244R produced more viral proteins, RNAs and progeny in the presence of E151 in Vero cells. However, both of RG/B5-wt and RG/B5-AR were still E151 sensitive in tested human and murine cells. On the other hand, in the absence of E151, RG/B5-AR exhibited growth attenuation with defective cell entry/exit. It had augmented binding affinity to sulfated glycans, cells and tissue/organs, which might trap the virions to reduce the viral dissemination and infection. Moreover, RG/B5-AR was also attenuated in 5-day old immunodeficient AG129 mice and its 50% lethal dose (LD\textsubscript{50}) was 355 times more than that of RG/B5-wt. However, it was highly immunogenic in adult AG129 mice to elicit EV-A71 specific neutralizing antibodies and protect their suckling mice from lethal EV-A71 challenges. The properties of RG/B5-AR contribute to the understanding of EV-A71 virulence and suggest a novel way of developing live attenuated EV-A71 vaccines.

**Results**

**E151 enhanced infection of Vero/B5-E151-P14 in Vero cells**

Food dye brilliant black BN (E151) has been previously identified to inhibit infection of EV-A71 and coxsackievirus A16 (CVA-16) (34). In this study, adaptation of EV-A71 isolates in Vero cells in the presence of E151 led to mutants whose growth was enhanced by E151. A clinical isolate EV-A71-B5, for example, produced about 1.67 x 10\textsuperscript{7} PFU/ml (plaque forming unit per milliliter) with a mean plaque diameter of 2.6 mm in the absence of E151, while 100 µM E151 reduced its mean plaque number and diameter to 317
PFU/ml and 0.34 mm, respectively (Fig. 1A to C). In contrast to the parental EV-A71-B5, passages of EV-A71-B5 gradually produced less and smaller plaques in the absence of E151, but more and bigger plaques in the presence of E151 during serially blind passage (data not shown). The 14th passage of EV-A71-B5 in Vero cells in the presence of E151, named Vero/B5-E151-P14, exhibited growth defects with decreased plaque number (5.17 x 10^6 PFU/ml) and diameter (0.40 mm) in the absence of E151. However, its infection was compensated and became more efficient with increased plaque number (5.83 x 10^8 PFU/ml) and diameter (3.23 mm) in the presence of 100 µM E151 (Fig. 1A to C). The inhibition of EV-A71-B5 and enhancement of Vero/B5-E151-P14 by E151 were dose-dependent. The growth titers of Vero/B5-E151-P14 were significantly promoted by E151 at concentrations of ≥6 µM and reached the highest level at 20 µM. Higher concentrations of E151 did not further increase viral growth (Fig. 1D).

**VP1-V238A,K244R responsible for the E151 enhanced infection of Vero/B5-E151-P14**

To identify the mutations determining the E151 enhanced infection of Vero/B5-E151-P14, its genome was sequenced and compared to that of the parental EV-A71-B5. The result indicated that two amino acid substitutions, VP1-V238A (substitution of A for V at VP1-238, GUG→GCC) and VP1-K244R (AAG→AGG), occurred at the binding site of E151 (Fig. 2A). The genomic sequences of less passaged viruses showed that VP1-K244R and VP1-V238A emerged at as early as passage P3 and P8, respectively (data not shown). The two mutations V238A (GUG→GCC) and K244R (AAG→CGC) with new genetic codes were introduced alone or together into an infectious clone of EV-A71-B5, named pJET-EV-A71-B5-wt (wild type), by site-directed mutagenesis. Every one of infectious
clones was transfected into Vero cells in duplicates. After removal of the transfection remnants at 6 h post-transfection, one well of each transfected clone was cultured in DMEM-10 and the other in DMEM-10 with 100µM E151 to rescue the RG viruses. After 3 days, the transfected cells were frozen-thawed thrice and the viruses in supernatant were inoculated into fresh Vero cells in the presence or absence of E151. Based on the cytopathic effects (CPE) induced by virus infection, RG/B5-wt, RG/B5-V238A, and RG/B5-K244R grew better in the absence of E151, while double mutant RG/B5-AR with VP1-V238A,K244R grew better in the presence of E151 and became to be enhanced by E151 (Table 1).

The second passaged viruses RG/B5-wt, RG/B5-V238A, and RG/B5-K244R propagated without E151, and RG/B5-AR propagated with E151 were harvested after >80% infected cells showed CPE and further titrated by plaque assay in Vero cells in the presence or absence of 100µM E151 (Table 2). The results showed that RG/B5-wt, similar to its clinical isolate, was highly sensitive to E151 with a PFU ratio (+E151 / -E151: the PFU in the presence of E151 divided by the PFU in the absence of E151) of ~10⁻⁵, while single mutants RG/B5-V238A and RG/B5-K244R were less sensitive to E151 with their PFU ratios of 5 x 10⁻³ and 10⁻¹ respectively (Fig. 2B), suggesting that amino acids at VP1-238 and VP1-244 determine the viral sensitivity to E151 in Vero cells. RG/B5-AR, however, became E151 enhanced as its PFU ratio reached ~23, which is more than 1 (Fig. 2B). The results of virus plaque size also indicated that the single mutation V238A or K244R reduced viral sensitivity to E151 with higher plaque diameter ratios (the mean plaque diameter in +E151 divided by the mean plaque diameter in -E151) than RG/B5-wt, but the ratios were still less than 1. Contrastingly, the double mutation V238A,K244R
converted EV-A71-B5 to be E151 enhanced as its plaque diameter ratio was about 8 (Table 2, Fig. 2C). Similar to the Vero/B5-E151-P14, the growth of RG/B5-AR was enhanced by E151 in a concentration dependent manner. After 24 h infection in Vero cells at a multiplicity of infection (MOI) of 0.1 PFU/cell (based on the titer in the presence of 100 µM E151), VP1 antigen (Fig. 2D), RNA copies (Fig. 2E) and progeny titers (Fig. 2F) of RG/B5-AR incrementally rose with the increased E151 concentrations and reached the highest level at 20 µM. In human rhabdomyosarcoma (RD) cells, however, all the three mutants were still strongly inhibited by E151 and failed to generate visible plaques in the presence of 100 µM E151 (Table 2).

The multiple protein alignment of VP1 proteins of enteroviruses indicated VP1-238V is highly conserved in human enteroviruses, including poliovirus and rhinovirus, while VP1-244K is only conserved in EV-A71 (Fig. 3A). Based on the crystal structure of an EV-A71 isolate (PDB identification number [ID] 3VBS), VP1-238V, VP1-242K, VP1-244K and VP1-246P, which determine the sensitivity of EV-A71 to E151, are all located at the HI loop near the vertex of the viral 5-fold axis (Fig. 3B and C).

**In vitro growth attenuation of RG/B5-AR in the absence of E151**

In the absence of E151, all mutants RG/B5-V238A, RG/B5-K244R and RG/B5-AR exhibited growth attenuation with fewer and smaller plaques in both Vero and RD cells when compared to RG/B5-wt (Table 2). RG/B5-wt and RG/B5-AR were further titrated in Vero, RD, mouse 3T3 expressing human SCARB2 (3T3-hSCARB2) and human SK-N-SH neuroblastoma cells using TCID₅₀ assay. Like the results of plaque assay, the titer of RG/B5-AR was much lower than that of RG/B5-wt in all four cell lines. Moreover, the lower titer of RG/B5-AR was compensated by E151 in the only Vero cells, but further
reduced in other three cell lines (Fig. 4A). Although serial passage of RG/B5-AR in the absence of E151 in Vero cells (P1 to P20) slightly increased its growth, it always exhibited lower infectivity than RG/B5-wt (Fig. 4B). For example, the plaque assay of the twentieth passage of RG/B5-wt and RG/B5-AR, named wt-P20 and AR-P20 respectively, indicated that AR-P20 produced less and smaller plaques than wt-P20 in Vero cells. The mean growth titer and plaque diameter of wt-P20 were $5 \times 10^7$ PFU/ml and 2.81 mm, while those of AR-P20 were $7.5 \times 10^6$ PFU/ml and 0.82 mm respectively (Fig. 4C and D). The results demonstrated that attenuation of RG/B5-AR was stable after the passage in the absence of E151, implying the potential use of RG/B5-AR as a vaccine candidate provided that the stability will also be confirmed after the passage in vivo.

