SOLVx therapeutics vaccine – Activate T-cell immunity using broad surveillance epitope strategy against mutant strains SARS-COV2

Md Shamsuddin Sultan Khan [*], John A. Catanzaro

Neo7Biosciene Inc, 16155 SW 117 Ave, Suite B15, Miami, FL 33177, USA

ABSTRACT

Highly mutable Coronavirus-19 continuously reconstructs its genome and renders prophylactic vaccines ineffective. The objective of the present study was to demonstrate the anti-viral efficacy and safety of the SOLVx therapeutics vaccine. The peptides were designed with Neo7Logix R&D and synthesized with Genescript GLP laboratory with 95 % purity. BALB/C mice were used to develop the HCoV-229E mutant coronavirus model and viral mRNA confirmation in the lung tissue was assessed with qPCR. Mice were euthanized and effects of treatment on various parameters (Viral mRNA in lungs, cytokine levels, PBMC differentiation, hematological and biochemical) were assessed with respective biological samples. Immuno-typing analysis of PBMCs by flowcytometry showed marked increase in T cell subsets, % of B cells and NK cell population in mice treated with SOLVx (Series 1) in a dose dependent manner. Serum immunoglobulin G, and M levels were increased significantly (P < 0.001). In the peptide treatment groups, there was a dose dependent statistically significant decrease in IL-6, IL-10 and TNF-α levels (P < 0.001). IFN-γ was elevated in treatment group significantly (P < 0.001). In conclusion, the qPCR results suggested that the SOLVx vaccine (Series 1) reduced the SARS-COV2 virus infectivity in a dose dependent manner. The humoral, cellular and functional activity of the SOLVx showed that it worked through multi-mechanistic targeting the virus evolution, offering immune response, defense and eradication of the SARS-COV2 virus.

1. Introduction

The recent Global pandemic of novel Coronavirus disease 2019 (COVID-19) is an emerging infectious disease, with its reported origin in China and later spread worldwide [1]. As of 3 January 2020, a total of 44 patients with pneumonia had been reported to the WHO by the national authorities in China. An average of around 910 people per day has died from COVID-19 in the U.S.A. [2]. The resurgence of COVID-19 has also been reported in places where lockdowns have already worked. Coronavirus-19 is a highly mutated RNA virus that has "jumped" from animals to humans and transmitted person-to-person. Its high mutation rate continuously reconstructs its genome in a host, which renders prophylactics ineffective. The disease is spreading at an alarming rate due to the unavailability of any effective anti-viral or immunomodulatory therapy. Though Coronavirus-2019 is rapidly mutable, it uses similar ways to infect humans, the therapeutics vaccine targeting the cellular processes might inhibit the mutated strain of the virus. In this scenario, therapeutics vaccine (i.e. small peptide construct with target inhibitory and immune modulation mechanisms) can serve as potential treatment option for SERS-COV2. It has been shown that peptides act as modulators in viral diseases [3,4].

For mitigation or gain control over COVID-19, therapeutic vaccines seem to be the only option preferred over therapeutic drugs. In the case of COVID-19, prophylactic vaccines could be ineffective due to their complicated nature [5,6]. COVID-19 is a fatal disease mainly because of the cytokine storm. Therefore, there is an urgent requirement to discover a therapeutic vaccine to have curative as well as prophylactic effects, to activate an immune response, to inhibit the "cytokine storm" and to reduce the inflammatory damage associated with this disease [7].

Several RNA-based anti-COVID-19 vaccine candidates have entered the clinical trial [8]. However, their efficacy and potency are still questionable, hence, studies must be evaluated and validated more stringently [9]. As evident from published literature vaccines have high failure rates [10,11], as an alternative, we have studied novel, custom-designed synthetic peptides, which are a solution-based therapeutic vaccine (SOLVx SARS-CoV2) against COVID-19.

The concept of therapeutic vaccine within all the immunizations is the ability of the vaccine to initiate an immune response in a faster mode

and inhibit the target cell activation than the pathogen itself. Conventional prophylactic vaccines are costly, allergenic, time consuming and require in vitro culture of pathogenic viruses leading to serious concern of safety [12]. Thus, the need for safe and efficacious vaccines is highly recommended. Peptide-based vaccines do not need in vitro culture making them biologically safe, and their selectivity allows accurate activation of immune responses [13,14]. The core mechanism of the therapeutic vaccines is built on the chemical method to synthesize the recognized B-cell and T-cell epitopes that are immunodominant and can induce specific immune responses. B-cell epitope of a target molecule can be linked with a T-cell epitope to make it immunogenic. The T-cell epitopes are short peptide fragments (8–20 amino acids), whereas the B-cell epitopes can be proteins [15–18]. Therefore, in this study, we aimed to design a highly safe and effective therapeutic vaccine constructed with the SARS-COV2 genome and proteins information using PBIMA/SMART pipeline that we hypothesized to treat the mutant or other strains with broad surveillance potency [19–21] and to demonstrate this emerging infection at inhibition of the entry points,

preventing endocytosis, viral unpacking and replication of its hyper-mutated stage and reprogram the immune system for neutralizing and/or eradicating the virus in any stage of infections.

2. Results

Peptides were designed based on our hypothesis described in the Introduction. The peptides were selected according to structural information of SARS-COV2 containing T and B cell epitopes with potential efficacy. Peptides for such vaccine are selected from the most conserved parts of the viral proteins which have a high-affinity to most HLA-types in the population. These peptide-based vaccine agents will avoid the Antibody-Dependent Enhancement (ADE) problem, because short sequences (9-mer) will only induce CD8 + /CD4 + response, and without autoantibodies generated by B-cell responses. R analysis of lung tissues was carried out before the initiation of treatment, Fig. 1 represents the viral mRNA confirmation in the lung tissues post 72hrs of infection (hpi) with a mean Cq value of 24.77 in comparison to the NTC. After the

