

Article

Oral Immunization with the C488A Live-Attenuated Mutant of Coxsackievirus B4E2 (CVB4E2) Induces Potent Immune Response and Protects Balb/c Mice Against Lethal Infection

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Abstract

Background/Objectives: Coxsackievirus B4 (CVB4), a member of the *Enterovirus* genus and the *Picornaviridae* family, is a significant pathogen causing several human diseases such as pancreatitis, myocarditis, cardiomyopathy and type 1 diabetes. Despite its clinical impact, no vaccines or specific antiviral therapies are currently available. This study investigates the attenuation of CVB4 virulence through targeted mutations in the domain V of the IRES (Internal Ribosome Entry Segment) sequence present in the 5' UTR (Untranslated Region) of the viral genome. **Materials and Methods:** We engineered six CVB4E2 mutants by introducing single nucleotide mutations in domain V of the IRES sequence using PCR-based site-directed mutagenesis assays. Mutants were rigorously evaluated in vitro for their replicative capacities on HeLa cell culture and for their in vitro translation efficiencies in standard rabbit reticulocyte lysates supplemented with HeLa cell S10 extracts. Using different strategies of immunization and lethal challenges in a Balb/c mice model, we evaluated the immune responses elicited by the most attenuated C488A mutant strain. **Results:** The obtained results demonstrated that the live-attenuated C488A mutant with the single mutation C to A at nucleotide position 488 of the viral IRES sequence exhibited a significant reduction in vitro of both viral productivity and translation efficiency. The oral immunization with the live-attenuated C488A mutant induced a potent immune response and protected Balb/c mice against lethal infection challenge with a pathogenic strain. **Conclusions:** These findings underscored the critical role of IRES in CVB4 virulence and highlighted the use of the live-attenuated C488A mutant strain as a promising candidate for developing a live-attenuated vaccine against CVB4 infections.

Keywords: CVB4; replication; translation; virulence; live-attenuated mutant; vaccine; immunization; Balb/c mice

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1. Introduction

Coxsackievirus B4 (CVB4), a member of the *Enterovirus B* genus within the

Picornaviridae family, is a non-enveloped, single-stranded, positive-sense RNA virus with a 7.4 kb genome. Human enteroviruses are classified into four different species; *Enterovirus A* to *D* are ubiquitous infection agents and infect many organs of the human body's systems. CVB4 is a significant human pathogen implicated in a spectrum of severe clinical conditions, including viral pancreatitis, myocarditis, cardiomyopathy (CMD) and type 1 diabetes (T1D) [1]. CVB4 infections are particularly concerning due to their association with the autoimmune destruction of pancreatic β -cells, which contributes to T1D onset in genetically susceptible individuals [2,3]. In addition, CVB4 has been linked to severe systemic infections, including meningitis and encephalitis, highlighting its broad tissue tropism and pathogenic potential [4,5]. CVB4, like all human enteroviruses, is mainly transmitted directly or indirectly among humans by the fecal–oral route. The absence of effective vaccines or specific antiviral therapies underscores the urgent need for novel strategies to mitigate human CVB4 infections and associated diseases.

The CVB4 genome consists of a unique ORF (Open Reading Frame), flanked by 5' and 3' UTRs (Untranslated Regions). The genome 5' UTR contains the Internal Ribosome Entry Sequence (IRES), a highly structured RNA element critical for cap-independent translation [6,7]. The IRES, particularly the stem-loop domain V, is essential for recruiting ribosomes and host translation factors such as PTB and PCBP2 to initiate viral protein synthesis [8,9]. This region's structural and functional conservation across enteroviruses makes it a promising target for modulating viral pathogenicity and virulence [10]. The viral genome of CVB4 encodes a single ORF (Open Reading Frame) to finally synthesize a viral polyprotein constituted by 2185 amino acids during translation. This polyprotein is cleaved by viral enzymes into viral sub-proteins P1, P2 and P3. Sub-proteins are cleaved then by viral proteases to produce several structural viral proteins (VP1 to VP4) and viral enzymes. The structural proteins VP1, VP2, VP3 and VP4 form protomers and five of these protomers assemble into a pentamer. An icosahedral capsid approximately 30 nm in diameter consists of 12 pentamers. VP4 is an internal protein, whereas VP1, VP2 and VP3 are exposed on the particle surface. The success of the Sabin poliovirus vaccines provides a compelling model for attenuating enteroviruses through targeted IRES mutations. Sabin poliovirus vaccines rely on single nucleotide substitutions in the IRES domain V to reduce neurovirulence while maintaining immunogenicity, allowing a safe and effective vaccine against poliovirus [11,12]. It is well demonstrated that the attenuating mutations in poliovirus Sabin strains disrupt IRES-mediated translation efficiency, particularly in neural cells, thereby limiting viral replication in critical neural tissues [13,14]. Given that CVB4 belongs to the same genus as poliovirus, we hypothesized that similar IRES mutations could attenuate its virulence, particularly its diabetogenic and cardiotropic properties, which are mediated by its ability to infect pancreatic and cardiac tissues.

The present study focuses on the CVB4E2 strain, a diabetogenic strain isolated from a child died with T1D [2]. The CVB4E2 strain is well-characterized for its ability to induce pancreatitis, myocarditis, and T1D-like symptoms in mice, making it an ideal in vivo model for studying pathogenesis and virulence attenuation [15]. Thereby, in the present study we introduced six different single mutations at different nucleotide positions in the IRES stem-loop domain V of CVB4E2 genome using the PCR-based site-directed mutagenesis assay. These mutations were designed to mimic the attenuating effects of Sabin poliovirus mutations, in addition to other mutations targeting conserved and critical domain V IRES structure and function [16]. Generated mutants were evaluated for their in vitro replicative capacities and translation efficiencies. The most attenuated mutant among generated mutants C488A was selected to be assessed for its im-

munogenicity to induce immune responses in Balb/c mice, a robust model for CVB4-induced pathologies [17–19]. The objectives of this study were threefold: design and generate CVB4E2 mutants with targeted IRES domain V mutations, then assess their in vitro replicative capacities and in vitro translation efficiencies compared to the CVB4E2 pathogenic strain, and finally evaluate the immune response elicited by the selected C488A live-attenuated mutant in the Balb/c mice model.

2. Materials and Methods

2.1. Ethics Statement

The Balb/c mice model study in this work has been carried out in strict accordance with the recommendations of the Committee of Ethics of the Scientific Research Deanship of King Faisal University (DSR-KFU) described in their guide for the use of experimentations with animal models on 1 October 2024 (License number KFUE-REC-OCT-ETHICS247). Balb/c mice experiments were conducted making all efforts to minimize suffering for the animals during oral immunization, blood collection and euthanasia. All Balb/c mice experiments were in compliance with the recommendations of the Committee of Ethics of the institution in a protocol approved and supervised by the committee. Balb/c mice were bred in the College of Science animal facility.