To understand the mechanism underlying the attenuation of RG/B5-AR, one-step growth kinetics of RG/B5-wt and RG/B5-AR in Vero cells at a MOI of 5 or 0.01 was compared. At the high MOI of 5, both of viruses produced indistinguishable amount of viral RNA and VP1 protein from 8 to 24 h post-infection. In contrast, at the low MOI of 0.01, RG/B5-AR generated lower levels of viral RNA and VP1 protein than RG/B5-wt from 16 to 48 h post-infection (Fig. 4E and F). The results indicated that the RG/B5-AR had the same replication rate but a lower transmission rate than RG/B5-wt in vitro.

**Reduced cell entry and exit efficiency of RG/B5-AR**

Cell entry by-pass assay was performed by transfecting genomic RNAs of RG/B5-wt or RG/B5-AR into RD and Vero cells at 200 copies/cell. At 10 h post-transfection, the percentage of VP1 antigen positive cells, level of viral RNA (the ratio relative to beta-actin mRNA) and progeny virions were quantified and found not to be significantly different between the two genomic RNAs (Fig. 5A to C), implying that the RG/B5-AR
and RG/B5-wt possessed the same efficiencies in viral protein expression, genome replication and virus assembly inside the two cell lines. In contrast, RG/B5-AR generated less percentage of VP1 positive cells, level of viral RNA and progeny virions than RG/B5-wt during virus infection assay, in which the cells were infected by RG/B5-AR or RG/B5-wt at 200 virions/cell for 10 h (Fig. 5A to C). Therefore, the cell entry efficiency of RG/B5-AR was lower than that of RG/B5-wt in both RD and Vero cells.

In order to evaluate the efficiency of virus exit or progeny release \textit{in vitro}, the ratios of extracellular to intracellular progeny virions of RG/B5-wt and RG/B5-AR were also quantified and compared by qRT-PCR in above two assays. RG/B5-AR had much lower ratios than RG/B5-wt in both assays (Fig. 5D), suggesting that progeny release of RG/B5-AR was defective. In another experiment, RD and Vero cells were infected at a MOI of 0.01 PFU/cell. When all cells exhibited CPE at 48 h post-infection, the extracellular and intracellular virus progeny were titrated by TCID\textsubscript{50} assay. For RG/B5-wt, the titer ratios of extracellular virus to intracellular virus were about 0.106 and 0.145 in RD and Vero cells respectively. For RG/B5-AR, the titer ratios significantly declined to 0.005 and 0.020 in RD and Vero cells respectively (Fig. 5E). Therefore, virus exit of RG/B5-AR was less efficient than that of RG/B5-wt \textit{in vitro}.

\textbf{Changed binding affinity of RG/B5-AR to viral attachment and uncoating factors}

As RG/B5-AR had defective cell entry/exit, its binding affinity to viral attachment and uncoating factors, including HS, SCARB2 and CypA, was further evaluated. Compared to RG/B5-wt, binding affinity of RG/B5-AR to HS-conjugated agarose resin increased 38\% (Fig 6A). Moreover, RG/B5-AR or single mutant RG/B5-K244R strongly bound to chondroitin sulfate (CS, a ubiquitous sulfated GAG on mammalian cell surface) and
dextran sulfate (DS, a common bacterial sulfated glycan), which are not attachment factors of EV-A71 wild type strains. Binding affinity of RG/B5-AR to CS and DS was particularly 16.27 and 92.97 times as many as that of RG/B5-wt, respectively (Fig 6A), implying that these sulfated glycans probably trapped RG/B5-AR to attenuate its infection. When xylosyltransferase 2 (XT2) gene, responsible for biosynthesis of CS and HS, was knocked out of RD cells (RD-XT2<sup>−/−</sup>) (35), cell attachment of both RG/B5-wt and RG/B5-AR became weak. However, RG/B5-AR remained stronger attachment efficiency in RD-XT2<sup>−/−</sup> cells than RG/B5-wt (Fig 6B), suggesting that it had higher binding affinity to other components on cell surface, perhaps those with sulfation or other negatively charged groups.

In pull down assays, RG/B5-AR and RG/B5-wt had the same binding affinity to recombinant protein SCARB2-Fc (Fig 6C and D), while binding affinity of RG/B5-AR to GST-CypA dropped to 42% compared with that of RG/B5-wt (Fig 6E and F). The results were consistent with that the mutations V238A and K244R of VP1 were at the viral binding site of CypA, but not SCARB2 (25, 34). For comparison, all viruses did not interact with negative control CTLA-4-Fc or GST protein. As SCARB2 is the major receptor and uncoating factor of EV-A71 (22), uncoating process of RG/B5-AR was not greatly affected by the weaker interaction with CypA in RD cells, which was corroborated by that the growth kinetics of RG/B5-AR and RG/B5-wt was almost the same at the high MOI of 5 (Fig. 4E and F).

**Virulence attenuation of RG/B5-AR in AG129 mice**

As EV-A71 with increased binding affinity to sulfated GAGs are attenuated *in vivo* (26-28, 30, 31), virulence of RG/B5-AR was further evaluated in AG129 mice. The mouse
mortality caused by EV-A71 infection has been reported to be age dependent (36, 37). Therefore, groups of 5-, 7-, 11- and 14-day old AG129 mice were first infected intraperitoneally by $10^8$ virions of RG/B5-wt or RG/B5-AR, and their survival rates and clinical scores were recorded for 3 weeks. All the 5-, 7- and 11-day old mice were killed by the parental wild type RG/B5-wt at 5, 7 and 12 days post-infection respectively, and the 14-day old mice were partially killed at 3 weeks post-infection (Fig. 7A and B). In contrast, the mutant RG/B5-AR only caused death in some of the 5-day old mice and completely failed to induce severe symptoms, like limb paralysis, and death in the 7-day old mice (Fig. 7C and D), implying that the virulence of RG/B5-AR was attenuated. 5-day old mice were further used to evaluate LD$_{50}$ of RG/B5-wt and RG/B5-AR by intraperitoneal inoculation of virus diluents. After three weeks infection, the LD$_{50}$ of RG/B5-wt was determined to be $5.6 \times 10^5$ virions, while the LD$_{50}$ of RG/B5-AR was $2.0 \times 10^8$ virions which is about 355 times higher than the former (Fig. 8A and C). Clinical scores also indicated that mice infected with RG/B5-AR exhibited milder clinical features than those infected with RG/B5-wt at later time points (Fig. 8B and D).