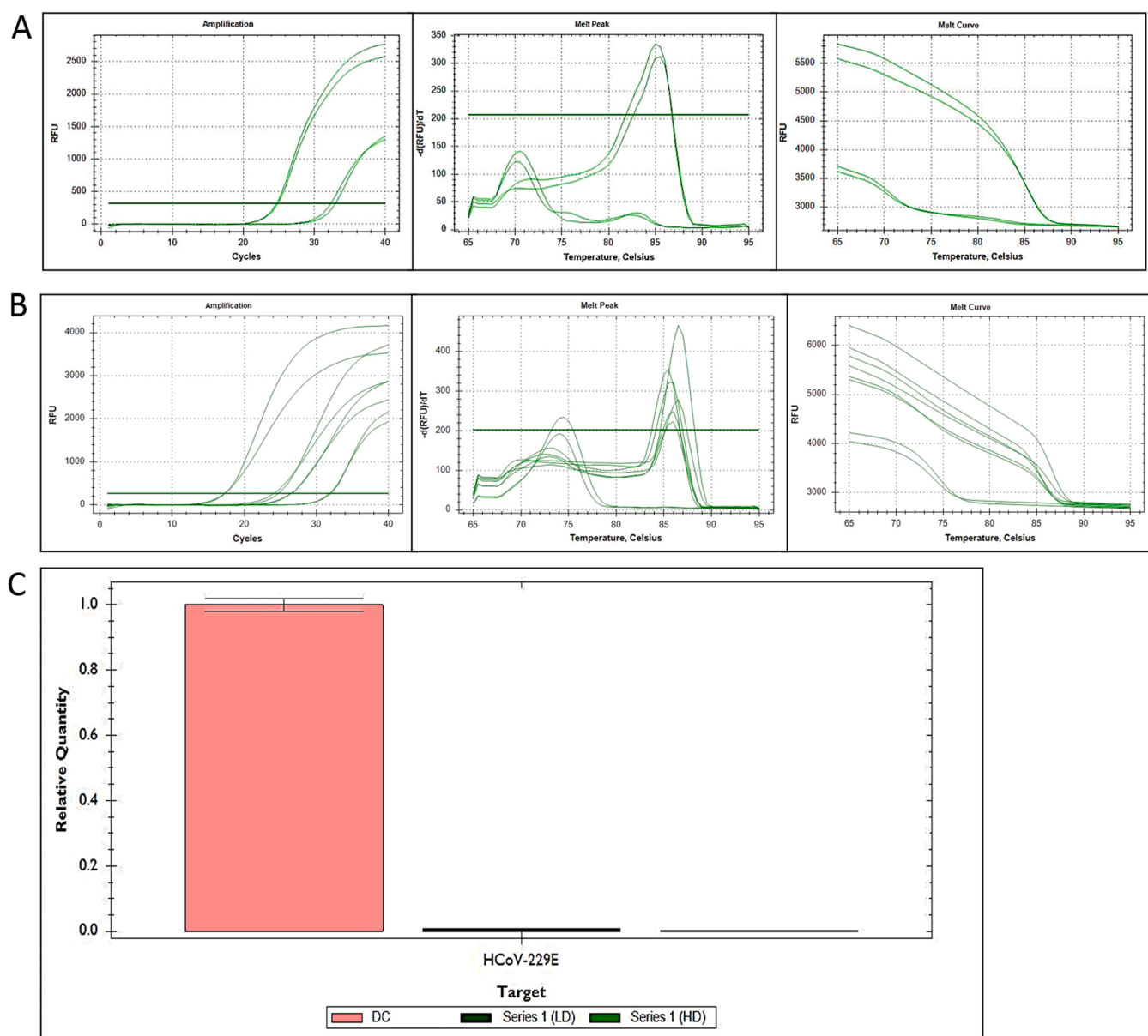**Fig. 1.** A–B) Cq (quantification cycle) determination of viral load (HCoV-229E) in lung tissues before and after treatment. Viral loads were compared by using the Cq (quantification cycle) value; with low Cq values resulting from large amounts of the amplified product indicating higher viral loads. (DC- Disease control); C) Relative expression in HCoV-229E viral load in Lung tissue at the end of the study by qRT-PCR.

confirmation of the infection in the lungs, animals were administered with test samples as per the predetermined treatment schedule. At the end of the study, mice were euthanized and effects of treatment on various parameters (Viral mRNA in lungs, cytokine levels, PBMC differentiation, hematological and biochemical) were assessed with respective biological samples.

At the end of the study, lung tissues from the mice from treatment groups were collected and processed for determination of HCoV-229E viral mRNA using qPCR. relative quantity of the viral mRNA was assessed in comparison with disease control with no treatment. The results suggest that the SOLVx peptide for HCoV-229E has shown dose dependent reduction in relative virus expression (Fig. 1). The qPCR analysis for HCoV-229E expression in lung tissues at the end of the study shows that the relative expression of HCoV at lower dose is 0.007 and 0.002 at higher dose compared to disease control (Fig. 1). The mice treated at 20 µg/animal has shown relative quantity of 0.007 with a mean Cq of 24.36 whereas, mice treated with 50 µg/animal has shown 0.002 with a mean Cq of 26.44 compared to disease control, normalized to 1 with a mean Cq of 17.24. overall, the results suggest the reduced viral mRNA in the lungs at the end of the study (Fig. 1). Overall, the qPCR results suggest that the test peptide Series 1 has reduced the virus infectivity in a dose dependent manner.

Immuno-typing analysis of PBMCs by flowcytometry shows marked increase in T cell subsets (Fig. 2). It was observed that the % of B cells were reduced when compared to un-infected mouse, 21.32 ± 0.94 and infected mouse with 4.96 ± 0.82 % cells. However, the analysis showed

the there is a distinct increase in % B cells and the data shows that there has been an increase in NK cell population as well in mice treated with SOLVx vaccine (Series 1) in a dose dependent manner (Fig. 2).

