Design of a Multivalent SARS-CoV-2 vaccine using Virus-like particles

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Abstract

The recent SARS-CoV-2 pandemic highlighted the urgent need for efficient vaccines that provide broad, durable immunity against multiple viral strains. Current SARS-CoV-2 vaccines have shown reduced potency against emerging variants and, for this reason, exhibit waning immunity over time. To address these limitations, we propose the development of a novel Virus-Like Particle (VLP) vaccine designed to present spike proteins from two different SARS-CoV-2 strains. VLPs are non-infectious, self-assembling structures that mimic native viruses and are known to stimulate robust humoral and cellular immune responses. For proof-of-principle, the proposed design incorporates spike proteins from the original Wuhan-1 strain and the Delta strain, along with the 4 SARS-CoV-2 proteins. This vaccine is intended for production using a plant-based expression system, specifically *Nicotiana benthamiana*. Plant systems offer advantages such as proper eukaryotic post-translational modifications necessary for VLP immunogenicity, high scalability, rapid production time, and significantly lower costs than mammalian cell cultures, while being free of mammalian pathogens. The pEAQexpress vector will be utilized for efficient transient protein expression in *N. benthamiana*. The vaccine's potential efficiency will be evaluated using an N-based genetic complementation system and in hACE2 transgenic mice *in vivo*. This approach aims to provide broader protection against multiple strains and elicit a more robust, longer-lasting immune response, offering a scalable and cost-effective platform for combating rapidly mutating viruses

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Aim and scope

The recent SARS-COV-2 pandemic shed light on the necessity for rapid development of efficient vaccines for new pathogenic species and strains. The Wuhan strain of SARS-CoV-2 was first identified in December 2019 in China and was associated with a pneumonia outbreak, leading to the World Health Organization (WHO) to declare COVID-19 a global pandemic in March 2020(Thiede et al.). Genomic analysis identified the role of the ACE2 receptor for viral infectivity, as the Spike (S) protein binds to human ACE2 (hACE2) for cell entry, with infection capable of causing severe outcomes, including acute respiratory distress syndrome (ARDS) and death(Bao et al.). As an RNA virus, it is characterized by a high genomic mutation rate and antigenic drift, resulting in the rapid and continuous emergence of Variants of Concern (VOCs)(Zhou et al.). VOCs include Alpha (identified September 2020 in the UK), Beta (May 2020 in South Africa), Gamma (November 2020 in Brazil), Delta (October 2020 in India), and Omicron (designated November 2021). The Delta variant was recognized for its increased transmissibility (reproduction number 5.08 vs. 2.79 for non-VOCs) and stronger transmission due to a P681R mutation, while Omicron exhibited a substantial number of mutations (e.g., 34 for BA.1) and infected human bronchus 70 times faster than Delta(Zhou et al.). This evolution has also led to reduced effectiveness of existing single-strain vaccines against these variants, especially Beta and Omicron, which showed the highest reduction in neutralizing titers and evasion from immunity. For example, Omicron demonstrated a 20-fold reduction in neutralization compared to the ancestral strain(Fiolet et al.), though they generally remained capable of preventing severe disease progression

The following proposal aims to design a more efficient SARS-COV-2 vaccine that provides immunity against multiple strains while retaining the durability of the immune response. The endeavor is to assess the potential for creating a Virus-Like particle (VLP) presenting spike proteins originating from 2 different SARS-COV-2 strains as first proof of principle. VLPs spontaneously assemble into structures that replicate the morphology, dimensions, and symmetry of native viruses while not containing the genetic material, eliminating the ability to reproduce(Mohsen and Bachmann). The analysis will unravel by providing all the relevant background information, building up to the design of the vaccine

SARS-COV-2 genome

Understanding the SARS-COV-2 genome is fundamental for genetic engineering a vaccine. Below is a summary of the crucial aspects.

The SARS-CoV-2 genome is a positive-sense single-stranded RNA virus and the largest known among RNA viruses, ranging from 27 to 32 kb. The 3' UTR of the SARS-CoV-2 genome contains an octameric sequence and a poly(A) tail. These regions contain cis-acting RNA elements necessary for replication. The genome is divided into several open-reading frames. ORF1a and ORFb are the largest ones, which occupy the 5' two-thirds of the genome. They encode non-structural proteins that form the replication-transcription complex (RTC), which plays an essential role in viral replication, subgenomic mRNA synthesis, and genome processing. The 3' ORF end encodes the structural proteins: Spike (S), Envelope (E), Membrane (M), and Nucleocapsid (N). These proteins are translated from subgenomic mRNAs and are critical for assembling new virions(Brian and Baric).

The S protein is a prominent transmembrane protein that recognizes cellular receptors, mediating fusion to the host cell. As the major antigen, the S protein makes for an ideal target for eliciting protective immune responses, with antibodies targeting its receptor-binding domain. It forms the characteristic peplomers on the surface of coronaviruses. Its composition consists of two subunits, S1 and S2; S1 is involved in viral entry into host cells and interacts with human proteins such as ACE2 or TLR4(Colonna). It is a highly varied protein amongst coronaviruses, particularly in their amino-terminal half, and this is the main reason that new viral strains evade antibody responses induced by infection or vaccination with former strains(Fischer; Focosi). The N protein packages the viral genomic RNA into a helical ribonucleocapsid while playing a crucial role in the coronavirus replication cycle and the host cell's response to viral infection.