The attenuation of RG/B5-AR in vivo was further investigated by determining the viral RNA levels in the tissues and organs of infected mice. Five-day old ARG129 mice were infected by $1.78 \times 10^6$ virions of RG/B5-wt (~3 LD$_{50}$) or RG/B5-AR. The brains and hind limb muscles were harvested for total RNA extraction at 4 and 8 days post-infection, and the levels of viral RNA and beta-actin mRNA (internal control) were then quantified by qRT-PCR (Fig. 8E). At 4 days post-infection, substantial copies of viral RNA were detected in the brains and hind limb muscles of both infected mice groups, but the viral RNA levels in the mice infected by RG/B5-AR were significantly lower than that of the...
mice infected by RG/B5-wt. At the later 8 days post-infection, the viral RNA levels in the brains and muscles of the RG/B5-wt infected mice increased as similar as in previous publications (36, 37), while the viral RNA levels in the brains and hind limb muscles of the RG/B5-AR infected mice declined to undetectable levels, suggesting RG/B5-AR replicated at a slower rate at the early stage of infection and was subsequently cleared.

EV-A71 and other viruses with higher binding affinity to sulfated GAGs (HS and/or CS) tend to be adsorbed and trapped by non-susceptible cells and extracellular matrix in host tissue/organs. Therefore, adsorption of RG/B5-wt and RG/B5-AR to insoluble fractions of homogenized mouse brain, hind-limb muscle, heart, lung, kidney or intestine was further examined. After incubation of the $10^6$ virions and 100 mg of homogenates at 4°C for 1 h, the non-adsorbed virions in supernatants were quantified by plaque assay. As both viruses are stable during incubation (data not shown), reduction of the viral PFU is proportional to the number of adsorbed virions. The results showed that 30 to 40% of input RG/B5-wt and 60 to 75% of input RG/B5-AR was adsorbed by the six different tissue/organ homogenates (Fig 8F), suggesting that this higher adsorption of RG/B5-AR might reduce its dissemination and contribute to its virulence attenuation in mice.

**High immunogenicity of RG/B5-AR in AG129 mice**

The immunogenic potential of RG/B5-AR was evaluated in adult AG129 mice. Four female groups (n = 5) were immunized with $10^9$ virions of live RG/B5-wt, $10^9$ virions of live RG/B5-AR, $10^9$ virions of UV-inactivated RG/B5-AR (UV-RG/B5-AR) or PBS. Sera collected 4 weeks post-immunization indicated that one dose of RG/B5-AR induced strong EV-A71 specific neutralizing antibodies against both homologous RG/B5-wt and heterologous EV-A71-C1 strains. Although the mean of neutralizing antibody titer (NAT)
elicited by RG/B5-AR was lower than that of RG/B5-wt, their NATs were not significantly different. In contrast, UV-RG/B5-AR did not elicit detectable neutralizing antibodies, implying that viral replication is necessary to elicit strong NAT in these immunized mice (Fig. 9A).

The immunized female mice were further used for breeding, their suckling mice were then challenged by 100 LD$_{50}$ of RG/B5-wt at 5 days old or 10 LD$_{50}$ of EV-A71-C1 at 14 days old. All pups from females immunized by RG/B5-AR or RG/B5-wt were completely protected from the lethal challenge through maternal antibodies and did not exhibit any symptoms, while all pups from the females immunized by UV-RG/B5-AR or PBS succumbed to the challenge (Fig. 9B and C). The survival percentages were concordant with the NAT results.

**Discussion**

EV-A71, as an RNA virus, has a high mutation rate and exists as quasispecies (38-40). During *in vitro* adaptation, it can evolve into different cell-adapted mutants or populations with various phenotypes. Propagation of a clinical isolate Tainan/5746/98 in Vero cells generates virulent progeny which kill transgenic mice with human SCARB2, while its passages in RD cells lose the virulence to kill the mice. Mutations E145G, V146I and S241L of VP1 at the vertex of the viral 5-fold axis are found in the RD adapted viruses and might be responsible for the virulence attenuation (41). In our previous studies, adaptation of EV-A71-B5 in RD cells in the presence of E151 leads to a E151 resistant mutant with E98K, G145E and P246A in VP1 (35). In contrast, its
adaptation in Vero cells in the presence of E151 resulted in a E151 enhanced mutant with V238A and K244R in VP1 as described in this study (Fig. 2). Interestingly, all mutations are also located at the vertex of the viral 5-fold axis, suggesting that the vertex is critical for infection of EV-A71 in vitro. It is well known that the vertex interacts with viral attachment factor HS and uncoating factor CypA in RD and Vero cells (24, 25). The VP1-V238A,K244R in RG/B5-AR greatly increased the viral binding affinity to HS (Fig. 6A), but reduced the viral binding affinity to CypA (Fig. 6E and F). However, the mutations did not affect the viral binding with the viral major receptor SCARB2 which interacts with the viral canyon region (Fig. 6C and D). Moreover, the vertex is also targeted by many anti-EV-A71 agents, including E151 (34). E151 retained interaction with RG/B5-AR, but it enhanced and inhibited the viral infection in Vero and RD cells, respectively (Table 2). The distinct mutations between the RD and Vero adapted EV-A71 mutants indicate that cellular components involved in the viral entry and infection differ in RD and Vero cells. This complicated virus-host interaction needs further investigation.

Efficiency in completion of each step of the viral life cycle is critical for EV-A71 to establish infection and cause diseases in the hosts. In the absence of E151, RG/B5-AR exhibited growth attenuation with defective cell entry/exit in all tested cell lines. This attenuation was compensated by E151 in Vero cells but not in human and murine cells (Table 2 and Fig. 4A). Our preliminary data indicated that E151 only enhanced cell entry of RG/B5-AR because addition of E151 in cell entry stage (0 to 3 h post-infection) but not virus intracellular replication stage (3 to 10 h post-infection) greatly increased percentage of infected cells and viral growth titer in Vero cells (data not shown). Mechanism underlying that infection of RG/B5-AR was enhanced by E151 in Vero cells
but inhibited by E151 in other cells will be studied in the future. Moreover, the
attenuation of RG/B5-AR was unchanged after serial passages in vitro (Fig. 4B to D),
implying that RG/B5-AR will be a good candidate for development of live attenuated
EV-A71 vaccines.

Positively charged amino acids R and K are usually found in sulfated GAGs binding
motifs (42, 43). VP1-242K and VP1-244K of EV-A71 are critical for the viral attachment
to HS on the surface of host cells (28, 29). R has stronger electrostatic interactions with
negatively charge molecules, such as sialic acids and sulfo groups, than K (43, 44).
Substitution of R for K at the VP1-244 enabled RG/B5-K244R to strongly bind to CS and
dS, which did not interact with RG/B5-wt as attachment factors. Moreover, combination
of K244R and V238A in VP1 of RG/B5-AR further increased the viral binding affinity to
all tested sulfated gylcans as well as other cellular surface molecules (Fig. 6A and B).
This augmented cell binding affinity of RG/B5-AR probably trapped it and harmed its
cell entry/exit (Fig. 5), resulting in growth attenuation with reduced efficiency of viral
transmission in vitro (Fig. 4).