2.2. Viral Strain and Cloning Vector

The CVB4E2 strain, a diabetogenic variant isolated from the pancreas of a child with T1D who died [2] kindly provided by the Enterovirology Laboratory, College of Medicine, University of Lille (France), has been used in this study. This viral strain was described as diabetogenic in a mice model and causes pancreatitis and myocarditis. The full genome strain (GenBank accession: AF311939), cloned into the pRib-CVB4E2/T7 vector under the control of a T7 promoter enabling in vitro RNA transcription, was kindly provided by the Virology research unit, College of Biotechnology, University of Monastir (Tunisia). The CVB4E2 strain is known for inducing pancreatitis and T1D-like symptoms in mice, making it ideal for studying virulence attenuation. The pRib-CVB4E2/T7 vector is a 10,548 bp double-stranded circular DNA plasmid containing an ampicillin-resistance gene (ampR) for bacterial selection and a T7 promoter for RNA transcription.

2.3. HeLa Cell Culture and Media

HeLa cells (epithelial cells purchased from American Type Culture Collection ATCC: CCL-2), known to support CVB4 strains' replication, were cultured in minimum essential medium (MEM, Invitrogen, California, USA) supplemented with 7% fetal calf serum (FCS, Invitrogen), 1% L-glutamine (Invitrogen), 50 µg/mL of streptomycin (Invitrogen), 25 UI/mL of penicillin (Invitrogen), 1% non-essential amino acids (Invitrogen) and 0.1% Amphotericin B (Invitrogen) in a humidified incubator with 5% CO₂ at 37 °C. Every 2–3 days, HeLa cells were sub-cultured to maintain sub-confluent monolayers for in vitro transcription, transfection and infection experiments. Supernatants were collected after inoculation, clarified by centrifugation and divided into aliquot stocks and stored at –80 °C until use.

2.4. Balb/c Mice

Female Balb/c mice aged 3 to 4 weeks (12–20 g body weight) were used under ethical approval from the King Faisal University Ethical Committee on 1 October 2024 (License number KFUE-REC-OCT-ETHICS247). Mice were housed in specific pathogen-free conditions with unlimited access to food and water. All procedures followed institutional guidelines to minimize suffering, including anesthesia during tissue collection.

2.5. Production of Mutant Viruses by PCR-Based Site-Directed Mutagenesis Assay

The initial material for all genetic and molecular biology experiments is the infectious cDNA clone pRib-CVB4E2/T7 under the control of a T7 promoter and containing the full-length genome of the CVB4E2 diabetogenic strain. The Δ pRib-CVB4E2/T7 monocistronic mutant clone was generated by deletion of a fragment sequence (nucleotides 2462 to 6310) from the parental clone (pRib-CVB4E2/T7). Six different single mutations were carefully introduced by PCR-based site-directed mutagenesis assay at different positions belonging to the nucleotide sequence of the IRES domain V of the CVB4E2 strain. The clones carrying single mutations were engineered using a PCR-based site-directed specific mutagenesis method involving two rounds of PCR of the Δ pRib-CVB4E2/T7 DNA. Introduced mutations were controlled regularly by the DNA automatic sequencing of the entire domain V sequences of the different mutants. A forward primer corresponding to nucleotide 28 to nucleotide 53 (5'-CCACAGGGCCCCACTGGGCGTTAGCAC-3') of the CVB4E2 viral cDNA and a reverse primer corresponding nucleotide 1126 to nucleotide 1106 (5'-CAACGTCTGGTTGGGTGGGT-3') were used in the first PCR round. The sequences of the forward and reverse primers used for introducing single mutations are presented in Table 1. These primers targeted specific nucleotides to create mutants E2-A486G (substitution of A to G at nucleotide 486), E2-G487A (substitution of G to A at nucleotide 487) and E2-C475T (substitution of C to U at nucleotide 475), mimicking attenuating mutations found in Sabin poliovirus strains, successively S1, S2 and S3. In addition, 3 other single mutations were introduced nearby to create E2-C488A (substitution of C to A at nucleotide 488), E2-A489C (substitution of A to C at nucleotide 489) and E2-C494T (substitution of C to T at nucleotide 494). The products of the first PCR round were purified and used as templates for the second PCR round. The first and second PCR rounds were carried out for 1 min 20 sec at 94 °C, followed by 40 cycles of 40 sec at 94 °C, 1 min at 63 °C, and 1 min at 72 °C, and a final phase of 10 min at 72 °C. All PCR reactions were conducted with a 50 μ L final volume with 10 mM deoxynucleotide triphosphates (dNTPs), 1.25 U of high-fidelity Pfu polymerase (Invitrogen), 25 pmol of each oligonucleotide (Invitrogen), and Pfu polymerase buffer (Invitrogen). The restriction fragments generated by double digestion with *BlnI/EcoRV* restriction enzymes (nucleotide 291 to 917 of CVB4E2 strain) were used to replace the counterparts of the Δ pRib-CVB4E2/T7. The fragments of the different Δ pRib-CVB4E2/T7 mutants generated by the double digestion with *BlnI/BglII* restriction enzymes (from nucleotide 219 to nucleotide 2042 of CVB4E2 genome) were introduced to the CVB4E2 entire cDNA. After clone screening, CVB4E2 expression plasmids for A486G, G487A, C475T, C488A, A489C and C494T mutations were successfully obtained, named pcDNA-E2 A486G, pcDNA-E2 G487A, pcDNA-E2 C475T, pcDNA-E2 C488A, pcDNA-E2 A489C and pcDNA-E2 C494T, respectively.

Table 1. Nucleotide primer sequences used for the generation of mutants by PCR-based site-directed mutagenesis assay.

Primers *	Sequences ** (5' → 3')	Substitution
A486G-F	CCTAACTGCGGGGACATGCCC	A → G (nt 486)
A486G-R	GGGCATGTGCCCCGAGTTAGG	A → G (nt 486)
G487A-F	CCTAACTGCGGAACACATGCCACAAACCA	G → A (nt 487)
G487A-R	TGGTTTGTGGGCATGTGTTCGCGAGTTAGG	G → A (nt 487)
C475T-F	CCTGAATGCGGCTAATCTAACTGCGGAGC	C → T (nt 475)
C475T-R	GCTCCGCGAGTTAGAAATTAGCCGCATTCAGG	C → T (nt 475)
C488A-F	AACTGCGGAGACATGCCCCAC	C → A (nt 488)

Primers *	Sequences ** (5' → 3')	Substitution
C488A-R	GTGGGCATGT <u>I</u> CTCCGCAGTT	C → A (nt 488)
A489C-F	CTGCGGAGC <u>CC</u> CATGCCCACAAACC	A → C (nt 489)
A489C-R	GGTTTGTGGGCATG <u>G</u> GCTCCGCAG	A → C (nt 489)
C494T-F	GGAGCACATG <u>T</u> CCACAAACCAG	C → T (nt 494)
C494T-R	CTGGTTTGTGG <u>A</u> CATGTGCTCC	C → T (nt 494)

* F: forward primer; R: reverse primer; nt: nucleotide. ** Mutations are indicated in bold and underlined.