Whole blood was processed for direct immunophenotyping using a cocktail of respective fluorotagged antibodies (details provided in experimental procedures). The results suggests that the test sample at 20 µg/animal and 50 µg/animal has shown the increase in Th1 cells to 18.73 ± 1.83 % and 25 ± 1.41 % respectively compared to disease control (15.54 ± 0.41 %) (Fig. 2). However, Th2 cell population is found to be relatively increased in the mice treated with test sample at 50 µg/animal compared to both disease control and mice treated at 20 µg/animal with 14.26 ± 0.1 % and 17.69 ± 0.98 % cells. Th17 is found to be increased in both the treatment groups with 16.15 ± 1.49 % cells and 27.46 ± 3.06 % cells compared to disease control with 13.11 ± 1.37 % cells. It is observed that% of Treg cells were decreased (0.53 ± 0.07 % cells) in mice treated at low dose compared to disease control (1.01 ± 0.13 % cells). However, marked increase (1.41 ± 0.07 %) of Treg cells were observed in mice treated with high dose. B cell population was observed to be significantly increased in both the treatment groups with 12.16 ± 0.65 % and 20.92 ± 0.55 %. The mice in the disease control group has shown significantly reduced B cell population (4.96 ± 0.82 %) compared to mice with no infection (21.32 ± 0.94%). NK (Natural killer) cell population was observed to be significantly increased in the treatment groups with 8.55 ± 0.46 % and 13.75 ± 0.98 % in low dose and high dose treatment groups respectively compared to both normal and disease control groups with 1.66 ± 0.2 % and 2.56 ± 0.06 % cells
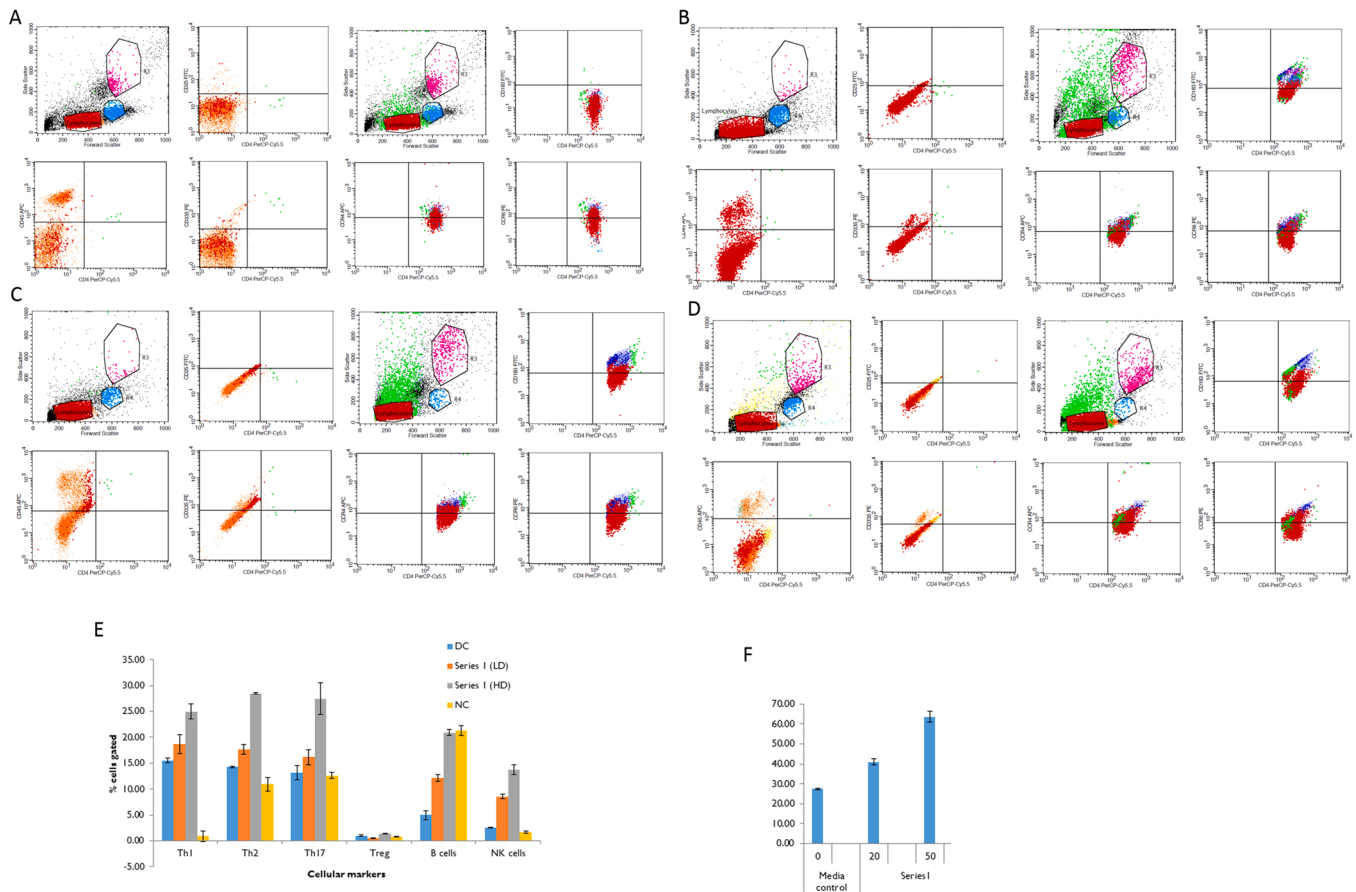
Fig. 2. SOLVx vaccine-induced immune response in animal models evaluating immune markers of SARS-COV2 outcomes. The present study assessed functional immune responses (e.g., neutralizing antibody) versus total antibody responses and Th1/Th2 balance in animals vaccinated with clinically relevant doses of the SOLVx vaccine. The PBMCs analysis of HCoV-229E infected mice was tested by Flow cytometry. A) Normal control group with no treatment or virus challenge; B) Disease control HCoV-229E model; C) SOLVx Low dose, 20 µg (series I-LD) group with HCoV-229E model; D) SOLVx High dose, 50 µg (series I-HD) group with HCoV-229E model; E) percent cell gated of cellular markers in PBMCs from mice infected with HCoV-229E virus; F) The functional activity of immune responses was evaluated in vitro in neutralization assays using Human PBMC. In-vitro immunogenicity (IFN-G) of therapeutic vaccine with 20 µg and 50 µg in Human PBMCs.

respectively (Fig. 2).

Immunoglobulins and cytokine levels were determined in serum from all the test groups and the results suggest that serum IgG and IgM levels were marginally elevated in the HCoV disease control group (Fig. 3). In the peptide treatment groups, at lower dose there was no statistically significant difference, however at higher dose there was a significant increase in IgG levels ($P < 0.001$) and there was a dose dependent statistically significant increase in IgM levels of 217.11 ($P < 0.05$) and 235.77 μg/ml ($P < 0.001$) at low and high dose respectively. Interleukin 6 (IL-6) levels were significantly elevated in the HCoV disease control group indicating a cytokine storm. In the peptide treatment groups, there was a dose dependent statistically significant decrease in IL-6 levels to 65.56($P < 0.001$) and 31.31 μg/ml ($P < 0.001$) at low and high dose respectively and TNF-alpha (TNF-α) levels were significantly elevated from 7.33 pg/ml in normal mice to 55.88 pg/ml in the HCOV disease control group indicating a cytokine storm. In the peptide treatment groups, there was a dose dependent statistically significant decrease in TNF-α levels to 31.27 pg/ml ($P < 0.001$) at high peptide dose treatment group. It was observed that there was a dose dependent statistically significant increase in IFN-γ levels to 167.20 ($P < 0.001$) and 185.08 μg/ml ($P < 0.001$) was observed at low and

high dose treatment groups respectively. In the peptide treatment groups, there was a dose dependent statistically significant decrease in IL-10 levels to 34.93 ($P < 0.005$) and 24.12 μg/ml ($P < 0.001$) at low and high dose respectively.

Complete blood count of study group animals showed the increase in WBCs (19.50 ± 0.56 10^3/cumm), Neutrophils (20.92 ± 0.55 %) and Lymphocytes (78.15 ± 1.56 %) in disease control group compared to normal and treatment groups (Fig. 4-D). Mice treated at 20 and 50 μg/ animal has shown dose dependent decrease with 13.17 ± 0.60 10^3/ cumm, 19.92 ± 0.49 %, 60.95 ± 1.51 % and 11.83 ± 0.95 10^3/cumm, 13.68 ± 0.72 % and 57.30 ± 1.41 % respectively. No observable difference was observed in Hb%, Platelet count and RBC count w.r.t treatment and disease control groups.

Clinical biochemistry analysis was carried out to evaluate the effects of test sample on the liver function and found that the disease control animals had relatively higher levels of BUN at 21.83 ± 1.59 mg/dl, 155.42 ± 0.97 IU/L SGOT and SGPT levels were observed to be 60.32 ± 0.78 IU/L compared to normal control animals (Fig. 4-E). No significant difference was observed in treatment groups compared to disease control group except SGPT levels were observed to be significantly decreased ($p < 0.001$) in mice treated with 50 μg/animal.