Augmented cell binding of viruses sometimes restricts their dissemination and attenuates
their virulence in vivo. Host cell adaptation of Japanese encephalitis virus (JEV), Murray
Valley encephalitis virus (MVEV) (45), yellow fever virus (YFV) (46) and EV-A71 (29)
significantly increases the viral binding affinity to sulfated GAGs, which are viral
attachment factors to enhance the viral infection in vitro. However, a high amount of
sulfated GAGs in the extracellular matrix can trap these cell-adapted viruses and prevent
them from further infection in vivo. For example, substitution of amino acid K or R for E
at the position 326 of the E protein of an attenuated YFV strain 17D gives the protein an
increased net positive charge to interact sulfated GAGs, makes the virus more sensitive to heparin and decreases the viral neuroinvasive ability in a SCID mouse model (46). For cell adapted EV-A71 strains, substitution of G or Q for E at VP1-145 make the vertex of the viral 5-fold axis more positively charged and increase the viral attachment affinity to HS and heparin (29). Similarly, EV-A71 strains with VP1-145G are less neurovirulent and fail to disseminate well in wild-type suckling mice (26, 30), transgenic mice expressing human SCARB2 (26) and cynomolgus monkeys (27, 31) compared to the strains with VP1-145E. In this study, the double mutation VP1-V238A,K244R in RG/B5-AR, which already has VP1-145G as RG/B5-wt, further augmented the viral binding affinity to sulfated glycans, cells and tissue/organs (Fig. 6 and 8F). As expected, RG/B5-AR was less virulent in suckling AG129 mice than RG/B5-wt (Fig 7 and 8).

Compared to RG/B5-wt, RG/B5-AR exhibited strong virulence attenuation with ~355 times higher LD50 in 5-day old AG129 mice (Fig. 8). Moreover, it was not lethal to older suckling mice even at very high challenge doses (Fig. 7). The replication of RG/B5-AR was also found to decrease in the mice, with its RNA detectable in the brains and hind limb muscles of infected mice at 4 days, but undetectable at 8 days post-infection. In contrast, viral RNA levels in the brains and hind limb muscles of RG/B5-wt infected mice were much higher and increased from 4 to 8 days post-infection (Fig. 8). This result implied that RG/B5-AR only replicated at the initial time points of infection in vivo, and the subsequent disappearance of RG/B5-AR could be ascribed to the host’s antiviral responses and/or the viral inability to disseminate and replicate well in the older mice. Although the replication of RG/B5-AR is self-limiting, its immunogenicity was not compromised in vivo. Female adult AG129 mice immunized with the RG/B5-AR
generated strong neutralizing antibodies at 4 weeks post-immunization and protected their pups from the lethal EV-A71 challenge (Fig. 9). However, pathogenesis and immunogenicity of EV-A71 in the immunodeficient AG129 mice are distinct from that in humans (33,36). Virulence attenuation and uncompromised immunogenicity of RG/B5-AR will need further confirmation in other immunocompetent animals and in human beings. Moreover, the genetic stability of RG/B5-AR must be evaluated because EV-A71 can quickly acquire host-adapted mutations due to its error-prone polymerase 3D. Additional mutations around the vertex of the 5-fold axis of RG/B5-AR might not only affect the viral immunogenicity (32, 33) but also reduce the viral augmented binding affinity to sulfated GAGs (28) which is thought to be one of reasons for the attenuation of RG/B5-AR. One possible solution could be to introduce high fidelity polymerase mutations into RG/B5-AR to increase its genetic stability and reduce its virulence reversion (20, 21).

In conclusion, a EV-A71 mutant RG/B5-AR with VP1-V238A,K244R at conserved sites dramatically reduced the viral infectivity and pathogenicity, but not significantly affected the viral immunogenicity. The mutant exhibited growth attenuation in vitro with reduced efficiency of cell entry/exit, but the attenuation was compensated by E151 in Vero cell line which has been approved for EV-A71 vaccine production. Moreover, the mutant acquired the augmented binding affinity to sulfated glycans, which supports a concept that viruses can be attenuated in vivo by trapping them with sulfated glycans to limit their dissemination and infection. Therefore, the mutant contributes to the further development of novel antiviral vaccines against EV-A71.
Materials and Methods

Cells, viruses and food dye

Human rhabdomyosarcoma (RD; ATCC® CCL-136™), human SK-N-SH (ATCC® HTB11™), African green monkey kidney (Vero; ATCC® CCL-81™) and mouse NIH/3T3 (ATCC® CRL-1658™) expressing FLAG tagged human SCABR2 cell lines were maintained in Dulbecco’s modified Eagles’ medium (DMEM) (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Biowest) and 1X antibiotic-antimycotic (Life Technologies) in a 37°C humidified incubator with 5% CO₂. An EV-A71 clinical isolate #NUH0083 (subgenogroup B5, GenBank accession No.: FJ461781) was originally propagated in Vero cells and named as EV-A71-B5. Food dye brilliant black BN (11220-25MG, Sigma-Aldrich) was dissolved in sterile deionized water (diH₂O) at 10 mM and then diluted to desired concentrations with DMEM.

Virus propagation and purification

RD or Vero cell monolayers with 80% to 100% confluency in T-flasks and plates (NUNC) were infected by each virus at a MOI of 0.1 to 1 and incubated at 37°C. When more than 80% cells detached and exhibited CPE after 2 to 3 days, the cells were frozen-thawed thrice and the supernatants containing viruses were harvested, aliquoted, and stored at 80°C for future experiments after the centrifugation at 3000 x g for 10 min. For virus purification, the supernatants containing virions were further centrifuged at 10,000 x g for 20 min at 4°C to remove small cell debris. The 20 ml of supernatants was then transferred into a polycarbonate ultracentrifuge bottle (Cat. #355618, Beckman Coulter) with 2.5 ml of 20% sucrose in PBS as cushion. The bottles were loaded into a Type 70 Ti rotor
(Beckman Coulter) and centrifuged at 120,000 x g for 3 h at 4°C. The supernatant and sucrose were decanted as much as possible after the ultracentrifugation, and then the virus pellet in each bottle was re-suspended in 2 ml of PBS or PBS containing 0.1% FBS for 2 to 6 h on ice with gentle agitation. The re-suspended virions were filtered through 0.22 µm sterile syringe filter (Millipore), aliquoted and stored at -80°C.

**Virus inactivation by ultraviolet light**

Purified RG/B5-wt or RG/B5-AR virion samples suspended in PBS in 35x10 mm cell culture dishes (Nunc) were inactivated by ultraviolet (UV) light in a biosafety class II cabinet (NuAire) for 30 min on a heating block cooled by ice. Complete inactivation was defined by no CPE after 3 blind passages of the samples in Vero cells.

**50% tissue culture infective dose (TCID_{50})**

The assay was carried out in cultured cells using the Reed and Muench formula as previously described (35). Briefly, serial 10-fold dilutions of each virus sample were transferred into octoplicated wells in 96-well flat bottom microtiter plates (NUNC) in which 10^4 of RD or Vero cells were seeded. No virus was added into control wells. The plates were incubated at 37°C in the incubator and the cells were observed daily for CPE up to 5 days post-infection.

**Plaque assay**

The assay was carried out according to a previous description (35). Briefly, serial 10-fold dilutions of virus samples were inoculated into RD or Vero cell monolayers in the presence or absence of 100 µM E151 in 6-well plates. After virus absorption at 37°C for 2 h, the monolayers were rinsed twice with PBS and overlaid with 1% UltraPure low
melting point agarose (Thermo Scientific) in DMEM-2 with or without E151. After the agarose solidified at room temperature, the RD and Vero cells were incubated for 4 and 5 days respectively at 37°C. The monolayer cells were then fixed with 4% formalin in PBS and further stained with 0.1% crystal violet in diH$_2$O. The plates with a ruler were imaged by a Gel-Imager ChemiDoc Touch (Bio-Rad), and the number and diameter of plaques were analysed by the Image Lab software.