2.6. In Vitro RNA Transcription and Cell Transfection Assays

The generated plasmid pcDNA-E2 from the full-length diabetogenic strain and from mutant strains was purified and then linearized using enzymatic digestion by *SalI* (nucleotide 7435 of pRib-CVB4E2/T7). In vitro RNA transcription was conducted as described previously [20,21]. The RNAs obtained by in vitro transcription were first purified before using them for HeLa cell transfections. Sub-confluent HeLa cell monolayers (10^6 cells/plate) were transfected with 1 µg of purified viral RNA mixed with 1 mg/mL DEAE-dextran (Invitrogen) in HBSS buffer for 30 min at 4 °C. After washing with MEM (without FCS), the RNA mixture was applied to HeLa cells for 30 min at room temperature, followed by incubation with MEM (2% FCS) at 37 °C in humidified incubator at 5% CO₂. Mock-transfected control cells were prepared using DEAE-dextran/HBSS without viral RNA. HeLa cells were subjected to three freeze–thaw cycles, then clarified by centrifugation and harvested for 72 h incubation until a total cytopathic effect (CPE) was observed. The viral titers of the mutant and pathogenic CVB4E2 strains were determined using the Reed and Muench TCID₅₀ assay as described previously [22].

2.7. In Vitro RNA Translation Assay

Standard rabbit reticulocyte lysates (RRL, Invitrogen) supplemented with HeLa cell S10 extracts were used for in vitro translation reactions as described previously [7]. Briefly, translation reactions were programmed with uncapped mRNAs at a concentration of 10 µg/mL and incubated for 1 h at 30 °C. Mutated and parental RNAs truncated from nucleotide 2462 to 6310 of the CVB4E2 genome were assayed in vitro in a cell-free translation system alone or supplemented with increasing percentages of S10 HeLa cell extracts (0%, 5%, 10% and 20%). RNase was used to stop reactions and then incubated for 10 min before the addition of blue solution (80% bromophenol blue, 20% β-mercaptoethanol). The viral polyprotein translation product was finally analyzed by SDS-PAGE. Quantification of translated viral polyprotein was conducted by densitometry of autoradiograms, using NIH image software (Image J 1.34s).

2.8. The One-Step Viral Growth Cycle Assay

HeLa cells were infected with virus strains (pathogenic and generated mutant strains) using an M.O.I (Multiplicity of Infection) equal to 20 TCID₅₀/cell. During different times post-infection (p.i.) 3, 5, 7, 10, 15, and 20 h, HeLa cells were frozen and thawed successively three times, and viral titers were quantified in supernatants using the Reed and Muench assay on a HeLa cell monolayer culture [22]. Experiments were performed in triplicate, and mean titers were used to generate growth curves for each virus strain.

2.9. Balb/c Mice Immunogenicity and Challenge Studies

The immunogenicity of the selected live-attenuated mutant was assessed in the Balb/c mice model according to an immunization and challenge strategy. Experimental procedures were conducted at the animal facility of the College of Science, King Faisal

University, and were realized to evaluate the immunogenicity and protective efficacy of the selected live-attenuated mutant in Balb/c mice. Balb/c mice were monitored daily for body weight, clinical signs and mortality. Thirty 4-week-old female mice were randomly assigned to six mice groups. Each animal group contained 5 mice ($n = 5$) and all mice groups were immunized or challenged by the oral (per os) route: Group 1 (G1) Balb/c mice received an oral prime-immunization at day 0 with a dose of 10^5 TCID₅₀ of the live-attenuated strain. Group 2 (G2) mice received a prime-boost-immunization at days 0 and 21 with the same dose of 10^5 TCID₅₀ of the live-attenuated strain. Group 3 (G3) mice received a prime-immunization with the live-attenuated strain, then were challenged at day 35 with 10^5 TCID₅₀ of the CVB4E2 pathogenic strain. Group 4 (G4) mice received a prime-boost regimen of immunization with the live-attenuated strain at days 0 and 21, then were challenged at day 35 with the CVB4E2 pathogenic strain. Group 5 (G5) mice were used as a positive control; mice of this group were orally administered 10^5 TCID₅₀ of the CVB4E2 pathogenic strain at day 0. Group 6 (G6), representing the negative control non-infected mice were administered PBS at days 0, 21 and 35. All survival group mice were euthanized at the end of the study (day 49). Mice experiments were conducted without blinding.

Blood samples were collected at days 14, 21, 28, 35, 42 and 49 from mice tails for specific neutralizing antibody and cytokine interferon gamma (INF- γ) quantification assays. At the end of the study (day 49), blood samples were collected directly from mice hearts during animal euthanasia. Body weight and mortality were monitored and recorded daily during the study. Mice fecal samples were collected every seven days to evaluate the stool viral shedding and to detect any reversion-mutation events in the viral genome. Heart and pancreas organs were collected from euthanized mice at the end of the study, rinsed with PBS and stored at -80°C in liquid nitrogen for virus quantification assays.

All mice experiments were performed by making efforts to minimize mice suffering and distress during oral immunization, blood collection from tails and euthanasia. All described mice experiments complied with the agreement of the Committee of Ethics of the DSR, KFU, on 1 October 2024 (License number KFU-REC-OCT-ETHICS247). The Ethics Committee approved and supervised the designed protocol.

2.10. Anti-CVB4 Neutralizing Antibody Titration Assay

HeLa cells were cultured in 96-well microtiter plates at 6×10^3 cells/well with 50 μL of cell culture medium (MEM with 7% of FCS, 1% L-glutamine, 50 $\mu\text{g/mL}$ of streptomycin, 25 UI/mL of penicillin, 1% non-essential amino acids and 0.1% of Amphotericin B). Cells were then incubated for 16 to 20 h at 37°C in a humidified incubator with 5% CO₂. Serum samples derived from immunized Balb/c mice were first inactivated at 56°C for 30 min, then diluted at serial two-fold dilutions with bovine serum albumin (BSA). The titrations of specific neutralizing antibodies present in serum samples were quantified by a micro-neutralization assay. The assay is based on HeLa cell viability as previously described [23]. Briefly, 50 μL of a two-fold serial serum dilution was incubated, respectively, with 50 μL of 100 TCID₅₀ CVB4E2 pathogenic strain at 37°C for 1 h, followed by the addition of HeLa cells (concentration of 6×10^3 cells/well). After 46 h of incubation at 37°C , cell viability was determined with alamar-blue reagent. Neutralizing antibodies were determined by calculating IC₅₀ (Inhibition Concentration) titers from sample-specific neutralization curves. The neutralizing antibody titer is the highest dilution of serum that inhibited the CPE (cytopathic effect) of the virus strain.