Furthermore, it functions as an RNA chaperone, facilitating viral RNA synthesis, which is located in double-membrane vesicles (DMVs) and convoluted membranes (CMs). E protein is a small, integral membrane protein involved in various stages of the viral life cycle, including assembly, budding, and pathogenesis. It is a viroporin, forming a pentameric ion channel in the ERGIC membrane. Mutations in the E protein can affect its oligomerization and, thus, its function. Its transmembrane domain forms a tight, cylindrical helical bundle that can be targeted by inhibitors(Cao et al.). The most abundant structural protein in the virion is the M protein, which is crucial for virus assembly. It interacts with other viral proteins, such as the S-protein, to assist in shaping the viral envelope. The carboxy-terminal extremity of the M protein is critical for virion formation(Kuo et al.). Understanding the intricacies of the SARS-CoV-2 genome, particularly the Spike protein, is paramount to developing effective vaccine strategies. To evaluate potential vaccine candidates and dissect the mechanisms of immune protection, researchers turn to animal models, especially mice. The following section will explore how immune responses are propagated and studied in mice, providing insights into developing a targeted vaccine proposal.

Mouse models of COVID-19

Mice offer several advantages that make them ideal for research. They are small, easy to handle, and have a short reproductive cycle, facilitating research. They have well-defined immune systems, which makes the study of immune responses significantly easier. Both mice and humans share a similar innate immune response involving receptor recognition, interferons, and inflammatory cytokines. Also, in terms of adaptive immune responses, there is high similarity in CD4+ and CD8+ T cell responses, while also B cell activation and antibody production. The last practical advantage serves the array of tools and techniques accessible for working with mice, including genetic modification technologies and immunological assays(Körner et al.)

Even though mice share immunological similarities with humans, the critical step of SARS-CoV-2 is the viral entry through the ACE2 receptor, which is inefficient in unmodified mice. Lack of effective binding to the murine ACE2 receptor means that unmodified mice are not readily susceptible to a SARS-CoV-2 infection. The paper “Human ACE2 Gene replacement Mice support SARS-CoV-2 Viral replication and Nonlethal Disease Progression” by Joshua M Thiede, presented that WT C57BL/6 mice showed no evidence for SARS-CoV-2 viral replication in either the lungs or the brain. Therefore, while mice offer numerous advantages for studying the disease, their resistance to SARS-CoV-2 infection presents a critical limitation that warrants using genetically modified mouse models that express the human ACE2 receptor (hACE2 mice)

Mouse models infected with coronaviruses exhibit a rapid activation of the innate immune system, including the activation of natural killer (NK) cells, which play a crucial role in antiviral responses. Type 1 interferon production by the NK cells is essential in controlling the antiviral response. Different mouse strains can have varying IFN responses, impacting disease severity. For example, A/J mice produce less type 1 IFN than resistant strains, leading to severe disease progression upon murine Hepatitis coronavirus strain one infection. A plethora of factors that are responsible for these different IFN levels are significantly reduced, like TLR7 levels and plasmacytoid dendritic cells (Körner et al.).

The adaptive immune system is also activated in mice with coronaviruses, similarly with the Human immune system, primarily by SARS-CoV-2, MERS-CoV, and MHV. Observed in hACE2 transgenic mice, memory T cells can patrol organs and infected tissues and provide protection even in distant

tissues(Colonna). Memory CD8+ T cells can protect substantially from severe acute respiratory syndrome coronavirus infection. CD4+ T cells can mediate neutralizing antibody responses and assist with persistent antibody responses and affinity-matured B cell memory(Sun et al.).

The antibody response in vivo is quite complex and dynamic, involving a complex mixture of antibodies targeting an abundance of viral proteins, like the different domains on a virus's envelope glycoprotein, neutralizing the virus. For SARS-CoV-2, a key target is the Spike (S) protein, specifically the Receptor Binding domain, which is crucial for ACE2 recognition and viral entry, which is the same for the immune response in Humans(Brian and Baric)

Current SARS-CoV-2 Vaccination Landscape

The urgency to combat the pandemic is reflected by the diversity of the vaccine types, ranging from mRNA vaccines, viral vectors, inactivated ones, and VLP vaccines, which will be analyzed in this paper. Approved vaccines present high efficacies, starting with mRNA vaccines, e.g. *"m`RNA-1273"* or SPIKEVAX by Moderna, introducing a >90% efficacy in preventing symptomatic cases against the original strain (Wuhan-1) in initial trials. The viral vector vaccines like "*AZD1222"* and protein subunit vaccines had demonstrated efficacies ranging from 50.6% to 92.3%, yet the specific strains they were tested against were not always specified. While initial vaccines, such as mRNA-1273 (Moderna) and AZD1222 (AstraZeneca/University of Oxford), demonstrated good efficacy against the original SARS-CoV-2 Wuhan-1 strain and earlier variants like Alpha (B.1.1.7), their potency has generally been reduced against later variants such as Beta (B.1.351) and Delta (B.1.617.2). For instance, mRNA-1273 showed 94.1% effectiveness against the original strain and 98.4% effectiveness against documented Alpha infection, but its neutralizing activity was considerably decreased against Beta, despite showing continuous protection against infection. Against the Delta variant, mRNA-1273's effectiveness against infection, while initially high at 94.1% (14 to 60 days post-second dose), declined to 80.0% after 151–180 days. Similarly, AZD1222 demonstrated 70.4% efficacy against symptomatic COVID-19 for the original strain and 73% effectiveness against documented Alpha infection, but its efficacy dropped significantly to 10.4% against the Beta variant in South Africa and 67% against the Delta variant in the UK(Fiolet et

al.)(Zhou et al.). This reduction in potency is attributed to the virus's ability to undergo antigenic drift and escape the host or vaccine-induced immune response(Kuo et al.; Katiyar et al.). Furthermore, a general decrease in immune protection has been observed at 6 months for current vaccinations." The rapid emergence of new variants calls for enhancing the current vaccination technologies and strategies. Overall, the current vaccinations bear the limitation of a decrease in immune protection at 6 months. The waning of immunity suggests a demand for vaccines that can elicit a more robust and longer-lasting immune response; VLPs have the potential to contribute to the durable response needed. (Fiolet et al.)[1]

Virus-Like particles in vaccine development

The vaccine candidate will be developed utilising VLP technology; this section will provide the background information on the nature of VLPs. VLP's are non-infectious, virus derived structures that mimic the size and shape of a virus but lack the viral genome. VLP's present multiple advantages over the current vaccines that make them ideal for optimization of the immunization of the masses.