**Quantitative reverse transcription PCR (qRT-PCR)**

Total viral RNA in infected cells and mouse tissues was purified by RNeasy mini kit (Qiagen), while the viral RNA genomes of virions in the supernatant were purified by E.Z.N.A.® Viral RNA Kit (Omega Bio-tek). The viral RNA was quantified by QuantiNova Probe RT-PCR kit using primers qRT-PCR-EV-F and qRT-PCR-EV-R and a Taqman probe qRT-PCR-EV-probe. Beta-actin mRNA, an internal control, was also quantified using qRT-PCR-β-actin-F, qRT-PCR-β-actin-R and qRT-PCR-β-actin-probe (Table 3). The RT-PCR reaction was carried out using a Rotor-Gene Q real time PCR cycler (Qiagen) according to a previous report (35).

**Western blot**

Viral and cellular proteins were dissolved in Laemmli SDS loading buffer, separated by 12% SDS-polyacrylamide gel, and transferred onto nitrocellulose membranes with a pore size of 0.2 μM (Bio-Rad) The membrane was first blocked by 5% non-fat milk in PBS with 0.05% Tween-20 (PBST), and Western blot was performed as previously described [13]. To detect VP1 protein of EV-A71, the membranes were blotted with a mouse monoclonal antibody 1D9 followed by HRP conjugated goat anti-mouse IgG antibodies
To detect cellular beta-actin, the membranes were blotted with HRP conjugated mouse anti-human beta-actin monoclonal antibody (sc-376421, Santa Cruz). To detect GST and GST tagged CypA, the membranes were blotted with mouse anti-GST sera followed by HRP conjugated goat anti-mouse IgG antibodies. To detect human Fc recombinant proteins SCARB2 and CTLA-4, the membranes were blotted with HRP conjugated rabbit anti-human IgG antibodies (P021402-2, Dako). After rinsed with PBST, the membranes were incubated with Clarity Western ECL substrate (Bio-Rad). Chemiluminescent signals were recorded and quantified by a Gel-Imager ChemiDoc Touch and Image Lab software (Bio-Rad), respectively.

Immunofluorescent assay

The viral VP1 antigen in EV-A71 infected or viral gnomic RNA transfected cells was detected as previously described (32). Briefly, the cells were fixed, permeabilized, washed and subsequently incubated at 4°C with guinea pig anti-VP1 sera for at least 3 h with gentle agitation. After PBST washing, the cells were incubated overnight with AF488 conjugated anti-guinea pig IgG antibodies (1:1000, Life Technologies). The cells were then stained by Hoechst 33258 in PBS at a concentration 1 µg/ml for 10 min. After another 3 PBST washes, the cells in PBS were observed under a UV microscope (Olympus).

Selection and sequencing of EV-A71-B5 mutants

E151 sensitive EV-A71-B5 was serially passaged in Vero cells in the presence of 10 µM E151 for 10 times and then in the presence of 100 µM E151 for another 4 times. The fourteenth passaged virus, named as Vero/B5-E151-P14, was selected as it became E151
enhanced in Vero cells. The genomic RNA of EV-A71-B5 and Vero/B5-E151-P14 was extracted using E.Z.N.A.® Viral RNA Kit (Omega Bio-tek), and the P1 genes were amplified by OneStep RT-PCR kit (Qiagen) using primers seqEV-A71-P1-F and seqEV-A71-P1-R. The RT-PCR thermal cycling conditions were applied at an initial incubation at 50°C for 30 min (reverse transcription), 95°C for 15 min (initial PCR activation step), followed by 40 cycles: 94°C for 30 s (denaturation), 58°C for 30 s (annealing), and 68°C for 4 min (extension), and a final extension at 68°C for 10 min. The PCR products were purified after gel electrophoresis and inserted into pJET1.2 vector (Thermo Scientific) for sequencing. The P1 sequences were compared, and mutations responsible for E151 enhancement were further confirmed by reverse genetics.

Construction and generation of EV-A71 mutants

The extracted EV-A71-B5 genomic RNA was amplified by RT-PCR and then put under the human RNA polymerase I promoter as previously described (47), and the infectious cDNA clone was named as pJET-EV-A71-B5. The mutations VP1-V238A and VP1-K244R were introduced into the pJET-EV-A71-B5 by site-directed mutagenesis with corresponding primers (Table 3) according to the In-Fusion protocol (Takara). The reverse genetics EV-A71-B5 wild type virus and mutants were generated by direct transfection of their corresponding infectious plasmids into Vero cells. Each plasmid transfection was carried out in duplicates, and then the media were replaced by fresh DMEM-10 for one or DMEM-10 with 100 µM E151 for the other at 6 h post-transfection. After 3 days, the transfected cells were frozen-thawed thrice, and the viruses in the supernatant were diluted 10 times and further propagated in Vero cells for another two passages in the presence or absence of 100 µM E151. The accuracy of VP1 genes of the
2nd passage viruses was confirmed by sequencing. All RG viruses used in subsequent experiments were obtained from a stock of the 2nd passage.

**Virus growth in the presence of different concentrations of E151**

Viruses EV-A71-B5, Vero/B5-E151-P14, RG/B5-wt and RG/B5-AR were diluted to a concentration of 2 x 10^4 PFU/0.5 ml in DMEM-10 with E151 concentrations ranging from 0 to 100 µM and incubated for 1 h at 37°C. Vero cells were seeded into 24-well plates at a density of 2 x 10^5 cells per well and incubated for 6 h in the incubator, and then the medium was replaced by 0.5 ml of virus diluents so that the cells were infected at a MOI of 0.1 PFU/cell. At 24 h post-infection, the viral protein, RNA and progeny were quantified by Western blot, qRT-PCR and TCID\textsubscript{50}, respectively.

**One-step growth kinetics of EV-A71**

Vero cell monolayers in 24-well plates were infected by RG/B5-wt or RG/B5-AR at a MOI of 5 or 0.01. After 1 h incubation at 37°C, infected cells were rinsed thrice with PBS to remove unbound viruses and further cultured with DMEM-1o in the incubator. Viral VP1 protein, RNA, and progeny were quantified at 1, 8, 16, 24, 32, 40 and 48 h post-infection.

**Cell entry by-pass assay**

RD or Vero cells were seeded into 24-well plates at a density of 10^5 cells per well and incubated overnight at 37°C. 2 x 10^7 copies of purified genomic RNA of RG/B5-wt or RG/B5-AR were transfected into cells by lipofectamine 2000 (Life Technologies). For comparison, 2 x 10^7 virions of RG/B5-wt or RG/B5-AR were inoculated into parallel wells. The cells were washed twice with PBS and cultured with fresh DMEM-10 after 3 h.
transfection or infection. The viral VP1 antigen, RNA, extracellular and intracellular virions were then quantified by IFA and qRT-PCR at 10 h post infection or transfection when one life cycle of EV-A71 finished.

**Titration of extracellular and intracellular viruses**

RD or Vero cells were seeded into 24-well plates at a density of $10^5$ cells per well and incubated overnight at 37°C, and then infected by 500 µl of RG/B5-wt or RG/B5-AR at a MOI of 0.01 in the presence or absence of 100 µM E151. At 48 h post infection, almost all cells exhibited CPE. For every infection sample, cell culture medium from infected wells was transferred into a centrifuge tube, and 250 µl of fresh medium was added into the wells. After centrifugation at 3000 x g for 10 min at 4°C, the supernatant containing extracellular viruses was transferred into a fresh tube, while the cell pellet was re-suspended by 250 µl of fresh medium and transferred back to the wells. The infected cells in wells were frozen-thawed for three times to release the intracellular viruses into the medium, and the supernatant was then collected after the centrifugation. The virus samples of RG/B5-wt and RG/B5-AR were then titrated in Vero cells in the absence and presence of 100 µM E151, respectively.