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
DC_HCOV vs NC C	5.960	4.730	Yes	***	2.713 to 9.207
DC_HCOV vs S1L L	0.2600	0.2527	No	ns	-2.391 to 2.911
DC_HCOV vs S1H H	-4.820	4.685	Yes	***	-7.471 to -2.169

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
DC_HCOV vs NC C	14.84	3.613	Yes	**	4.256 to 25.42
DC_HCOV vs S1L L	-10.34	3.083	Yes	*	-18.98 to -1.698
DC_HCOV vs S1H H	-29.00	8.648	Yes	***	-37.64 to -20.36

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
DC_HCOV vs NC C	117.8	73.83	Yes	***	113.7 to 121.9
DC_HCOV vs S1L L	80.91	62.09	Yes	***	77.55 to 84.27
DC_HCOV vs S1H H	115.2	88.38	Yes	***	111.8 to 118.5

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
DC_HCOV vs NC C	24.38	3.221	Yes	*	4.876 to 43.88
DC_HCOV vs S1L L	-58.82	9.518	Yes	***	-74.75 to -42.89
DC_HCOV vs S1H H	-76.62	12.40	Yes	***	-92.55 to -60.69

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
DC_HCOV vs NC C	16.09	10.69	Yes	***	12.21 to 19.97
DC_HCOV vs S1L L	5.070	4.125	Yes	**	1.902 to 8.238
DC_HCOV vs S1H H	15.88	12.92	Yes	***	12.71 to 19.05

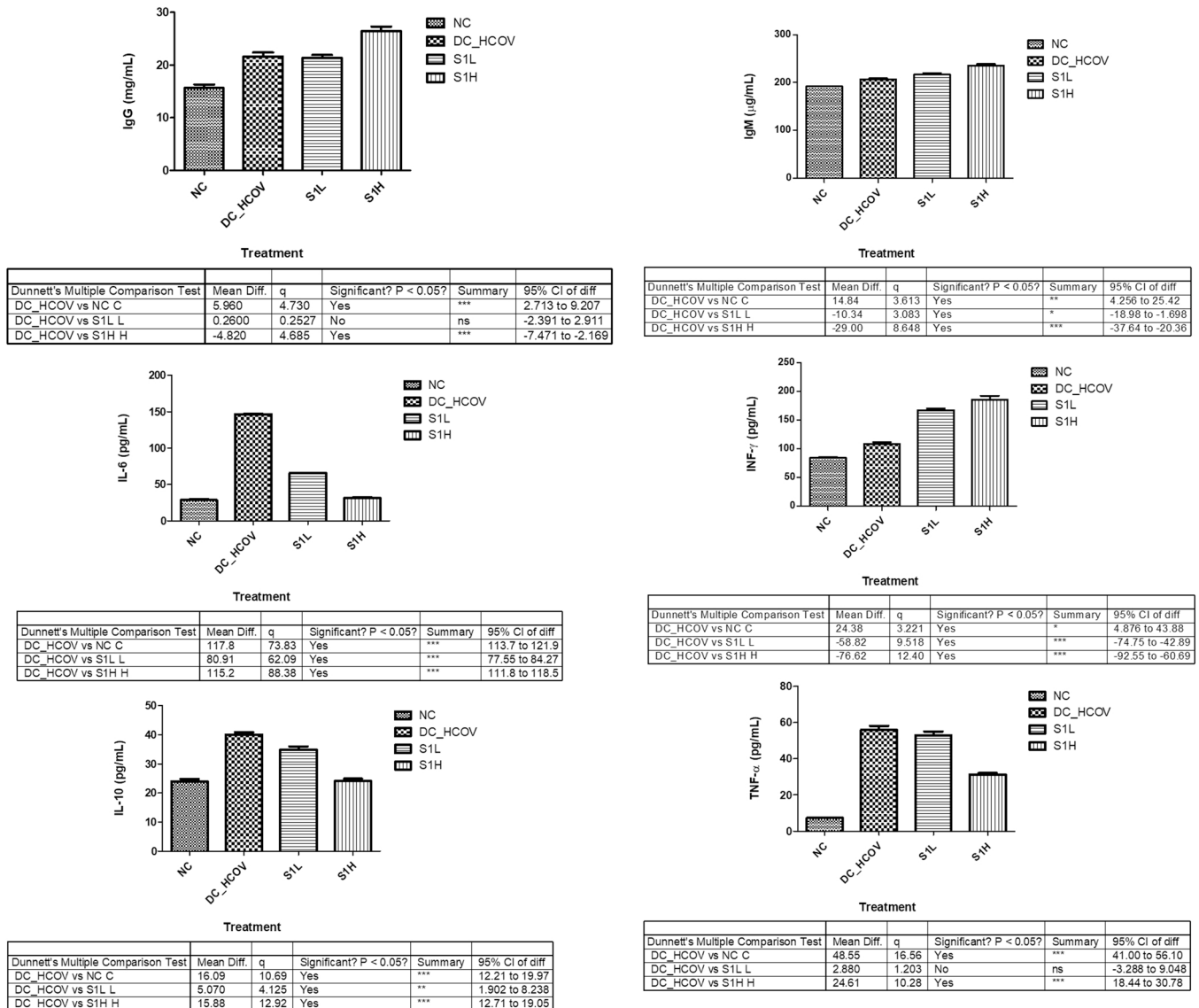| Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
DC_HCOV vs NC C	48.55	16.56	Yes	***	41.00 to 56.10
DC_HCOV vs S1L L	2.880	1.203	No	ns	-3.288 to 9.048
DC_HCOV vs S1H H	24.61	10.28	Yes	***	18.44 to 30.78