2.11. IFN- γ Concentration Assay

Sera from immunized and challenged Balb/c mice groups and from naive non-inoculated Balb/c mice groups were collected at days 14 to 49 after prime-immunization as described below. Cytokine interferon gamma (IFN- γ) concentrations were evaluated by ELISA assay, using a murine IFN- γ platinum ELISA kit (Thermo Fisher, Waltham, MA, USA) as described previously [24].

2.12. Viral Titration in Mice Organ Tissues and Stools

Heart and pancreas tissues from euthanized Balb/c mice at day 49 were removed from animals and rinsed with PBS. Mice stools were collected throughout the study. Snap-frozen organ tissues and collected stools were weighted, crushed using a tissue homogenizer (Qiagen, Germantown, MD, USA), centrifugated at $2000\times g$ for 10 min at 4 °C and homogenized in PBS with 1% antibiotics (penicillin and streptomycin) and stored at -80 °C in liquid nitrogen for virus titrations. HeLa cells were seeded at 3×10^6 on a six-well plate in MEM (Invitrogen) supplemented with 7% of FCS (Invitrogen), 1% L-glutamine (Invitrogen), 50 $\mu\text{g/mL}$ of streptomycin (Invitrogen), 25 UI/mL of penicillin (Invitrogen), 1% non-essential amino acids (Invitrogen) and 0.1% of Amphotericin B (Invitrogen) in a humidified incubator with 5% CO₂ at 37 °C for 24 to 48 h until 90% confluence. HeLa cells were inoculated with viral supernatants from centrifuged snap-frozen mice tissues. Cultures were then examined daily by light microscope for the enterovirus cytopathic effect (CPE) until day 7 post-infection. Virus titers were determined by the Reed and Muench assay [22] and expressed as TCID₅₀ by mg tissue values. Blind passages were systematically performed for negative samples, and non-infected HeLa cell cultures were used as a negative control.

2.13. Statistical Analysis

The results are presented as mean values $\pm$ SD (standard deviation). Statistical analyses were performed using a one-way analysis of variance ANOVA test, and the significance of the difference between means was determined by an independent Student's t-test, using GraphPad Prism 8.0 software. A p -value < 0.05 was considered statistically significant. All of the experiments with a quantitative analysis described in the figures have been performed in triplicate.

3. Results

3.1. Generated Mutants Reveal Different Levels of Reduced Replicative and Translation Efficiencies

To determine whether the structure of the stem-loop domain V of the CVB4 IRES is important for IRES activity, six different mutants were designed and constructed using the PCR-based site-directed mutagenesis assay from the cloned full-length cDNA of the CVB4E2 pathogenic strain. The six mutant plasmids (pcDNA-E2 A486G, pcDNA-E2 G487A, pcDNA-E2 C475T, pcDNA-E2 C488A, pcDNA-E2 A489C and pcDNA-E2 C494T) were successfully constructed, each carrying a single nucleotide mutation in IRES domain V as shown in Figure 1A. The integrity of the plasmid constructs was always confirmed by 1% agarose gel electrophoresis of restriction-digested plasmid sizes (Figure S 1, Supplementary Materials). The introduced mutations were verified and confirmed during all steps of mutant construction by DNA Dye-Terminator sequencing of the *BlpI/EcoRV* fragments (nucleotide 291 to 917), and the sequencing results revealed no unintended mutations detected in the targeted genome region.

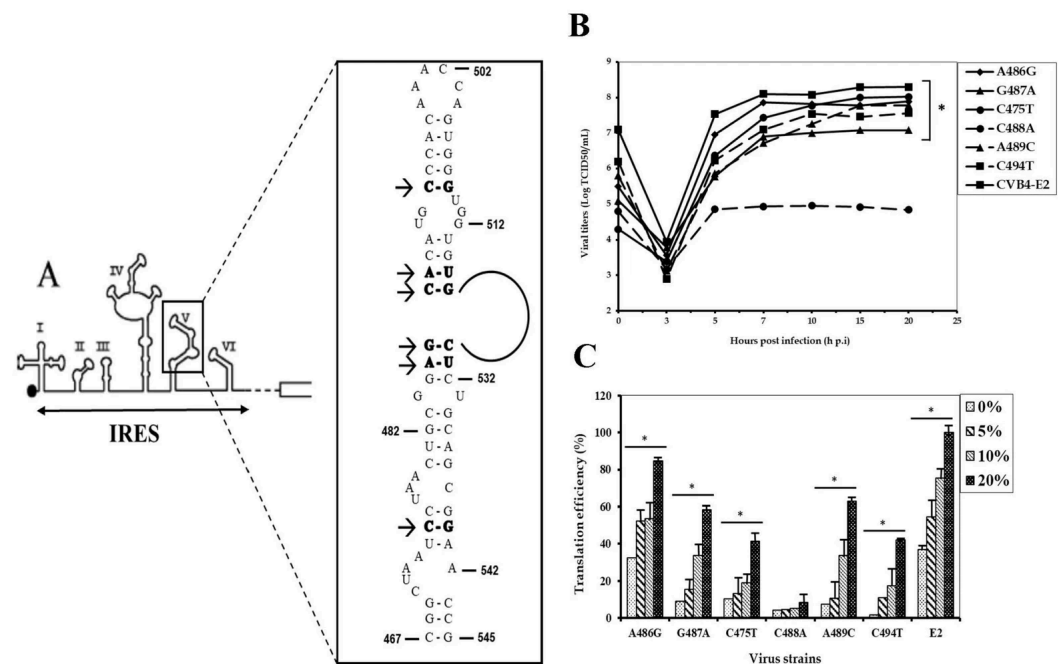


Figure 1. In vitro replicative capacities and translation efficiencies of generated CVB4E2 mutants. (A) Structure and nucleotide sequence of the IRES domain V of CVB4E2. The 6 introduced single mutations by PCR-based site-directed mutagenesis assay are indicated by arrows. (B) Evaluation of the in vitro replicative capacities of the generated mutants in comparison with the parental CVB4E2 strain using the one-step cycle assay. HeLa cells were infected with the pathogenic and generated mutant CVB4E2 strains at an M.O.I of 20 TCID₅₀/cell at 37 °C. Viral production was calculated in log TCID₅₀ titers at the indicated times post-infection (3, 5, 7, 10, 15 and 20 h p.i.) by Reed and Muench assay. (C) In vitro translation efficiencies of the generated mutants and the parental CVB4E2 RNAs at different percentages of S10 HeLa cell extracts. Densitometric quantifications of viral polyproteins are revealed on SDS-PAGE gel. The data shown are the mean $\pm$ SD from three replicated experiments ($n = 3$). The p value of the statistical analysis in each assay was marked, * $p < 0.05$ compared vs. control, Student's t test.