**Binding of EV-A71 to sulfated glycans**

HS (H7640), CS (C4384) and DS (D4911) purchased from Sigma were first linked to epoxy-activated agarose lyophilized powder (E6632, Sigma). 0.5 g of the powder resin was soaked and washed thrice with double distilled water; and suspended in 5 ml of distilled water. 5 mg of each sulfated glycans dissolved in 1 ml of coupling buffer (0.1 M Na₂CO₃, 0.15 M NaCl, pH 13) was mixed with 0.5 ml of the suspended resin at room
temperature overnight for coupling reaction. The resin was then washed with PBS five times and incubated with 1 ml of quenching buffer (1 M ethanolamine, pH 8) at room temperature for 6 h. The resin was further washed thrice with acetate buffer (0.1 M CH₃COONa, 0.5 M NaCl, pH 4) followed by Tris buffer (0.1 M Tris-HCl, 0.5 M NaCl, pH 8), and stored in 0.5 ml of PBS with 0.02% NaN₃ at 4°C for following experiments.

100 µl of the sulfated GAGs conjugated or negative control resin was blocked by PBS containing 1% BSA at 4°C overnight and washed twice with PBS. It was then mixed with 10⁷ RG/B5-wt, RG/B5-V238A, RG/B5-K244R or RG/B5-AR virions in 1 ml of binding buffer (20 mM Tris-HCl, 150 mM NaCl, 0.2% Igepal CA-630, 0.1% BSA, pH 7.4) and incubated at room temperature for 2 h with gentle agitation. After incubation, the resin was washed 3 times with PBST and the bound virions were quantified by qRT-PCR.

**Attachment of EV-A71 to EDTA detached cells**

RD and RD-XT2⁺ cells (35) in T-flask were first detached with PBS containing 5 mM EDTA, washed thrice with PBS and re-suspended in DMEM-10. After chilled on ice for 10 min, 10⁶ cells were incubated with 2 x 10⁸ of ice-cold RG/B5-wt or RG/B5-AR virions at 4°C for 1 h. Unbound virions were removed by rinsing the cells thrice with ice-cold PBS, total RNA of attached virions and cells were then purified using RNeasy mini kit (Qiagen). The viral RNA and cellular beta-actin mRNA were quantified by qRT-PCR.

**Binding of EV-A71 to uncoating factors**

Purified 10⁹ virions of RG/B5-wt or RG/B5-AR were first diluted in 500 µl of PBS+ (PBS, 0.2% Igepal CA-630, 0.1% BSA, pH 7.4), and then mixed with 50 µl of BSA pre-treated protein A/G sepharose beads (Santa Cruz) containing 3 µg of SCARB2-Fc (R&D
systems), or CTLA-4-Fc (R&D systems), or 50 µl of BSA blocked Glutathione-sepharose beads (Thermo Fisher) containing 30 µg of GST-CypA or GST (34). After incubation at 4°C for 1.5 h with gentle agitation, the beads were washed thrice with ice-cold PBS containing 0.01% Tween-20 and the bound proteins and viral VP1 antigen were analysed by Western blot.

**Ethics statement**

Mice were housed in individually ventilated cages inside ABSL2 laboratory for animal care and use. All animal experiments were carried out in accordance with the Guides for Animal Experiments of the National Institute of Infectious Diseases (NIID), and experimental protocols were reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of the Temasek Life Sciences Laboratory Ltd, Singapore. The IACUC Approved Project was “TLL-18-016: Pathogenicity and immunogenicity of drug dependent human enterovirus 71 variants in AG129 mouse”.

**Determination of virulence of RG/B5-wt and RG/B5-AR**

AG129 mice (129/Sv mice without interferon alpha/beta/gamma receptors) breeders were purchased from B&K Universal (United Kingdom). Usually, one male and two females were housed in a ventilated cage for breeding and observed daily. After 3 days from the birth of pups, the mother and pups were transferred into a new cage for later experiments.

Groups of AG129 mice (n = 6 to 8) at 5-, 7-, 11- and 14-day old were first intraperitoneally infected by 10⁸ virions of purified RG/B5-wt or RG/B5-AR, and their survival rates and clinical scores were recorded for 3 weeks as previously described (32). As the RG/B5-AR failed to kill 7-day and older mice, determination of LD₅₀ of both
viruses were further carried out in 5-day old mice. 50 µl of 10-fold serial dilutions of RG/B5-wt (10^5 to 10^8 virions), RG/B5-AR (10^7 to 10^9 virions) or PBS was intraperitoneally inoculated into mice, and their survival rates and clinical scores were recorded for 3 weeks.

Quantification of viral RNA in mouse organ/tissue

To study the replication and dissemination of RG/B5-wt and RG/B5-AR in 5-day old mice, the mice were intraperitoneally infected by 10^6.25 virions of RG/B5-wt (~3 LD_{50}) or RG/B5-AR. At 4 and 8 days post-infection, hind limb muscles and brains (n = 6 to 8) were harvested, frozen and weighed after euthanasia of the mice. The total RNA purification of mouse samples was performed using a RNeasy Mini Kit (Qiagen). Briefly, about 50 mg of the samples were homogenized in 1 ml of Buffer RLT containing 1% β-mercaptoethanol by using a TissueLyser LT homogenizer (Qiagen). After centrifugation of the lysates at 16,000 x g for 3 min, 100 µl of the supernatant of each sample were transferred into a new Eppendorf tube by pipetting for RNA extraction in duplicate. The purified RNA was eluted by nuclease free water, and amount of viral RNA and mouse beta-actin mRNA were quantified by qRT-PCR.

Virus adsorption by mouse organ/tissue

Mouse brains, hind-limb muscles, hearts, lungs, kidneys and intestines were collected and homogenized by the TissueLyser LT in DMEM-2. Insoluble fractions of homogenates were washed with DMEM-2 three times, and 100 mg (wet weight) of them were incubated with 10^6 virions of RG/B5-wt or RG/B5-AR in 1 ml of DMEM-2 at 4°C for 1 h with gentle agitation. After centrifugation at 3,000 g for 20 min, non-adsorbed virions in
supernatants were subjected to plaque assays for virus quantification. Input virions less non-adsorbed virions equals adsorbed virions.

Lethal EV-A71 challenge of suckling mice from immunized adult AG129 females

10 to 14 weeks old AG129 females (n = 5) were intraperitoneally immunized with 300 µl of $10^9$ live RG/B5-wt virions, $10^9$ live RG/B5-AR virions, $10^9$ UV inactivated RG/B5-AR virions or PBS. At 4 weeks post-immunization, the sera were collected for EV-A71 neutralizing antibody titration. The females were then used for breeding to produce pups. The suckling pups were intraperitoneally challenged by 100 LD$_{50}$ of homologous RG/B5-wt strain (5.6 x $10^7$ virions) at 5 days old or by 10 LD$_{50}$ of heterologous EV-A71-C1 strain (1.5 x $10^8$ TCID$_{50}$ in RD cells) at 14 days old (32), and their survival rates and clinical scores were recorded up to 3 weeks.

Neutralizing antibody titration

The sera were heat-inactivated in a 56°C water bath for 30 min and 2-fold serially diluted by DMEM-2 in 96-well plates. 50 µl of diluted sera were mixed with 50 µl of DMEM-2 containing 100 TCID$_{50}$ of RG/B5-wt or EV-A71-C1, and then incubated at 37°C for 1 h. 50 µl of DMEM-10 containing $10^4$ Vero cells were then inoculated into 96-well plates, and the plates were incubated at 37°C up to 5 days for CPE development. Each serum sample was duplicated, and its neutralizing antibody titer were defined as the reciprocal of the highest dilution fold which completely prevented CPE caused by virus infection in both wells. Every sample was performed in two independent experiment and its average value was represented.