Fig. 3. ELISA assay was conducted to characterize the humoral response of SOLVx vaccine. Analysis of serum immunoglobulins and cytokines in HCOV treatment groups.
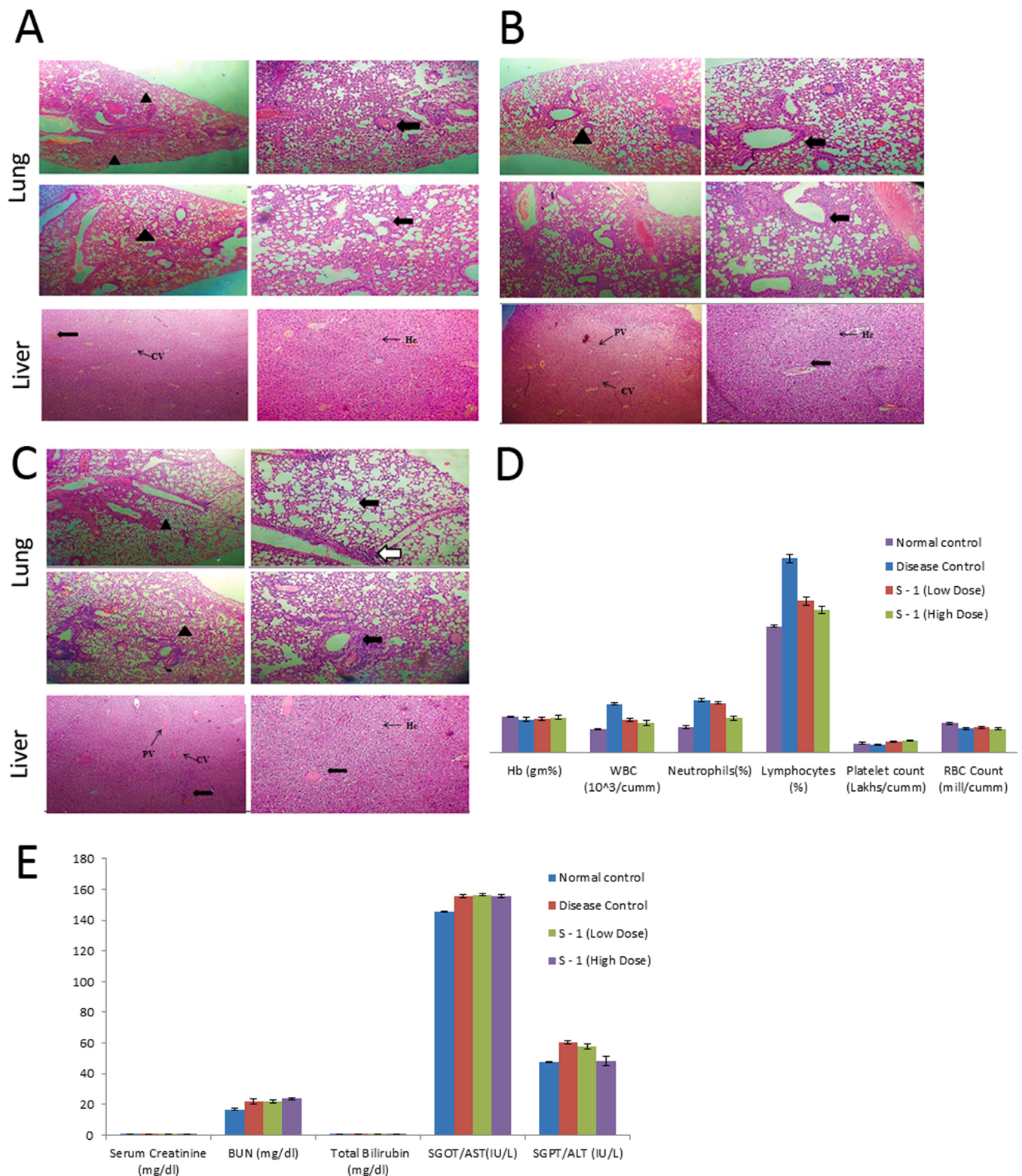
Fig. 4. Lung histopathology (e.g. evaluation of eosinophilic lung infiltration) associated with SARS-COV2 and Biochemical parameters to demonstrate the safety of SOLVx. A) Disease control Lung: Focal inflammation in lung observed (Arrow head) and Infiltration of inflammatory cells in alveolar septa (Black Arrow); Severe congestion observed (Arrow head) and Lung deformation with enlargement of alveolar duct and alveolar collapse (Black arrow); Liver: Mild congestion (Bold Arrow) - CV: Central Vein, PV: Portal Vein, Hc: Hepatocytes; B) SOLVx Low dose (series 1-LD) Lung: Severe congestion observed (Arrow head), Infiltration of lymphocytes (Black Arrow), Lung deformation with enlargement of alveolar duct and alveolar collapse with infiltration of inflammatory cells (Black arrow), Liver: Haemorrhages and congestion observed (Black Arrow); C) SOLVx High Dose, Lung: Mild congestion (Arrow head), Alveolar thickening (Black Arrow), Infiltration of inflammatory cells (bold arrow), Congestion observed (arrow head); Liver: Mild haemorrhages and congestion observed (Black Arrow), Migration of inflammatory cells in inter alveolar septa (White arrow); D) Mean complete blood count in HCOV treatment groups; E) Serum biochemical analysis in HCOV treatment groups.

The H&E staining of Lungs and Liver was carried out in Histological sections of mice treated with or without SOLVx vaccine (Series 1) at low dose (20 μg/mice) and high dose (50 μg/ml) (Fig. 4 A–C). The gross histological analysis of lung tissue from Disease control revealed that there is a severe congestion and lung deformation observed along with enlargement of alveolar duct and alveolar collapse. The tissue sections also reveal the infiltration of inflammatory cells in alveolar septa and focal inflammation in lungs. The Liver sections shows the mild congestion and the tissue section shows no other marked pathology.

It is observed that, in the treatment group of SOLVx vaccine (series 1) at 20 μg/ml, there is a severe congestion and lung deformation (Fig. 4 A–C). Lung histological sections of animals from low dose group suggests the increased thickness of the alveolar septa and alveolar collapse is observed compared to the histological sections in the treatment group of series 1 with high dose (50 μg/mice), in which there is a marginally lesser wall thickening is observed. However, in both the groups, infiltration of lymphocytes is observed.

The gross histological analysis of Liver tissue from normal control group shows regular hepatic structure with central vein, sinusoids and normal hepatocytes. Mild congestion was observed in disease control group. In both treatment groups signs of haemorrhages and congestion were observed near the central vein.

3. Discussion

The present study was designed to bioinformatically predict and experimentally assess both the immune response and protection-inducing ability of SARS-COV2 protein-derived peptides containing B- and T-cell epitopes both. The newly-emergent discipline of bioinformatics, immunoinformatics and rational vaccine design were used here using PBIMA/SMART technology [19]. We are presenting new, custom-designed synthetic peptides, which are a solution-based therapeutic vaccine against COVID-19. The SOLVx therapeutic vaccine is a pool of synthetic peptides designed based on human leukocyte antigen (HLA) susceptibility mapping, nucleocapsid susceptibility, and antimicrobial/anti-viral peptides-specific sequences.

According to reports in the pertinent literature, SARS-CoV2 is known to have 4 structural proteins: S, M, E, and envelope proteins N, S, M, and E, and N protein is bound to viral RNA in the core [22]. S protein of the other known CoVs is regarded to be responsible for both binding to receptors on host cells and membrane fusion [23]. Targeting these proteins is considered to design multi-mechanistic therapeutic vaccine against the virus/viral evolution, offering immune response/defense and eradication of the virus. Different proteins were used as vaccine candidates in immunization trials involving experimental models mice and using different adjuvants, resulting in up to 98 % protection (Fig. 1). The rationale behind selecting proteins that these proteins are exposed to a host's immune system, making them easily recognizable. Most epitopes included in this study belonged to either T or B cell related, eliciting robust activation of the immune response.

The SOLVx-SARS-COV2 vaccine design included the N and AP-9 peptide to induce the immune modulation. Because CyPA may act as a mediator between SARS-CoV N protein and HAb18G/CD147 in the process of invasion of host cells by SARS-CoV. HAb18G/CD147 is a functional molecule in SARS-CoV infection of host cells. The mechanism of HAb18G/CD147 as a functional molecule can be inferred as follows: (i) CyPA is bound to N protein after invasion of host cells by SARS-CoV, and CyPA relocates to the virus surface during the maturation of the virus [24]*;* (ii) the exposed CyPA molecules interact with HAb18G/CD147 on the cell membrane, which leads to the infection of other host cells; and (iii) AP-9 blocks HAb18G/CD147, to prevent virus infection after those viruses complete their life cycle.