A first advantage would be the immunogenicity it stimulates. Their structure allows efficient recognition by the immune system, eliciting a robust immune response. VLPs stimulate both humoral and cellular immunity through the MHC class 1 and 2 pathways.

Secondly, they can also be loaded with immune modulators (i.e, adjuvants)to provoke more effective immune responses.

Thirdly, VLPs permit great versatility because they are engineered, allowing the expression of specific antigens, which will be utilized in this review to generate a multistrain vaccine.

The last yet significant advantage is safety; VLPs do induce a strong immune response, allowing immunization without posing any danger since they lack a productive viral genome, thus being unable to divide, making them non-infectious and safe for use in vaccines.

Production of VLPs can be carried out using various expression platforms, each presenting different production characteristics. Prokaryotic systems like *Escherichia coli* are used for their high protein yields

[1] Fiolet T, Kherabi Y, MacDonald CJ, Ghosn J, Peiffer-Smadja N. Comparing COVID-19 vaccines for their characteristics, efficacy and effectiveness against SARS-CoV-2 and variants of concern: a narrative review. Clin Microbiol Infect. 2022 Feb;28(2):202-221. doi: 10.1016/j.cmi.2021.10.005. Epub 2021 Oct 27. PMID: 34715347; PMCID: PMC8548286.

and cost-effectiveness. There is a wide variety of eukaryotic systems that are used to produce complex proteins carrying mammalian posttranslational modification or requiring folding chaperones, ranging from insect cell systems like the Sf-9 system to mammalian cell cultures and plant systems.

The purification and characterization process of the VLPs usually involves the following steps. The procedure is initiated by cloning and expressing the viral structural genes in the chosen system. Then, harvesting and lysing cells, followed by purification and clarification, are performed to isolate in an intact manner the VLPs. A formulation step may be followed to add adjuvants and other ingredients to achieve a safe and effective vaccine. To characterize the VLPs and ensure the success of the experiment, multiple methods may be applied, such as mass spectrometry, dynamic light scattering, and immunoassays.(Nooraei et al.)

Optimization of SARS-CoV-2 vaccine

This analysis aspires to provide a structured hypothesis for a vaccine that elicits a more robust immune response than the already approved vaccines while possessing multivalency for recognizing several viral strains. This vaccine shall be generated utilizing VLP technology as described above; the multivalency will originate from the expression of two spike proteins from two different Sars-CoV-2 strains. The aim is to introduce this novel concept, designed for the original Sars-CoV-2 strain (Wuhan) and the delta strain. If successful, the system can be readily adapted for more and different spike proteins.

Contents of Host and Vector

The choice of host organism is important, as explained in the paragraph "*Virus-like particles in vaccine development"*. In the current proposal, a plant-based system will be used. Plant-based systems appear to be the optimal choice for producing VLPs. They offer proper eukaryotic post-translational protein modifications, which are pivotal for the correct folding and immunogenicity of VLPs, like the glycosylation of the S protein or the phosphorylation of the N protein(Cheng et al.). Plants provide a highly scalable and low-cost production system and extremely fast production time, making them ideal for quick responses to outbreaks. The *Nicotiana Benthamiana* VLP production system, for example, takes

approximately 3-7 days post-agroinfiltration to produce the VLP(Hemmati et al.). Finally, the cost of utilising plant systems is 0.1% of the cost of mammalian cell culture systems and 2-10% of the microbial ones, while bearing non-existent safety risks since they are free of endotoxins, oncogenes or are at risk of mammalian pathogens. The specific organism that will be used is *Nicotiana benthamiana,* which has already successfully been used to produce VLPs mimicking SARS-CoV-2.

However, certain considerations arise due to the nature of the specific VLP design. Our vaccine displays a complex production due to the inclusion of many SARS-CoV-2 proteins (N,E,M) and two different Spike proteins; even so, plant systems have demonstrated the ability to produce complex structures. Furthermore, multiple sources reference the production of chimeric VLPs, for example, chimeric hepatitis B surface antigen VLPs presenting antigens from HCV and HIV, displaying the compatibility with the vaccine candidate.(Hemmati et al.)

Every host system has a specific set of vectors that can be applied. In this paper, the proposed vector is pEAQexpress (Genbank accession:GQ497230), a vector specifically designed for the efficient transient expression of multiple proteins in plants, particularly for *N. benthamiana(Hemmati et al.),* without viral replication. The pEAQ vector has a modular design permitting the insertion of multiple coding sequences on the same T-DNA to enable the co-expression of multiple proteins from one T-DNA. T-DNA or transfer DNA, refers to the specific segment of DNA from the *Agrobacterium tumefaciens* plasmid that is transferred from the bacterium to the host plant cell for production. These key features ensure that multiple genes can be delivered into one cell by containing all genetic information in one vector. There are three main components in the vector: the CPMV-HT expression cassette, the P19 silencing suppressor, and the antibiotic resistance markers. CPMV-HT refers to a modified version of the Cowpea mosaic virus, which is used as a common expression system in plants. It offers cloning flexibility since multiple expression cassettes can be inserted in the transient expression vector but also two cassettes can be added simultaneously through multipart ligations, consequently addressing the challenge of delivering multiple genes into the same cell.(Hemmati et al.)