Analysis of viral protein sequence and structure
Viral nucleotide and protein sequences were analyzed using Lasergene 13 package (DNASTAR). The crystal structure data of 4AED (EV-A71) were obtained from Protein Data Bank. The molecular graphics and analyses of proteins and virions were done using UCSF Chimera.

Statistics

All quantification of viral amounts and binding assays was performed in duplicates or triplicates. Their values were compared using Student's t-test, ANOVA test or nonparametric Mann-Whitney test in Excel (Microsoft) and/or GraphPad Prism version 8.0.1 (GraphPad software, USA). Kaplan-Meier survival curves and mean clinical score curves were analyzed by GraphPad Prism using the log-rank test and the Wilcoxon test, respectively. Two-tailed p values <0.05 were considered statistically significant (* p <0.05, ** p <0.01, *** p <0.001).

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Figure Legends

Fig 1. Selection and characterization of Vero/B5-E151-P14. EV-A71-B5 was passaged in Vero cells in the presence of E151. Growth of the 14th-passage virus Vero/ B5-E151-P14 was enhanced by E151. Plaque morphology (A), PFU (B) and plaque diameter (C) of EV-A71-B5 and Vero/B5-E151-P14 in the presence or absence of 100 µM E151 (+E151 or +E151). A representative image of triplicated independent experiments for each condition is shown in A. The data in B and C are shown as the mean values ± standard errors (means ± SEM) of three independent experiments (n=6 in B, n=30 in C, two-tailed unpaired Student’s t test). (D) E151-concentration dependent growth of Vero/B5-E151-P14 in Vero cells. 2 x 10⁵ cells were infected by EV-A71-B5 or Vero/B5-E151-P14 at a MOI of 0.1 for 24 h in the presence of E151, whose concentration ranged from 0 to 100 µM. The virus progeny of EV-A71-B5 and Vero/B5-E151-P14 were titrated in Vero cells in DMEM-10 and DMEM-10 containing 100 µM E151, respectively. The lines indicate the means ± SEM (n=3, one-way ANOVA with Dunnett’s posttest compared to those at 0 µM E151 for each virus).

Fig 2. Identification of mutations responsible for E151 enhanced infection of Vero/B5-E151-P14. (A) Comparison of VP1 sequences of EV-A71-B5 and Vero/B5-E151-P14 indicated two amino acid mutations V238A and K244R (highlighted by red rectangles). The ratios of average PFU (B) and average plaque diameter (C) of B5 variants in the presence of 100 µM E151 (+E151) to those in the absence of E151 (-E151) presented in Table 2. (D to F) E151-concentration dependent replication of RG/B5-V238A,K244R (RG/B5-AR). 2 x 10⁵ Vero cells were infected at a MOI of 0.1 PFU/cell (based on the titer in the presence of 100 µM) for 24 h in 24-well plates in 500 µl of
DMEM-10 containing different concentrations of E151. Its VP1 antigen (D), RNA copies (E) and virus progeny (F) were quantified by Western blot, qRT-PCR and TCID\(_{50}\) assay, respectively. A representative figure and the average relative density VP1 to beta-actin of 3 independent experiments in D are shown. The lines in E and F indicate the means ± SEM (n=3, one-way ANOVA with Dunnett’s posttest compared to those at 0 µM E151).

**Fig 3. Conserved VP1-238A and VP1-244K in EV-A71.** (A) Multiple protein alignment of VP1s of enteroviruses. The VP1 proteins of 8 EV-A71 strains (EV-A71-A, EV-A71-B2, EV-A71-B4, EV-A71-B5, EV-A71-C1, EV-A71-C2, EV-A71-C4, EV-A71-C5) and 3 CVA16 strains (CVA-16-MOH1, CVA-16-G10, CVA-16-OS) were previously sequenced (32). The VP1 sequences of CVA-10 (ARU09507), CVA-6 (AUF49646), CVA-4 (ATP75726), CVB-3 (AIZ97146), PV-1 (CAA24461, poliovirus type 1), PV-3 (AAN85444, poliovirus type 3), EV-D68 (ALJ53630, enterovirus D68), RV-A40 (AFK65738, rhinovirus A40) and RV-B35 (AFK65739, rhinovirus B35) were retrieved from the NCBI database. All sequences were aligned by Clustal W method. (B) Three-dimensional structure of a VP1 pentamer of an EV-A71 virion (PDB ID 3VBS). The solid surface of four VP1 proteins and the rounded ribbon of one VP1 protein are presented. A white pentagon indicates the vertex of the viral 5-fold axis. Amino acids at VP1-145 (red), VP1-238 (yellow), VP1-242 (cyan), VP1-244 (blue) and VP1-246 (green), which modulate the interactions among E151, EV-A71 and host cells, are highlighted. (C) An enlarged view of a white rectangle in B. Highly conserved VP1-238V, VP1-242K, VP1-244K and VP1-246P in the ball and stick form are all located at the HI loop near the vertex of the viral 5-fold axis.
Fig 4. In vitro attenuation of RG/B5-AR in the absence of E151. (A) In the absence of E151, the titers of RG/B5-AR (AR) was lower than those of RG/B5-wt (wt). The P2 viruses wt and AR were titrated in Vero, RD, 3T3-hSCARB2 and SK-N-SH cells in the presence (+) or absence (-) of 100 μM E151. The TCID50 was calculated based on viral antigen positiveness in IFA in SK-N-SH and CPE in other three cell lines at 3 days post-infection in three independent experiments (n=3, two-tailed unpaired Student’s t test; N.D.: not detectable). (B-D) Attenuation of AR was stable. Plaque morphologies (B) of different passages P1, P5 P10 and P20 of wt and AR in Vero cells. The PFU (D) and plaque diameter (E) of the twentieth passage wt-P20 and AR-P20 were presented as the means ± SEM of three independent experiments (n=6 in D, n=30 in E, two-tailed unpaired Student’s t test). (E-F) One-step growth kinetics demonstrated the growth attenuation of AR at a low but not high MOI. Vero cells were infected by wt or AR at of a MOI of 5 or 0.01. The viral RNA (E) and VP1 protein (F) were quantified at different hours post-infection by qRT-PCR and Western blot, respectively. The lines in E indicate the means ± SEM (n=6, two-tailed unpaired Student’s t test). A red rectangle in F shows VP1 protein expression of AR was less than that of wt from 24 to 40 h post-infection when MOI was 0.01.

Fig 5. Reduced cell entry and exit of RG/B5-AR. (A-D) RG/B5-AR had less efficient cell entry and exit than RG/B5-wt in cell entry by-pass assay. RD and Vero cells were transfected with purified viral genomic RNAs at 200 copies/cell or infected by viruses at 200 virions/cell. At 10 h post transfection or infection, the percentage of VP1 positive cells, relative viral RNA level, intracellular and extracellular progeny virions were quantified by IFA or qRT-PCR for two independent experiments (n=6, two-tailed
unpaired Student’s t test; n.s.: not significant). (E) Virus progeny release of RG/B5-AR was less efficient than that of RG/B5-wt. Cells were infected by RG/B5-wt or RG/B5-AR at a MOI of 0.01 for 48 h. Extracellular and intracellular virus progeny were titrated in Vero cells and their ratios were calculated for two independent experiments as the means ± SEM (n=6, two-tailed unpaired Student’s t test).