HeLa cells were used in this study to experimentally evaluate the replicative capacities of the generated mutants compared to the parental CVB4E2 pathogenic strain. This cell line is known to support productive enterovirus infections, especially coxsackievirus serotypes. All produced mutants seemed viable upon in vitro transfection of HeLa cells with the different generated RNA mutants. The titration results of virus mutants revealed a 3 Log drop in infectivity for the C488A mutant strain with a viral titer of 1.2×10^3 TCID₅₀, whereas the viral titers of the mutants A486G (5.5×10^6 TCID₅₀), G487A (1.8×10^5 TCID₅₀), C475T (5.3×10^5 TCID₅₀), A489C (8.8×10^5 TCID₅₀) and C494T (5×10^5 TCID₅₀) demonstrated a drop of just 1 Log compared to the infectivity of the CVB4E2 pathogenic strain with a viral titer of 7.1×10^6 TCID₅₀ (Table S1, Supplementary Material S).

The one-step growth cycle method was used to evaluate the viral productivity of the different produced mutants during their first viral cycle and to compare their growth properties in HeLa cell cultures with that of the parental CVB4E2 strain. We followed the replicative capacities of the generated mutant strains during the first 20 h post-infection (p.i.), using an M.O.I of 20 TCID₅₀/cell (i.e., 20 infectious viral particles that infect one HeLa cell). At different times post-infection, at 3, 5, 7, 10, 15 and 20 h p.i., supernatants from infected HeLa cells were clarified and stored at -80 °C. Viral titers were determined by the end point dilution of the Reed and Muench method. As demonstrated in Figure 1

B, the parental and mutant viruses exhibited logarithmic growth by 3 to 7 h p.i., and maximum virus production was achieved after 7 h of infection. Interestingly, viral supernatant from cells infected with the C488A mutant strain demonstrated a significantly decreasing titer with a difference of 3 log at the different times starting from 5 h p.i. compared with the parental CVB4E2 pathogenic strain. However, supernatants from cells infected with the other five mutants A486G, G487A, C475T, A489C and C494T revealed productive viral titers comparable to that of the parental CVB4E2 strain at the different times post-infection. All mutant strains showed approximately similar virus production at all times post-infection, with the exception of the C488A mutant. The results of the one-step growth cycle study of the different produced mutants suggest that the introduction of different single mutations in the IRES domain V of the CVB4E2 genome leads to different levels of replicative capacity of the mutants. Interestingly, the single mutation C488A reveals the most attenuation in replicative capacity. However, the mutants generated by the single mutations A486G, G487A, C475T, A489C and C494T were not severely disabled in HeLa cell cultures.

To determine whether the replicative phenotypes of the mutant strains observed in HeLa cell cultures were correlated with a reduced translation efficiency of the corresponding mutated RNAs, we conducted *in vitro* translation assays using an RRL system for all mutants and parental RNAs with the same starting concentration of RNA (10 µg/mL). Mutated and parental RNAs were assayed in a cell-free *in vitro* translation system alone or supplemented with increasing percentages of S10 HeLa cell extracts (0%, 5%, 10% and 20%). The translated viral polyprotein products of the different assays were analyzed on 15% polyacrylamide gels by SDS-PAGE and the autoradiograms were scanned and treated with NIH image software version 1.63 to obtain a quantitative analysis. The results of the *in vitro* translation assay revealed different levels of translation capacity between the different generated mutant RNAs and the parental RNAs. Interestingly, the RNA mutant C488A directed translation at a low efficiency in reactions supplemented with the different percentages of S10 HeLa cell extracts. Indeed, the RNA mutant C488A revealed a 91.66% increase in comparison with the parental CVB4E2 RNA level. However, the translation of the other five RNA mutants A486G, G487A, C475T, A489C and C494T revealed a moderate decrease in translation efficiency, evaluated successively at 15.44%, 41.55%, 58.49%, 37.24% and 57.87%. In addition, to determine the effects of RNA concentration on the translation efficiency, we conducted similar *in vitro* translation assays using the same system of RRL but with a constant concentration of HeLa extract S10 (20% of the final volume) and with increasing mutant and parental RNA concentrations (1.25, 2.5, 5 and 10 µg/mL). The results demonstrated that the mutation C488A provoked a similar reduction in translation efficiency, measured at 86.12% compared to the parental CVB4E2 RNA (Figure S2, Supplementary Materials). The results of the *in vitro* translation efficiency of the generated RNA mutants suggest that the introduction of different single mutations in the IRES domain V sequence of the CVB4E2 genome leads to a different levels of translation efficiency in the cell-free system with a notably dramatic decrease shown in the case of the single mutation C488A.

Taken together, our *in vitro* one-step growth cycle and translation efficiency results demonstrate that the notable and severely handicapped replication in HeLa cell cultures of the generated mutant C488A was correlated with a dramatic reduction in the translation of the corresponding mutant RNA, leading to a live-attenuated mutant strain. Interestingly, all generated mutants were always and continued to replicate in HeLa cell culture, encouraging us to evaluate the use of the most attenuated C488A mutant as a live-attenuated candidate vaccine in the Balb/c mice model.

3.2. The Live-Attenuated C488A Mutant Immunization Elicits Robust Levels of Neutralizing Antibodies and IFN- γ Cytokine Responses in Balb/c Mice

To determine whether the attenuated phenotype of the selected C488A CVB4E2 mutant strain could induce immune responses in an animal model, female Balb/c mice groups were orally prime-inoculated (G1) and prime-boosted (G2) by the C488A live-attenuated strain successively at days 0 and 21. Blood samples were collected from mice tails at different days post-prime-immunization: days 14, 21, 28, 35, 42 and 49. Specific neutralizing antibodies' anti-CVB4E2 titers and cytokine IFN- γ concentrations were assessed in sera collected from the inoculated mice groups G1 and G2, from the positive G5 mice group, and from the negative control G6 mice group as described in Section 2.