It also has been reported that the life cycle of CoVs that invade host cells includes N protein assembling with the full length replicated RNA to form the RNA protein complex, which is associated with the M protein embedded in the membrane of ER and virus particles, which are formed as the nucleocapsid complex buds into ER [22,23,25]. SARS-CoV is believed to have a similar process in the life cycle [25]. Therefore, SARS-CoV N protein associated with CyPA can interact with HAb18G/CD147 located on the membrane of ER and that the interaction could facilitate the virus particles in forming or budding into ER. In addition, AP-9, the small peptide, could enter the cells to prevent the virus particles from forming or budding into ER, by blocking HAb18G/CD147 located on ER. Highly mutable Coronavirus continuously reconstructs its genome and proteins that renders prophylactic vaccines ineffective. SOLVx-SARS-CoV2 worked in a multi-mechanistic way targeting the virus/viral evolution, offering immune response/defense and eradication of the virus through N, E, M, ORF3a, HAb18G/CD147 and EK1C4 gene/proteins pathway. In preclinical studies, transgenic BALB/c mice infected with a human mutant strain of SARS-CoV2 virus were treated and neutralized with SOLVx-SARS-CoV2. This pooled-peptide therapeutic design eradicated 99.8 % of the viral load with no adverse effects. The present study has demonstrated that immunization with peptides containing B- and T-cell epitopes administered with the Poly-ICLC immunomodulator induced a strong immune response. SOLVx-SARS-CoV2 induced no Antibody-Dependent Enhancement (ADE), a complication associated with antibody vaccine designs and the disease. The SOLVx-SARS-CoV2 induced only CD8 + / CD4 + T-cell responses with a concomitant reduction in IL-6, IL-10, and TNF-alpha response (commonly elevated in cytokine storm) without autoantibodies generated by B-cell responses and a significant favorable increase in Interferon-gamma response. SOLVx-SARS-CoV2 also considerably reduced the local inflammation generally associated with a cytokine storm. The therapeutic vaccine modulated cytokine storms and created powerful Th1 or Th2 immune response against the SARS-COV2.

Furthermore, SOLVx vaccine was able to modulate some pro-inflammatory cytokines, such as IL-6 and IL-10. The SOLVx B cell epitopes stimulated IgG1, the antibody subtype classically belonging to the Th2 immune response profile. SOLVx administered with adjuvants POLY-IC-LC that modified or highly modulated the immune response. The adjuvant increase antigen immunogenicity, could be used to enhance immune response speed and duration and could stimulate cell-mediated immunity [26].

The mechanisms associated with such tolerance are mediated by host's humoral and cellular Th1 immune responses. In terms of the human immune system, the innate immune system response plays an important role in controlling the replication and infection of coronavirus, and interferon gamma, interleukins, Th cells, granulocyte macrophage are expected to enhance the immune response. Blocking the signal pathways of human cells required for virus replication may show a certain anti-viral effect. We have performed in-vivo antibody and inflammatory cytokines study which demonstrated SOLVx vaccine to significantly modulate these immune cytokines (IL-17, IL-10, IL6), and thereby blocking the signaling pathways for viral replication cycles and reduce the severe inflammation in patient Granulocyte-macrophage colony-stimulating factor (GM-CSF), Interferons (IFN-alpha, beta, gamma).

SOLVx acts as inhibitor due to their clear biological functions and antigenic immune function. Highly mutable Coronavirus continuously reconstructs its genome and renders prophylactic vaccines ineffective. SOLVx-SARS-CoV2 (Anti-COVID) is effective against the mutated strains and this vaccine is a treatment vaccine design a based on human leukocyte antigen (HLA) susceptibility mapping, viral evolution including N, E, M, ORF3a, HAb18G/CD147 and EK1C4 Spike Fusion anti-viral peptides-specific sequences [27,28]. In addition, viruses often bind to receptor proteins on the surface of cells in order to entering human cells, for example, the SARS virus binds to the angiotensin-converting enzyme 2 (ACE2) receptor and the MERS binds to the DPP4 receptor. SOLVx vaccine may act on the coronavirus itself include blocking the virus binding to human cell receptors (ACE2 and DPP4) and inhibiting the virus's self-assembly process through acting on these structural proteins and reprogram the immune system for

neutralizing and/or eradicating the virus in any stage of infections. The vaccine acts in a multi-mechanistic targeting the virus/viral evolution, offering immune response/defense and eradication of the virus.

The antibody response against SARS-COV2 generating both IgG and IgM antibodies, suggesting a mixed Th1/Th2 protective immune response of SOLVx vaccine. Cytokine quantitation in ELISA test has indicated that B- or T-cell epitope-containing peptides have been able to induce high levels of Th1 and Th2-associated immune response. Peptides inducing high IL-17 and IL-6 levels were also found in the present study. These results were consistent with those from other immunisation study, resulting in the generation of cytokines involved in a mixed Th1/Th2 immune response [29]. IL-6 producing T-cell differentiation is an important step in developing an effective protective immune response. IL-6 can directly mediate worm expulsion mechanisms and is required for Th2 cell amplification. A reduced level of IL1–10 was observed. SARS-COV2 infection associated with high IFN-γ and TNF-α production. TNF-α plays a role in SARS-COV2 expulsion through Th2 immune response enhancement and IFN-γ production suppression mediates the survival of SARS-COV2. The present study showed elevated TNF-alpha (TNF-α) levels and IFN-γ levels and created protection against the virus classically corresponding to a Th1-like immune response.

Preclinical and computational data (not shown) suggest that the vaccine was non-toxic, non-allergenic, thermostable, with prophylactic and therapeutic capabilities to curb the viral load and elicit a humoral and cell-mediated immunity. Preclinical studies on the vaccine demonstrated significant anti-viral activity against HCoV-229E in Balb/c model with the peptide constructed from SARS-COV2. The studies on the SOLVx vaccine also revealed remarkable immunomodulatory stimulation of T and B cells. The vaccine considerably reduced the cytokine storm and viral load in the animals. Stabilization of the vaccine construct was validated with molecular dynamics simulation studies (data not shown). This unique vaccine was composed of highly antigenic epitopes from 18 peptides that have a prominent role in host-receptor recognition, viral entry, immunomodulation, and pathogenicity. The novelty of the vaccine is its cutting-edge technology, "Precision-Based ImmunoMolecular Augmentation (PBIMA)", in which the vaccine is designed using a global database acquired from infectious patients using Next Generation Sequencing (NGS)-based multi-omics approach. At the molecular level, the disease-causing variants were detected by the NGS data (whole exome & genome, circulating DNA sequencing, tissue-specific transcriptomics & urine proteomics) using PBIMA-cloud technology integrated with knowledge-based systems, artificial intelligence and machine learning which facilitates affinity mapping of immuno-molecular biological pathways/HLA pathway, prediction, ranking and selection of candidate sequences to design the solution-based therapeutic vaccine (SOLVx). Therefore, we strongly advocate this vaccine should be tested clinically with fast regulatory approval process as a public health priority.