Fig 6. Changed binding affinity of RG/B5-AR to viral attachment and uncoating factors. (A) RG/B5-AR had stronger binding affinity with sulfated GAGs than RG/B5-wt. Purified RG/B5-wt, RG/B5-V238A, RG/B5-K244R or RG/B5-AR was incubated with HS, chondroitin sulfate (CS) or dextran sulfate (DS) conjugated agarose resin. The percentages of input virions bound to sulfated GAGs were quantified by qRT-PCR for two independent experiments and are presented as the means ± SEM (n=4, two-tailed unpaired Student’s t test). Ratios of bound RG/B5-wt to bound RG/B5-AR were also shown above brackets. (B) RG/B5-AR had higher binding affinity with RD and RD-XT2 than RG/B5-wt. Ice cold virions and EDTA-detached cells were incubated at 4°C, cell attached virions were quantified by qRT-PCR for two independent experiments (n=6, two-tailed unpaired Student’s t test). (C and D) Binding affinity of RG/B5-AR with human SCARB2 remained the same as that of RG/B5-wt. (E and F) Binding affinity of RG/B5-AR with human CypA was lower than that of RG/B5-wt. Virions were incubated with recombinant protein SCARB2-Fc, CTLA-4-Fc, GST-CypA or GST. The proteins interacting virions were pulled down by protein A/G- or glutathione-sepharose beads and quantified by Western blot. A representative image of two independent experiments under each condition was presented using ImageLab (C and E, i: 20% of input, b: bound,
+: addition, -: no addition) and the percentages of bound virions are presented as the means ± SEM (n=4, two-tailed unpaired Student’s t test; N.D.: not detectable).

Fig 7. Age-dependent mortality of AG129 mice infected by RG/B5-wt or RG/B5-AR.
5, 7, 11 or 14 days old AG129 mice were intraperitoneally infected by 10^8 virions of RG/B5-wt (A and B) or RG/B5-AR (C and D). Control mice were administrated with PBS (mock). The mice were observed daily. Survival percentages (A and C) and clinical scores (B and D) were recorded up to 21 days post-infection.

Fig 8. Attenuation of RG/B5-AR in 5-day old AG129 mice. (A-D) RG/B5-AR was less virulent than RG/B5-wt. The 5-day old AG129 mice were intraperitoneally injected with serially diluted doses of RG/B5-wt (A and B) or RG/B5-AR (C and D) ranging from 10^5 to 10^9 virions/mouse. Control mice were administrated with PBS (mock). Survival percentages (A and C) and clinical scores (B and D) were recorded up to 21 days post-infection. (E) RG/B5-AR was less effective to replicate and/or disseminate than RG/B5-wt. Relative viral RNA levels in the brains and hind limb muscles of 5-day old mice infected with 1.78 x 10^6 virions of RG/B5-wt (unfilled circles and triangles) or RG/B5-AR (black filled circles and triangles) were quantified at 4 and 8 days post infection. Black solid and dash lines indicate means (n = 6-8, Mann Whitney test). (F) RG/B5-AR was easier to be adsorbed by mouse tissue/organs. After the input 10^6 virions of RG/B5-wt or RG/B5-AR were incubated with 100 mg of insoluble fractions of six different tissue/organ homogenates in DMEM-10 at 4°C for 1 h, non-adsorbed virions in supernatants were quantified by plaque assay. Percentages of input virions adsorbed by tissue/organs are presented as the means ± SEM (n=6, two-tailed unpaired Student’s t test).
Fig 9. High Immunogenicity of RG/B5-AR in AG129 mice. Adult females (n=5) were immunized with purified RG/B5-wt, RG/B5-AR, UV-inactivated RG/B5-AR (UV-RG/B5-AR) or PBS, and then subjected to serum collection and breeding 4 weeks later. (A) RG/B5-AR elicited neutralizing antibodies equivalent to RG/B5-wt. Sera were tested in neutralizing antibody titer assay against homologous RG/B5-wt (circles) and heterologous EV-A71-C1 (dash diamonds). Solid and dash lines indicate means, and results are significant at p values < 0.05 (n=5, Mann Whitney test). (B-C) Immunization of RG/B5-AR in females protected their pups from lethal EV-A71 challenge. Pups (n = 6) of immunized females were intraperitoneally challenged with 100 LD$_{50}$ of RG/B5-wt at 5-day old (B) or 10 LD$_{50}$ of EV-A71-C1 at 14-day old (C). Survival percentages were recorded up to 21 days post-challenge.

Table 1. Severity of CPE of Vero cells transfected or infected by EV-A71-B5 RG variants in the presence or absence of 100 µM E151. Vero cells were transfected with RG viral infectious clones or infected with RG viruses in the presence or absence of E151. Effect of E151 on the CPE induced by viral infection was observed and recorded daily up to 72 h.

<table>
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<tr>
<th>RG variants</th>
<th>Transfection</th>
<th>Passage 1</th>
<th>Passage 2</th>
<th>Effect of E151 on CPE</th>
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<tr>
<td>V238A</td>
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<td>-</td>
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<td>K244R</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>V238A,K244R (AR)</td>
<td>-</td>
<td>+/-</td>
<td>+/-</td>
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</table>

+++: CPE in >50% cells at 24 h post-infection or -transfection

++: CPE in >50% cells at 48 h post-infection or -transfection

+: CPE in >50% cells at 72 h post-infection or -transfection

+/-: CPE in few cells at 72 h post-infection or -transfection

-: no CPE
Table 2. Plaque assay of EV-A71-B5 RG variants (the second passage) in Vero and RD in the presence or absence of 100 µM E151

<table>
<thead>
<tr>
<th>RG variants</th>
<th>PFU/ml a</th>
<th>Plaque diameter (mm) b</th>
<th>PFU/ml</th>
<th>Plaque diameter (mm)</th>
<th>PFU/ml</th>
<th>Plaque diameter (mm)</th>
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<td>+E151</td>
<td>-E151</td>
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<tr>
<td>wild type (wt)</td>
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<td>2.58 ± 0.12</td>
<td>2.50 ± 0.58 x 10^3</td>
<td>0.28 ± 0.05</td>
<td>4.75 ± 0.96 x 10^6</td>
<td>1.50 ± 0.07</td>
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<tr>
<td>V238A</td>
<td>0.48 ± 0.10 x 10^7</td>
<td>2.20 ± 0.09</td>
<td>2.75 ± 0.96 x 10^3</td>
<td>1.14 ± 0.07</td>
<td>0.43 ± 0.15 x 10^6</td>
<td>0.83 ± 0.06</td>
<td>0</td>
<td>Nil</td>
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<tr>
<td>K244R</td>
<td>1.23 ± 0.52 x 10^7</td>
<td>1.00 ± 0.07</td>
<td>1.20 ± 0.54 x 10^4</td>
<td>0.34 ± 0.06</td>
<td>2.25 ± 0.96 x 10^6</td>
<td>0.41 ± 0.05</td>
<td>0</td>
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<tr>
<td>V238A,K244R (AR)</td>
<td>0.65 ± 0.10 x 10^7</td>
<td>0.43 ± 0.06</td>
<td>1.50± 0.56 x 10^4</td>
<td>3.23 ± 0.1</td>
<td>0.63 ± 0.13 x 10^6</td>
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aMean ± SEM of two independent experiments (n = 6) for each virus.
bMean ± SEM of randomly selected plaques (n = 20) for each virus.
Table 3. List of primers used in this experimental study.

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<td>B5-VP1-V238A-R</td>
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A

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B

C