Once the choice of vector, model organism, and expression system has been made, genetic engineering of the insert can commence. Apart from the genes that will be expressed for the production of the VLP, numerous structural components will be included to guarantee a stable and accurate production. The construct starts with a 5’ UTR (Untranslated region): GCCCCACC. The

5’ UTR plays a crucial role in translational efficiency. The Kozak sequence upstream of the start codon enhances ribosomal binding, improving protein expression. Then, the Pr1 signal peptide is included “ATGGTGAAGACTTACGAGGAAGAATTTGGTGTCTTTTGTTCAGGAAGTTTATGTTGCCGTTGTTAGTGG AATTCTTTAG”. This Pr1 originates from the pathogenesis-related protein 1 of *Nicotiana tabacum.* Its primary role is to direct newly synthesized proteins to specific cellular compartments, particularly the endoplasmic reticulum, which is fundamental for protein folding, post-translational modifications, and trafficking to the final destination. For the proper expression, folding, and assembly of the SARS-CoV-2 proteins, separate expression cassettes for each structural protein within a single T-DNA construct will be used. That will assist since protein folding will occur autonomously, the organelle-specific targeting will remain effective, and stoichiometric control will be more achievable since expression levels can be influenced by changing the promoter strength. After all genes are implemented, five more structural components will be incorporated: 3’ UTR, Poly (A) tail (~100nt), CaMV 35S promoter, terminator, T-DNA borders, and suppressor of silencing (P19). The 3’ UTR, like the 5’ UTR, promotes mRNA stability and translation efficiency; it contains regulatory elements that affect how long mRNA persists in the cell and how efficiently it gets translated to protein. The poly (A) tail protects the mRNA directly from degradation and promotes efficient translation. The CaMV 35S promoter is crucial in the construct owing to the fact that it drives high levels of expression in *N. benthamiana* but also is part of the CPMV-HT cassette, which was explained earlier. Overall, this promoter is significant for achieving high levels of VLP-related protein expression in Nicotiana benthamiana. T-DNA borders are a requirement for *Agrobacterium*-mediated transformation; they flank the insert for it to be introduced in the plant genome. Finally, the *P19* suppressor of silencing is fundamental due to the broad issue of gene silencing in plant models. *P19* is a viral suppressor of gene silencing that assists in increasing expression levels of target genes(Sainsbury et al.; Hemmati et al.).

Experimental methodology

The experimental procedure is divided into 4 phases: E. coli-mediated amplification of the vector, Agrobacterium-mediated transformation to *N.benthamiana*, and drug production and

Biochemical-Biophysical characterization. Once the edited vector is produced, E. coli transformation can initiate. This organism is chosen for amplification due to its rapid growth rate and simplicity, along with very low costs. The selection of successfully transformed cells is achieved by plating them with kanamycin. The pEAQexpress vector contains a resistance gene for this antibiotic. Then, colony PCR and sequencing should be performed to examine the presence of the insert in the vector. The pEAQ expression construct should be introduced into *A. tumefaciens*, specifically the AGL1 strain(Saeger et al. 2020). Agrobacterium suspension is a well-established tool for transferring genes into plant cells, and AGL1 is the most common strain used. Introducing the agrobacterium suspension into *N.benthamiana* leaves allows for the transient expression of the SARS-CoV-2 proteins. VLPs are typically composed of one or more structural proteins so when they are expressed heterologously, they spontaneously self-assemble into the VLP structures without incorporating plant proteins.

Once VLP production is done, biochemical and biophysical characterization can be initiated. For biochemical characterization, the proposed test would be Reverse Phase-High Performance Liquid chromatography (RP-HPLC), a technique that will determine the purity, integrity and molecular weight of the VLP. It is useful for characterizing post-translational modifications in viral glycoproteins. It can support both stages of product purity monitoring and product formulation and stability development in the VLP-based vaccine development(Rathore et al. 2024). This is achieved by the separation of hydrophobic peptides, the detection of site-specific PTMs through retention time shifts and UV profile changes, but it also quantifies glycoform heterogeneity. Overall, utilizing RP-HPLC is a good choice as it provides an accurate and reproducible method for purity assessment.

For biophysical assessment, two techniques are proposed. The first one is Atomic Force Microscopy, which measures particle size and size distribution. It's a powerful technique that measures the mentioned variables even in ambient conditions while attaining the advantage of imaging single particles without a high cost.

The second is Electrospray differential mobility analysis, which measures VLP particle size. It's a valuable technique due to the fact that it's a rapid and quantitative method that can characterize multimodal VLP distributions meaning samples containing distinct populations of particles with different sizes and detect subtle changes in the size or other characteristics of internal packaging distributions meaning the heterogeneity in what is encapsulated inside the VLP or the chimeric composition of VLP surface proteins. Chimeric composition refers to the surface of the VLP, which is composed of spike proteins of two different strains rather than a population of Spike proteins from a single strain

Lastly, Mass spectrometry (MS) can be utilized to analyze the molecular mass along with their protein sequences and their amino acid composition. The combination of MS with liquid chromatography will

provide well-rounded structural information making it a powerful tool for characterizing the specific protein and their ratios in the VLP surface(Nooraei et al.).

When the positive output is received from the tests mentioned, the VLP can move to the efficiency testing phase. Both in vitro and in vivo methodology will be proposed

Vaccine efficiency in mouse models

The proposed technique is called *"N-based genetic complementation system"* and has been developed by Xiaohui Ju et.al in 2021. This novel cell culture system is based on a modified virus named SARS-CoV-2 GFP/ΔN trVLP, which lacks the N gene, which is crucial for genome packaging and virome assembly and is replaced with the GFP gene. This modification ensures that the virus cannot continue its life cycle individually. Three modified cell lines are used in the original paper, but only one will be used in this analysis. CACO-2-N cells are obtained from an immortalized cell line derived from human colorectal adenocarcinoma. It's modified to stably express the N protein, complementing the missing N gene of the modified virus, permitting the SARS-CoV-2 GFP/ΔN trVLP to undergo a complete life cycle within these cells. This technique provides a significant advantage in biosafety, allowing experiments to be conducted under BSL-2 conditions and overcoming the limitations imposed by the BSL-3 classification of SARS-CoV-2.