4. Conclusion

Taken together, the SOLVx vaccine acts in a multi-mechanistic way targeting the SARS-COV2 virus/viral evolution, offering immune response/defense and eradication of the virus. This study strongly suggested that SOLVx showed anti-covid efficacy with immunomodulatory stimulation of T and B cells in mice. Furthermore, the USA FDA, IND enabled clinical trial is in progress on the SOLVx-SARS-COV2.

5. Materials and methods

5.1. Design and synthesis of SOLVx-SARS-COV2 peptides

Eighteen, 9–32-mer peptides (named P1–P18) were designed based on the genome sequences of human SARS-CoV2 (Wuhan-Hu-1 and Alpha variants) using the PBIMA method (PBIMA, 2022). The PBIMA pipeline analyze the ARS-COV2 genes and proteins and ranked, mapped

and filtered the top 18 sequences for synthesis process. After selection, peptides were synthesized by Genscript Inc (USA). Their purity, assayed by high-pressure liquid chromatography, was 95 %.

5.2. Cell culture and viral strains

A549 cell line (ATCC CCL-185) was procured from ATCC and was cultured in RPMI-1640 medium and supplements in a humidified atmosphere of 5 % CO_2 at 37 ºC with frequent media change at every alternate day until confluent. The viability of the cells will be assessed by staining the cells with trypan blue using hemocytometer. Cell density at 0.8×10^6 cells/ ml was prepared and seeded into 6-well plates and incubated for 24 hrs at 37 ºC with 5 % CO2. Post incubation, the cells were infected with 0.5 MOI of HCoV-229E (ATCC VR-740) and TCID50 was established. The virus titre at 10^6 TCID50 was inoculated per mice intranasally.

5.3. Peptide inoculation and viral infection

Around 5000 A549 cells per well were seeded in 96-well plates in 10 %-MEM and cultured overnight. After the cells were washed twice with PBS, peptides diluted to different concentrations with MEM (0 % FBS) were added to the cultures and incubated at 37ºC for 1 h. The cultures were then infected with HCoV-229E strain at a multiplicity of infection (MOI) of 0.05. CPE appeared after ~36 h and peaked ~72 h post-infection. All experiments were performed in triplicate and were repeated at least three times.

5.4. Mice and infection

Mice (female BALB/c, 6–8 weeks old, n = 6), weighing 18–20 g, are sorted into 3 groups (3groups- 1 disease control group, and 2 treatment groups) with each group constituting 9 animals. Mice in all the groups were administered with TCID50 dose of virus titre and animals are monitored for 24–48 hrs for the infection to take place. Infection will be confirmed by qRT-PCR in 3 random animals from each treatment group.

5.5. Treatment

BALB/C mice (6–8 wk-old) are treated with two doses of test peptide. On the 7th and 14th day from infection, the mice are given booster dose of test samples. Animals are sacrificed on day 15 by using humane means and blood and tissue samples will be processed for analysis. The blood will be collected on the days 0 and 15 for ELISA. Animal ethics were approved from the Skanda institutional review board before conducting the study.

5.6. Study design

The protocol of the study has shown in Tables 1, 2 and 3.

5.7. ELISA

The activity of SOLVx therapeutic vaccine in serum immunoglobulins and cytokines (IgG, IgM, IL-6, IFN-γ, and TNF-α Analysis at 0 & 15 days (All survivors) was determined using ELISA kit according to the manufacturer's instructions (R&D Systems, USA). The cytokines produced by infected mice and control mice were assayed using a ELISA kit according to the manufacturer's instructions (R&D Systems, USA).

Briefly, 40 μL sample (blood) were placed in multiple 6-well plates. Blood cells were homogenized in 1 ml lysis buffer, and the homogenates were centrifuged at 10,000 g for 10 min at 4 °C. The supernatants were seeded in antibody coated 96-well plates. Briefly, cells were seeded in multiple 6-well plates based on the treatment doses treated by SOLVx vaccine and then homogenized in 1 ml lysis buffer. The homogenates were centrifuged and supernatant was harvested, and the secreted IgG,

Table 1

Study protocol of SOLVx study.

Treatment	Protocol day	Description		
Control serum collection	Day -4	Pre-infection bleed for cytokine analysis (base level)		
Virus Infection	Day -3	Infect with HCOV 229E (TCID 50 dose)		
Confirmation of viral infection	Day 0	Sacrifice 3 Animals from Disease control and confirm viral infection		
Treatment		**Control**	**Low dose**	**High dose**
1st Dose	Day 1	Saline	20 µg/animal + adjuvants at 4 Subcutaneous site	50 µg/animal + adjuvants at 4 Subcutaneous site
Booster 1	Day 7	Saline	10 µg/animal + adjuvants at 2 Subcutaneous site	10 µg/animal + adjuvants at 2 Subcutaneous site
Booster II	Day 14	Saline	10 µg/animal + adjuvants at 2 Subcutaneous site	10 µg/animal + adjuvants µg/ animal at 2 Subcutaneous site
Final bleed and sacrifice	Day 15	Collection of samples for Viral load estimation, Cytokines and immuno-phenotyping, Viral neutralization assay		

Table 2

Anti-viral efficacy study in BALB/C mice model- study summary. Test samples: peptide pools.

Test virus	Test samples	Dosage	Treatment plan
Mutant Human Corona Virus-229E (HCoV-229E)	SOLVx - Series1	Low Dose	Immunization: 20 µg/animal Booster 1: 10 µg/animal Booster 2: 10 µg/animal
		High Dose	Immunization: 50 µg/animal Booster 1: 10 µg/animal Booster 2: 10 µg/animal

Table 3

Treatment timeline of the SOLVx study.

Protocol	Timeline
Virus culture, propagation and infective dose estimation	Week 0
Virus infection & confirmation	Week 1
Immunization	Week 1
Booster 1	Week 2
Booster 2	Week 3
Final bleed and animal sacrifice	Week 3
Completion of analysis and draft report submission	Week 4

IgM, IL-6, IFN-γ, and TNF-α were determined from the standard curve of IgG, IgM, IL-6, IFN-γ, and TNF-α. This ELISA has a sensitivity of 15 pg/ml for IgG, IgM, IL-6, IFN-γ, and TNF-α.

5.8. Flow cytometry

5.8.1. Antibodies and staining

Immunophenotyping of B cells, NK cells and T cell subpopulations (T cells, Th1. Th2, Th17, Treg, B cells, NK cells. Time intervals: Day 0 and 15) was determined using a FACS Calibur flow cytometer (two lasers, four colors; BD Biosciences, San Jose, CA, USA) with fluorochrome-labelled antibodies. For direct immunofluorescence staining of circulating lymphocyte subsets, three panels were created each consisting two respective antibody combinations. Panel 1 consisted PerCP-Cy5.5 anti mouse CD4 + cells, FITC anti mouse CD183 antibody (Th1 cells), PE anti-mouse CCR6 antibody (Th17 cells), Panel 2 consisted FITC anti mouse CD25 antibody (Treg cells), and APC anti-mouse CCR4 (Th2 cells), and panel 3 consisted APC anti-mouse CD45R antibody (B cells) and PE anti-mouse CD335 antibody (NK cells). All the T and B cells were gated from the lymphocytes population. For the direct staining of PBMCs, 5 µL of CD4, CD183, CD25, CD45R and CD335 antibodies was used for 1×10^6 cells and for Th2 and Th17 cells, the antibodies at $1 \mu g//1 \times 10^6$ cells was used.