The results presented in Figure 2B demonstrate that the titers of specific neutralizing antibodies that are anti-CVB4E2 strain (in U/ μ L) of inoculated mice groups—except the naive control group—exhibited a significant increase after prime inoculation with the live-attenuated C488A strain. Interestingly, the level of antibodies in the sera of the prime-boosted mice group G2 exhibited a significant increase revealed from day 28 post-prime-immunization due to the boost regimen at day 21. The quantification results of the produced IFN- γ cytokine in mice group sera presented in Figure 2C clearly demonstrate that the IFN- γ concentrations (in pg/mL) of the prime-immunized mice group G1 increased significantly from day 14 post-prime-immunization. Interestingly, in parallel with specific neutralizing antibodies, the cytokine IFN- γ titers from the prime-boosted mice group G2 demonstrated a significant increase from day 28 post-prime-immunization in comparison with the prime-immunized mice group G1 and the naive control mice group G6.

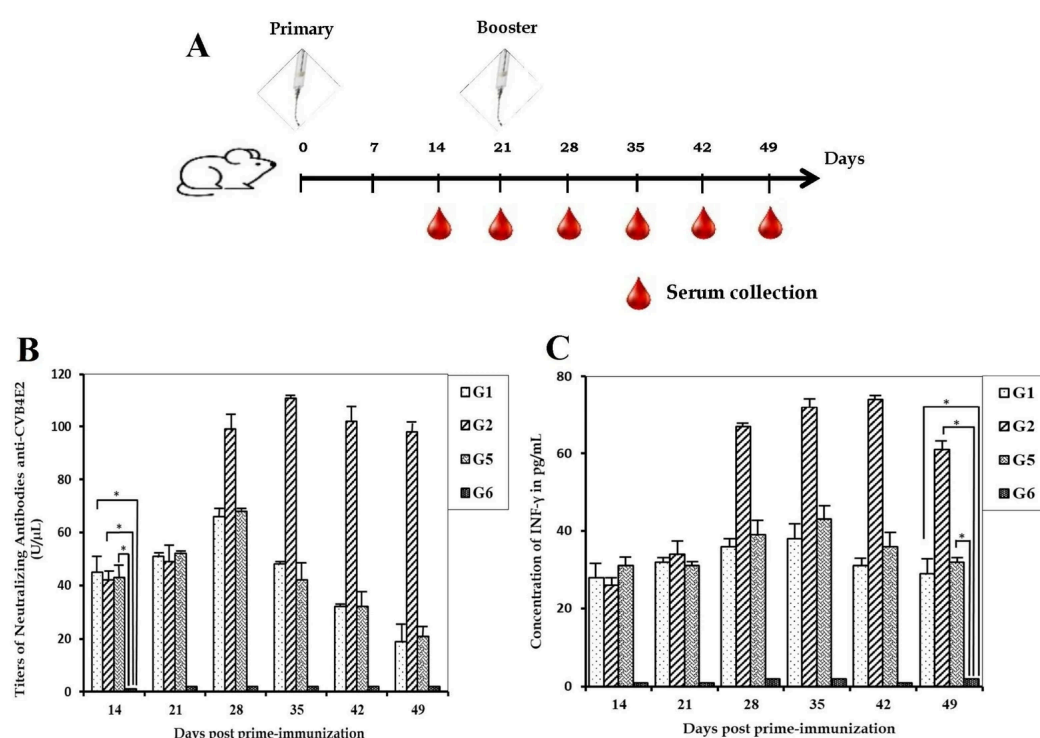


Figure 2. Specific neutralizing antibodies and cytokine IFN- γ production during the immune response induced by the live-attenuated C488A mutant in Balb/c mice. (A) Immunization schedule of mice groups (G1, G2, G5 and G6). G1 mice group were prime-immunized at day 0 with a dose of 10^5 TCID₅₀ of the C488A live-attenuated mutant vaccine candidate. G2 mice group were prime-boost-immunized at days 0 and 21 with the same dose of 10^5 TCID₅₀ of C488A live-attenuated mutant. Positive control mice G5 received a primary inoculation with a dose of 10^5 TCID₅₀ of the

CVB4E2 pathogenic strain. Naive control mice G6 were inoculated at days 0, 21 and 35 with PBS. Titers of specific neutralizing antibodies anti-CVB4E2 (**B**) and concentrations of the cytokine IFN- γ (**C**) produced in the sera of the different studied mice groups (G1, G2, G5 and G8) were measured at days 14, 21, 28, 35, 42 and 49 post-prime-immunization. The data shown are the mean $\pm$ SD from three replicated experiments ($n = 3$). * $p < 0.05$ compared vs. control mice group, Student's t test.

The results of the assessment of neutralizing antibodies and IFN- γ titers in sera of the different studied mice groups demonstrated that prime-immunization with the live-attenuated C488A mutant strain elicits an effective and potent humoral and cellular immune responses in the Balb/c mice model. Immune responses are enhanced when a prime-boost regimen is adopted in the Balb/c mice model.

3.3. Vaccination with Live-Attenuated C488A Mutant Protects Balb/c Mice from CVB4E2 Lethal Viral Challenge

To determine whether the live-attenuated C488A strain protects Balb/c mice or not from lethal virus challenge with the CVB4E2 pathogenic strain, we evaluated the immunogenicity elicited by both prime-immunization and prime-boost-immunization with the vaccine candidate. All Balb/c mice groups were included in this evaluation study, and the adopted immunization strategy is shown in Figure 3A. The mice groups G1, G2, G3 and G4 were prime-immunized at day 0 with a dose of 10^5 TCID₅₀ of the vaccine candidate. G2 mice received a boost-immunization at day 21 with the same dose. G3 mice were challenged with the pathogenic CVB4E2 strain on day 35. G4 mice received a boost-immunization at day 21, then challenge with the pathogenic CVB4E2 strain at day 35. G5 and G6 mice served successively as a positive and naive control and were subjected to the same immunization schedule as described below.