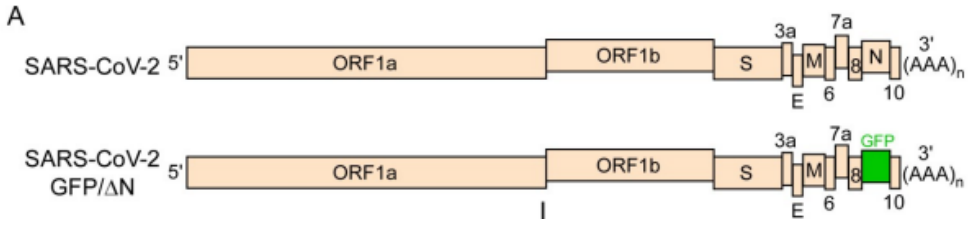

Figure 1: SARS-CoV-2 GFP/ΔN construct. The construct on top represents the genome of SARS-CoV-2, containing the two open reading frames and the four proteins: Spike, Envelope, Membrane, and Nucleocapsid. The second construct represents the edited SARS-CoV-2 GFP/ΔN *construct,* which has replaced the Nucleocapsid protein with GFP

To evaluate the antibody response, serum samples should be collected from the mouse subjects vaccinated with the multivalent vaccine at different time points post-vaccination. The proposed time points are the following: Day 0,4,7,10,14,28,42. Following this timeline early innate-phase response, early plasmablast activity, peake of primary IgG response, and durability response will be evaluated(Quan et al.)(Hodgins et al.2017)

SARS-CoV-2 GFP/ΔN trVLP of both strains should be mixed with the collected serum, allowing antibodies in the serum to bind to the viral particles. Applying the serum-virus mixtures to CACO-2-N cells ensures that any reduction in infection is due to neutralization, since this cell line solely allows the complete viral life cycle of the trVLP. These cell lines will be named Group A, and two other groups will be generated, Group B, which will be the positive control for infection, and Group C, which will be the negative control for infection. For Group B the same methodology will be applied, simply without combining the serum with the viral particles and applying only the viral particles. This control will establish a baseline level of viral infection and GFP expression for both strains in the absence of any neutralising antibodies. The capability of the cells to be infected by these strains will be confirmed, along with the ability to express the GFP reporter gene under the experimental conditions. Group C consists of only the CACO-2-N cells without any addition at all, which will determine the background level of GFP fluorescence or any other signal emitted in the absence of viral infection, excluding any fake positive results.

GFP expression levels should be measured using flow cytometry; the SARS-CoV-2 GFP/ΔN trVLP is engineered such that, with successful viral entry, and potentially subsequent replication, it leads to the production of GFP within the infected cells. Therefore, detecting GFP f.0luorescence in the CACO-N cells indicates viral infection. Reduction of the GFP signal will indicate the reduction in infection due to neutralization, indicating the vaccine's success. Quantitative vaccine efficiency will be calculated by comparing GFP expression levels between groups A and B.

In vivo analysis will be conducted by utilizing a mouse model proposed by Linlin Bao et. al in the paper. As explained above, normal mice are susceptible to coronaviruses, yet they are not susceptible to SARS-CoV-2 due to the absence of the ACE2 receptor. Genetic engineering allowed for the creation of a mouse strain that has integrated the human ACE2 receptor, permitting studies for SARS-CoV-2 infection to be conducted.

The in vivo phase will be split into two stages. The first one will assess the “neutralizing power of mouse antibodies,” and the second will evaluate the effects on disease progression by the VLP vaccine.

The VLP vaccine will be administered to the hACE2 mice through intramuscular administration. Serology will be performed every day for up to 20 days to observe the antibody production rate. The presence of antibodies recognizing both Spike proteins will be ensured using an ELISA specific for antibodies against Spike proteins of the Wuhan-1 and Delta strains. The serum collected should be mixed with the previously edited SARS-CoV-2 GFP/ΔN trVLP and administered to the CACO-2-N cell cultures while performing the exact same methodology. This way, the mouse-produced antibodies' neutralization capabilities will be evaluated.