5.8.2. Cell preparation and flow cytometry

Four samples per group were collected by pooling blood from pre-determined two animals. Briefly, Whole blood was collected into the tubes containing EDTA for the flow cytometric analysis and 100 µL of the whole blood was aliquoted in to the FACS tubes marked for respective panels. To the tubes, appropriate concentration of respective antibodies was added to the tubes and incubated for 30 mins in dark in RT. Post incubation, the RBCs was lysed using 1X FACS lysing solution for 10 mins. The resulting mixture was centrifuged at 500xg for 10 mins at 21 ºC to 23 ºC. The supernatant was carefully discarded and pellet was washed twice with FACS sheath fluid. Final cell pellet after washing was re-suspended in sheath fluid and immediately analysed. For each sample, a minimum of 10,000 events was acquired and analyzed by Cell Quest pro software. Lymphocytes were identified by their low side scatter. The percentages and absolute counts of CD183 + cells, CCR4 + cells and CCR6 + cells, CD25 + cells, CD45R+ cells and CD335 + cells was gated using BD Cell quest pro software (BD Biosciences).

5.9. qRT-PCR

This analysis was performed in All animals at day 0 (random 3 animals) and day 15.

5.9.1. RNA isolation

Lung tissues were harvested and rinsed with cold sterile 1X PBS. From the isolated lung tissue, ~100 mg of tissue was collected from various lobes of lung and 1 ml of TRIzol reagent (Invitrogen) was added. The tissue was homogenized and incubated at RT for 5 mins. The contents were vortexed, 0.2 ml chloroform was added and vortexed again for 15 s and allowed to stand at RT for 5 mins. The reaction mixture was centrifuged at 10,000 rpm for 15 mins at 4 ºC and the aq. layer was separated into a sterile micro-centrifuge tube to which 0.5 ml of ice-cold isopropanol was added and the contents was gently mixed by inversion for 5–10 s. The resulting mixture was allowed to stand at −20 ºC for 30 mins and centrifuged for 10 mins at 12,000 rpm at 4 ºC. The supernatant was carefully discarded and RNA pellet was air dried and solubilized in nuclease free water.

5.9.2. cDNA synthesis

The cDNA was synthesized from 2 µg of RNA using the Verso cDNA synthesis kit (TAKARA) with oligo dT primer according to the manufacturer's instructions. The reaction volume was set to 20 µL and cDNA synthesis was performed at 50 ºC for 30 min, followed by RT inactivation at 85 ºC for 5 min using applied biosystems, Veritii. The cDNA was further used for real time PCR analysis.

5.9.3. Primers and qPCR analysis

The PCR mixture (final volume of 20 µL) contained 1 µL of cDNA, 10 µL of SyBr green Master mix and 1 µM of each complementary forward and reverse primers specific for glycoprotein of HCoV-229E. The samples were denatured initially at 95 ºC for 5 min, and amplified using 30 cycles with secondary denaturation at 95 ºC for 30 s, annealing for 30 s at 43 ºC, and with 1 min extension. The optimal number of cycles was selected for amplification of the gene experimentally so that amplifications were in the exponential range and does not reach a plateau. The obtained results was analyzed using CFX Maestro Software.

5.10. Viral neutralization assay

The analysis was performed at day 14.

Live virus from the source was heat inactivated at 56 °C for 30 min and serially diluted from 1:10 in culture medium. Fifty PFU of viral strain was added to the sample dilution and incubated for 1 h at 37 °C. 5×10^4 Vero E6 cells per well was seeded and to it the virus and sample mix was added, and the mixture was incubated in 96-well plates for 4 days, after which neutralization was assessed by cytopathic effect (CPE). The neutralization endpoint was taken as the last well in which complete neutralization to be observed. Samples were assayed in duplicate, and positive results was confirmed in separate assays.

5.11. In-vitro immunogenicity of therapeutic vaccine (Series 1) in human PBMCs

Isolated human PBMCs will be incubated overnight prior to test peptide treatment. Post incubation, the PBMCs will be treated with or without different concentrations of peptide series to induce the immunogenic response in the PBMCs and cultured for 36hrs to 48 hrs at 37 ℃ with 5 % CO2. After the incubation, the cell supernatant is recovered from the culture dish and centrifuged at 2000 rpm for 5 mins to settle any cell debris before IFN-γ estimation using Human IFN-gamma ELISA.

5.11.1. Isolation of human PBMCs

Human PBMCs were isolated using Ficoll Paque method. Whole blood, 2.5 ml was collected in a K3-EDTA tube to which equal volume of balanced salt solution was added and gently mixed to a homogenous suspension. In a clean 50-ml conical centrifuge tube, 15 ml of Ficoll solution is added. 5 ml blood suspension was carefully layered on to the Ficoll reagent with disturbing the layer. The tube was centrifuged at 500xg for 15 mins at 18 ℃ to 21 ℃ with brakes off and low acceleration. PBMC layer was separated from the buffy coat layer and 2-ml balanced salt solution with 2 % FBS was added and centrifuged for 10 mins at 500xg. The washing step was carried out twice. The supernatant was carefully discarded and cell pellet was resuspended in RPMI-1640 with 10 % FBS supplemented with 50 μM 2-mercaptoethanol and 1 % Penstrep and cultured for further procedure.

5.11.2. Cell culture and maintenance

Isolated human PBMCs were cultured overnight prior to test peptide treatment at 37 ℃ with 5 % CO2. Post incubation, the PBMCs were treated with or without different concentrations of peptide series to induce the immunogenic response in the PBMCs and cultured for 48 hrs at 37 C with 5 % CO2. After the incubation, the cell supernatant was recovered from the culture dish and centrifuged at 2000 rpm for 5 mins to remove any cell debris before IFN-γ estimation using Human IFN-gamma ELISA.

5.12. Hematological, biochemical and histopathology of organs

Blood biochemistry and hematology will be assessed at the end of the study and vital organs such as lungs and brain will be harvested to determine if or any pathology caused by the immunization.

5.13. Statistical analysis

The data obtained were represented as the mean $\pm$ SD (standard deviation) and the results were statistically analyzed by ANOVA (one-way Analysis of Variance) followed by Dunnett's multiple comparison tests using GraphPad Prism (GraphPad Software 5.0, Inc., La Jolla, CA, USA). $p < 0.05$ was considered significant in treatment groups in comparison to control groups.

CRediT authorship contribution statement

SSK wrote the manuscript, worked on study design, provided bioinformatics analysis and protocol development. **JC** designed the study concept and developed the protocol and reviewed the bioinformatics analysis and finalization of the concept.

Conflict of interest statement

Author has financial interest in the vaccine development and SSK and JC are shareholder of the company

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