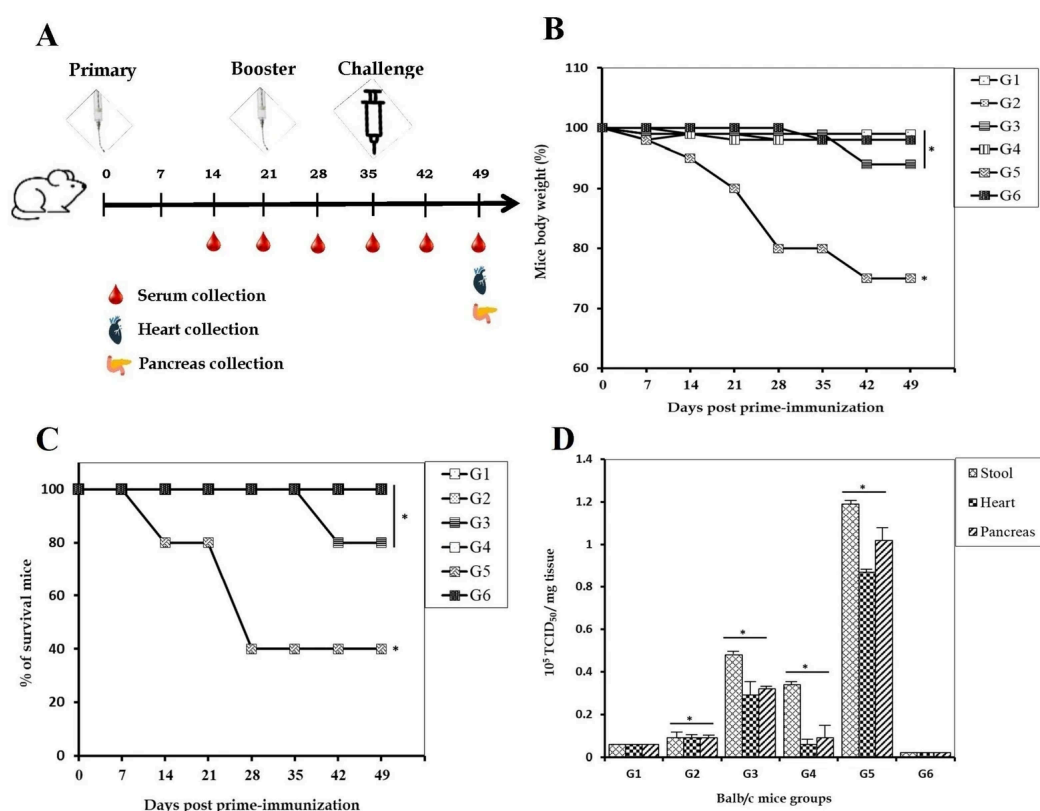


Figure 3. Balb/c mice's protection against pathogenic CVB4E2 lethal challenges by immunization with the live-attenuated C488A mutant. (A) Schedule of mice immunization and challenge. Balb/c

mice groups G1 and G3 received prime-immunization at day 0 with a dose of 10^5 TCID₅₀ of the live-attenuated C488A strain. Mice groups G2 and G4 were prime-boost-immunized at days 0 and 21 with the same dose of the vaccine candidate. Mice groups G3 and G4 were then challenged at day 35 with a dose of 10^5 TCID₅₀ of CVB4E2 pathogenic strain. G5 and G6 mice represent successively the positive inoculated and the naive control non-inoculated mice. **(B)** The percentages of mice body weight changes. **(C)** The percentages of survival in mice at different times post-prime-immunization with the vaccine candidate. **(D)** The viral yields at day 49 post-prime-immunization in mice heart, pancreas and stool. Viral titers of parental and CVB4E2 mutant strains were determined using HeLa cell cultures in TCID₅₀/mg of tissues. The data shown are the mean $\pm$ SD from three replicated experiments ($n = 3$). * $p < 0.05$ compared vs. control mice group, Student's t test.

All Balb/c mice groups were monitored daily throughout the study for disease clinical signs, body weight changes and mice mortality. The results of the mice body weight study presented in Figure 3B demonstrate that the average body weight of the positive control mice group G5 inoculated with the CVB4E2 pathogenic strain decreased substantially at day 28 post-inoculation. Mice of this group lost 25% of their weight at days 42 and 49 due to the virus pathogenesis, making mice feel sick and thus eat less. Mice of the group G3 receiving just a prime-immunization at day 0 with the live-attenuated strain, then challenged at day 35 with the pathogenic CVB4E2 strain, showed only a 5% decrease in body weight. No significant loss of body weight was observed for the other mice groups (G1, G2 and G4) prime-immunized or prime-boost-immunized with the vaccine candidate. The naive control G6 group mice maintained their weights throughout the study. The results of mice group mortality presented in Figure 3C are in accordance and confirmed our results of the body weight loss among mice groups. The rapid evolution of disease and the loss of body weight of the mice groups caused the death of some mice. The results demonstrated that the percentage of mice survival was variable among the different mice groups. Interestingly, the mice group G4 prime-boost-immunized with the live-attenuated strain at days 0 and 21, then challenged with a lethal dose of the CVB4E2 pathogenic strain at day 35, demonstrated a protective efficacy of 100%. The mice group G3 just prime-immunized at day 0 with the vaccine candidate, then challenged at day 35 with a lethal dose of the CVB4E2 pathogenic strain, demonstrated a protective efficacy of only 80%. The mice groups G1 and G2 successively prime- and prime-boost-immunized with the vaccine candidate and the control naive mice group G6 showed no significant mortality throughout the study. However, the positive mice group G5 inoculated with a lethal dose of the CVB4E2 pathogenic strain revealed the most elevated percentage of mortality at day 28 with a protective efficacy of only 40%.

To correlate the percentages of mice mortality and body weight loss with the pathogenic viral severity of infection in mice, we assessed the viral load in the heart, pancreas and stool of the different mice groups at the end of the study (day 49 post-first-immunization). CVB4E2 viral titers were calculated in TCID₅₀ by a given weight of organ tissues (TCID₅₀/mg of tissues) as described in Section 2, using the Reed and Muench assay [22]. The results of viral loads in mice tissues at day 49 are presented in Figure 3D, demonstrating that the viral yields in hearts, pancreas and stool of the prime-immunized mice group G3 and prime-boost-immunized mice group G4 with the vaccine candidate then challenged with the CVB4E2 pathogenic strain at day 35 were significantly lower than the viral loads of the positive non-immunized mice group G5. Interestingly, the viral yields at day 49 revealed a substantial reduction of 40%, 36% and 31% successively in the stool, heart and pancreas of the mice group G4 that were prime-boost-immunized. As expected, the tissue viral load of the naive control PBS mice group G6 was negative. The results of mice tissue viral titrations demonstrated that prime-boost-immunization with the live-attenuated vaccine candidate at days 0 and 21 could enhance the protective im-

immune response and protect mice from the viral lethal challenge.