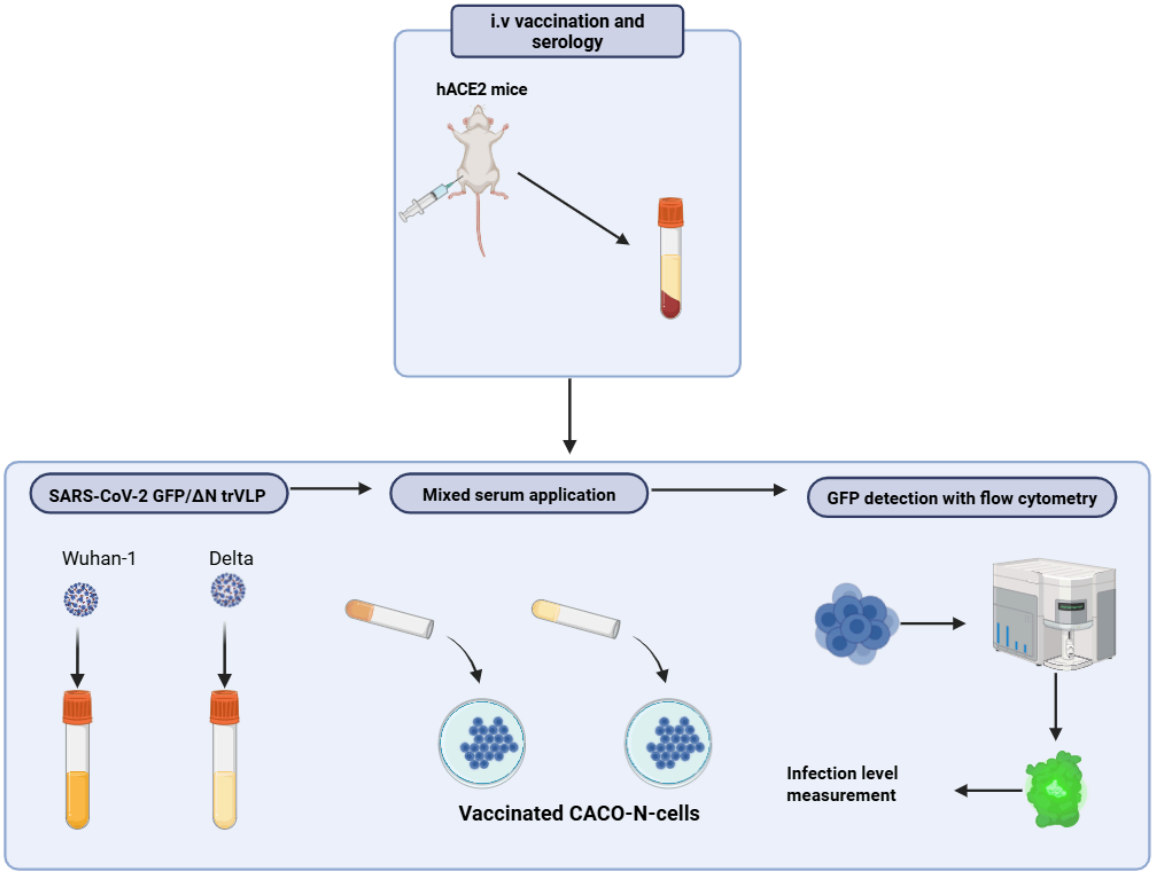

Figure 2: Created in Biorender.com. In phase 1, in vivo efficiency, pro-intramuscular vaccination of the mouse models, and antibody effectiveness are evaluated by mixing the isolated sera with the genetically modified SARS strains. Infection of the genetically edited Caco-N-cells by the edited SARS will induce the expression of GFP. Thus, the application of the mixed sera with the cells will evaluate the effectiveness of the antibodies

On the condition that the previous phase exhibited positive results, phase 2 can start. The paper by *M. Thiede et al* in 2024, provides evidence on the safety of the model. SARS-CoV-2 infection in ACE2 transgenic mice leads to mild, non-lethal disease without CNS involvement and has the capacity to mount an immune response.

Two groups will be utilized for the following methodology: the vaccinated Group A and the control Group B. Group A will be immunized with the vaccine candidate intramuscularly, and Group B will be given the intramuscular placebo. For the induction of SARS infection, the same methodology as the paper will be followed, with the alteration of both Groups being split into 2, receiving intranasally the *"Wuhan-1"* strain and the other subgroup the *"delta"* strain in both nostrils.

Four different methods will be used to monitor disease progression: daily monitoring of clinical signs, quantification of viral load in lung tissue and nasal turbinates, histopathological analysis of these tissues, and assessment of serological response. These methods will allow the comprehensive evaluation of the efficacy of the candidate vaccine in the ACE2 mouse model by comparing disease progression.

Clinical signs of disease can be ataxia, paralysis, labored breathing, and weight loss. Based on the study, unvaccinated mice showed no detectable weight loss; vaccinated individuals maintained weight and exhibited fever while showing fewer clinical signs than the unvaccinated group(Thiede et al.).
Viral load quantification in tissues requires the sacrifice of the mice. At three time points, subsets of the mice should be sacrificed from both groups, and tissues of interest, specifically the lungs, should be harvested and tested. The first time will be 3 days post-infection to assess the initial stages of viral replication, the second should be 7 days post-infection, and the last should be 12 days post-infection to assess viral clearance, resolution of pathology, and the development of the adaptive immune response. Lower viral loads in tissues would indicate that the vaccine efficiently reduces viral replication. Histopathological analysis should be done using the same methodology as the paper(Thiede et al.). Microscopic examination would allow the complete assessment of both groups' inflammation, tissue damage, and other histological changes. Lastly, serological assessment would allow the monitoring of antibody response after viral infection through blood samples, including an evaluation of inflammatory cytokines as well.

Levels of key pro-inflammatory cytokines like IL-1β, IL-6, and TNF-α should be measured in order to assess whether infection in vaccinated individuals leads to an inflammatory state, meaning increased levels or a balanced state(Sun et al.). Importantly, type 1 interferons (IFN-α and IFN-β) will be evaluated since they are crucial first-line antiviral cytokines. Early and robust type 1 IFN responses are very important for controlling the replication of the virus and regulating the inflammatory responses. Apart from these, interleukin-4 (IL-4) is associated with antibody-mediated immunity and leads to the production of IgG1 in mice. Measurement of the previously mentioned cytokines will assist in the understanding of the mechanisms apart from blocking viral entry. IL-1β, IL-6, and TNF-α are produced mainly by APCs-like dendritic cells. Their presence indicates the initiation of a robust innate immune response, which is crucial for shaping the adaptive immune response. Their presence contributes to recruiting more immune cells, like CD4+ and CD8+ T cells. T-cell responses are a significant component of cellular immunity. By observing their activation and differentiation through the cytokine levels, the ability of the vaccine to initiate a cellular immune response can be inferred. The kinetics of the pro-inflammatory cytokines will reflect on the severity of the disease. Certain cytokines are found at elevated levels in severe COVID-19 cases and at low levels in asymptomatic ones; thus, by measuring the cytokines longitudinally after the vaccination, correlations will be possible. Apart from that, cytokines are linked to effective immune control, like for example IFN-γ, which activates T cells and macrophages; thus, information will be provided on how immunity is mediated.(Thiede et al.; Grifoni et al.)

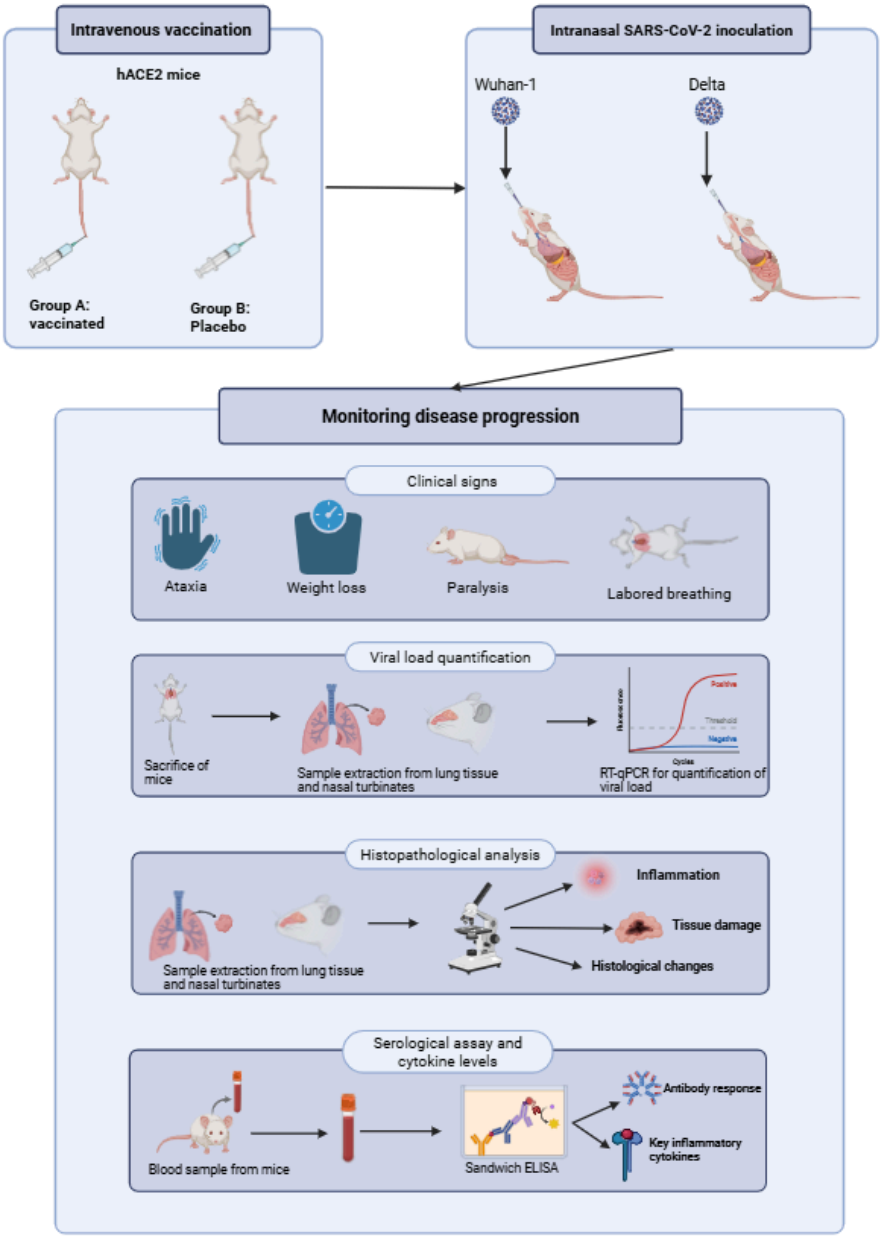

Figure 3`1 : Made in Biorender.com Phase 2 of in vivo efficiency, Disease progression monitoring through clinical signs, Viral load quantification, histopathological analysis, serological assays and cytokine levels

Risk mitigation

The experimental pipeline for the development of the multistrain VLP vaccine is a linear process, meaning that it entirely depends on the success of the previous steps. Suboptimal results at any time point of the procedure will trigger the risk mitigation system, which consists of 2 steps. First is the re-evaluation of VLP quality, morphology, and stability; repetition of the biochemical and biophysical characterization would reconfirm the VLPs' integrity. Utilization of the RP-HPLC would assess the purity, integrity, and molecular weight of the VLP while also detecting site-specific PTMs. Then AFM or electrospray differential mobility analysis will accurately measure VLP particle size and size distribution. Once the issues have been identified, the first step would be optimizing the extraction, purification, and storage conditions. Proper handling of the VLPs after their production will prevent aggregation or loss of structural integrity. Secondly, the plant-based production system should be optimized. If VLP yields are consistently low or quality is compromised, the transformation efficiency should be improved. This can be achieved through the optimization of Agrobacterium strains used or infiltration parameters. Assessment of the post-translational modifications should be investigated since *Nicotiana Benthamiana* may be able to provide Eukaryotic modifications, yet specific patterns may not be identical(Hemmati et al.). If the VLPs immunogenicity gets compromised due to the PTM differences, antibody neutralization may be directly impacted in the in vitro assay. Another limitation is the potential for VLP instability. VLPs are complex macromolecular structures prone to instability under certain conditions, which could occur in extraction, purification or storage. These factors are temperature, pH, shear forces, and the presence of proteases that could lead to aggregation or degradation of the VLPs or loss of structural integrity. The paper “Platform Process for an Autonomous Production of Virus-like Particles” by Simon Baukmann et al suggests using a membrane pump to reduce shear forces, which should be considered.