4. Discussion

Vaccines are commonly used to reduce the incidence of infectious diseases. One of the most successful technologies for viral vaccines is to immunize animals or humans with a weakened or live-attenuated strain of the virus. Due to limited replication after immunization, the live-attenuated strain does not cause disease. However, the limited viral replication is sufficient to express the full repertoire of viral antigens and generate potent and long-lasting immune responses to the wild-type virus. These generated live-attenuated vaccines are among the most successful vaccines used in public health. Ten of the sixteen viral vaccines approved for sale in the USA are live-attenuated vaccines. Enteroviruses include an IRES within the 5' UTR that is required for efficient cap-independent translation of the viral coding region. Mutations within the IRES can seriously impair or abrogate translation. The IRES domain V sequence among the genome of enteroviruses is a critical regulator of enterovirus translation and replication, as demonstrated by its pivotal role in the attenuation of Sabin poliovirus vaccines [16]. It has been demonstrated in some viral RNAs that the local secondary structure is highly involved in modulating IRES function [25]. Attenuated Sabin strains of poliovirus exhibit a decreased efficiency of translation in neural cells compared to wild-type neurovirulent strains. This difference in translational efficiency was found to correspond to their major attenuating mutations within the IRES of poliovirus wild-type strains [26]. The present study provides compelling evidence that a single nucleotide substitution in the IRES domain V of the CVB4E2 pathogenic strain significantly impairs viral replication and abolishes translation *in vitro*, offering a promising approach for developing a live-attenuated CVB4 vaccine. Our results of the *in vitro* replicative capacities of the six generated mutants demonstrated that the C488A mutant caused a notable reduction in viral titer (3-fold lower than the CVB4E2 pathogenic strain) in HeLa cell cultures. In contrast, the other five mutants A486G, G487A, C475T, A489C and C494T exhibited only marginal reductions in replication (1 to 1.5-fold lower titers). The results of the *in vitro* translation efficiencies of the different generated mutants confirmed the attenuated phenotype of the mutant C488A. The results demonstrated that the RNA mutant C488A revealed a 91.66% increase in comparison with the parental CVB4E2 RNA and the other five RNA mutants which revealed moderate decrease in translation efficiency. Together, the results of *in vitro* replicative capacities and translational efficiencies suggest that the mutation C488A introduced into domain V of the CVB4E2 IRES sequence could disrupt essential molecular interactions critical for viral fitness, likely involving RNA secondary structures or interactions with host translation factors, thereby limiting viral protein synthesis and replication, and thus abolishing the initiation of translation for the C488A mutant as observed in previous studies on related enteroviruses like CVB3 [27–30]. However, biochemical assays such as RNA pull-down or electrophoretic mobility shift assays could elucidate the specific host factors affected by the C488A mutation. Structural analyses, such as cryo-electron microscopy or NMR spectroscopy, could provide insights into how this mutation alters IRES conformation or protein binding [10].

The molecular attenuation of the generated CVB4E2 C488A mutant, evidenced by the significant decrease in replicative capacity and translation efficiency, is particularly promising for live-attenuated vaccine candidate development by evaluating its immunogenicity in a mice model. Preliminary data from related and similar studies suggested that attenuated enteroviruses can elicit immune responses, including neutralizing antibodies and T-cell activation [11,17]. Using different strategies of immunization and lethal challenge in the Balb/c mice model, we evaluated the immune humoral and cellular re-

sponses elicited by the live-attenuated C488A mutant as a vaccine candidate. The mode of natural contamination with CVB4 strains is mainly the fecal–oral route (digestive tract); thus, we have chosen the oral route in the present study for mice immunization and challenge. Our results of per os (oral inoculation) prime-immunization with the live-attenuated C488A vaccine candidate found that it could effectively induce the synthesis of specific neutralizing antibodies against the CVB4E2 pathogenic strain and the production of IFN- γ . Interestingly, the titers of both specific neutralizing antibodies and the cytokine IFN- γ increased significantly after boost-immunization at day 21 post-prime-immunization, thus enhancing both the humoral and cellular immune responses. On the other hand, we evaluated the protection of immunized Balb/c mice against CVB4E2 pathogenic strain lethal challenge. Our results of mice body weight changes and percentage of survival in mice demonstrated that the body weight loss among positive control mice inoculated with the CVB4E2 pathogenic strain was significant. However, no body weight change was observed in prime-boost-immunized or challenged mice. In addition, our results on the mice survival rates revealed that the prime-boost-immunized and challenged mice groups demonstrated a protective efficacy of 100%. However, a significant death rate was observed among mice inoculated with the CVB4E2 pathogenic strain and non-immunized with the vaccine candidate, suggesting that prime-boost-immunization with the live-attenuated C488A mutant could protect Balb/c mice against lethal challenge with the CVB4E2 pathogenic strain. This protection of mice by immunization with the vaccine candidate was confirmed by the results of viral load in mice heart, pancreas and stool at day 49, revealing a substantial reduction in viral yields in the tissues of mice prime-boost-immunized with the vaccine candidate. In addition, genomic sequencing of isolated virus from mice organs and stools confirmed the stability of the introduced C488A mutation and did not reveal any reversion events in the IRES sequences.

The broader public health implications of this research are significant. CVB4 is a major cause of viral pancreatitis and myocarditis, and its association with T1D underscores the need for preventive strategies. A live-attenuated CVB4 vaccine could reduce the burden of these diseases, particularly in high-risk populations. However, our study limitations include its focus on a single cell line (HeLa cell) and animal model (Balb/c mice), which may not fully recapitulate human infection dynamics. HeLa cells, while sensible and permissive to CVB4, do not represent the diverse cell types targeted by the virus in vivo, such as pancreatic β -cells or cardiomyocytes [15]. Balb/c mice, while a robust model for CVB4-induced pathology, may not fully reflect human immune responses [31]. Future studies should address these limitations by testing the live-attenuated C488A mutant in additional cell lines (e.g., pancreatic primary cell lines) and animal models (e.g., NOD mice for T1D studies) and conducting comprehensive genomic analyzes to rule out secondary mutations.

5. Conclusions

Preventing enterovirus infections and the diseases they cause with vaccines is possible. Polio vaccines have already made it possible to eradicate poliomyelitis and to eliminate wild-type poliovirus strains from most countries. The protection conferred by these vaccines is based on neutralizing antibodies at the mucosal and systemic level (live-attenuated vaccine, administered orally). The effectiveness of these vaccines has encouraged the development of other vaccines to prevent enterovirus infections. In this study, we highlighted the efficacy of the designed live-attenuated C488A mutant to induce an immune response and protect Balb/c mice against lethal challenge, making it a potential vaccine candidate. Further investigations regarding the expression of CD4+ and CD8+ T cells could clarify the mechanism of the immune response induced by immunization

with this vaccine candidate.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/doi/s1>, Figure S1: Agarose gel 1% of the restriction digestion of pRib-CVB4E2/T7 mutant clones; Table S1: Viral titers of CVB4E2 parental and generated mutant strains in TCID₅₀ calculated by Reed and Muench method.

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Informed Consent Statement: Not applicable

Data Availability Statement: The original contributions presented in this study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author(s).

Conflicts of Interest: The authors declare no conflicts of interest.

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