The last limitation is the potential for immunodominance. Immunodominance is the phenomenon where multiple antigens are presented to an immune system, leading to the mounting of a stronger response towards certain antigens while neglecting others. In the context of this multivalent vaccine, the immune system may preferentially recognize, e.g, the Wuhan-1, leading to insufficient or no protection against the other strain. If immunodominance is present during the experimentation, it will be countered by utilizing adjuvants. Adjuvants are tools that modulate and enhance immune responses, thus assisting in the activation of both humoral and cellular immunity, meaning that the responses will be boosted for the less dominant epitope. (Vogel and Bachmann; Nooraei et al.)

By methodically addressing these potential issues, the root of the issue may be identified, corrective measures will be implemented, and the quality of the VLP product will be confirmed, allowing for confidence in the subsequent steps.

Limitations of the Proposed Plant-based VLP Production System

While the proposed methodology is highly promising, it is vital to acknowledge the system's limitations and the subsequent downstream processing. Five main limitations have been identified: firstly, the downstream processing, VLP morphology, size, and polydispersity; secondly, immunogenicity and Post-translational Modifications; thirdly, plant defense mechanisms; fourthly, transformation efficiency; and lastly, the potential for VLP instability.

As already mentioned in this paper, the downstream processing and subsequent steps after the VLP production pose certain limitations, referring to complex processes that involve multiple stages. In the paper (Baukmann et al.), downstream processing is carefully analysed in the context of utilizing mammalian cells. Yet, these steps of VLP harvesting, product purification, and concentration, as well as their transferring to a suitable buffer, generally apply to VLP methodologies regardless of the expression system. Potential issues are highlighted, like filter fouling during transgential flow Depth Filtration (TFDF), which is a membrane technique used to initially harvest and clarify the VLP-containing solutions, due to particle accumulation, and the need for dynamic concentration adjustment during ultrafiltration/Diafiltration UF/DF, which is a two-part membrane separation used for further purification, concentration, and buffer exchange of the VLP product, to ensure a robust process. These challenges may be relevant for plant-produced VLPs, considering the complex mixture of plant components that would have to be separated from the VLPs

The second main limitation is the VLP morphology, size, and polydispersity. In the paper "VLP Formation" by (Nooraei et al. 2021) , what's emphasized are the biophysical parameters of VLPs, and the difficulty of achieving consistent VLP characteristics in any expression system. In addition, visualization like TEM (tomographic emission microscopy) may lead to particle deformation during sample preparation, potentially leading to misinterpretation of particle size, marking the importance of encompassing highly specialized techniques in the methodology, like Cryo-EM and AFM, which are safer in terms of sample deformation.

Immunogenicity and post-translational modifications may not be limitations of this proposal, yet they are essential to consider since they can be technically challenging. The quaternary structure of viral capsid proteins can differ in different expression systems due to post-translational modifications. *N. benthamiana* can perform eukaryotic modifications, but the specific types and patterns might not be identical. Differences in PTMs, like glycosylation, can potentially affect the immunogenic potential of the vaccine via receptor binding or immune cell recognition

Plant defense mechanisms are the fourth limitation since the system relies on transient expression in *N.benthamiana* using Agrobacterium-mediated transformation; limitations of this process can indirectly impact VLP production. The paper by (De Saeger et al.) analyzes the multifaceted plant defense system that Agrobacterium needs to circumvent for successful transformation. The plant defense system includes pattern-triggered immunity, inducing MAPK signaling, and reactive oxygen species production, both resulting in the release of phytohormones that induce anti-viral responses in the plant. Even though Agrobacterium can circumvent them, the plant defense system can still limit transformation efficiency, meaning that while it's a well-established method, reaching high and consistent yields may be challenging. Furthermore, even though *N. benthamiana* is considered amenable to *Agrobacterium transformation;* optimization like increasing bacterial transformation efficiency or reducing plasmid recombination, may still be required, and the efficiency could potentially limit the VLP yield

Advantages of the optimized vaccine

This proposal aims to address the current limitations of the SARS-CoV-2 landscape by introducing a novel concept: a multivalent VLP-based vaccine that aims to confer broad resistance against multiple SARS-CoV-2 strains. Current SARS-CoV-2 vaccines have undoubtedly been crucial in controlling the pandemic, demonstrating high efficacy. Yet, their effectiveness faced challenges from the emergence of Variants of Concern (VOCs), such as the Delta strain. Another significant limitation is the observed decrease in immunity over time(Fiolet et al.). The proposed VLP-based vaccine is specifically designed to address these issues. Multivalency for broader protection is the core novelty of the proposed vaccine. Incorporating spike proteins from two different SARS-CoV-2 strains will provide broader protection

against multiple viral strains, aiming to counteract the reduced efficacy observed with current single-strain vaccines against variants

The virus-like particle platform itself provides two powerful advantages. First is safety, VLPs are non-infectious since they lack the viral genetic material required for replication. The inherent safety profile of this vaccine is a key advantage, especially for immunocompromised individuals. Second, there is enhanced immunogenicity due to their size and shape, which resembles the SARS-CoV-2 virus. VLPs can elicit both humoral and cellular immune responses, thus stimulating robust protection in the patients(Nooraei et al.; Hemmati et al.).. With the success of this vaccine model, doors toward incorporating more spike proteins will be opened. The plant-based production system,N.benthamiana, offers several significant benefits. Plants provide the necessary machinery for eukaryotic post-translational protein modifications, which are crucial for proper folding and the immunogenicity of the VLP. Notably, the scalability and cost-effectiveness of the plant system offer significantly lower costs than mammalian cell culture systems. Plant-based systems are highly appealing for rapid and large-scale vaccine manufacturing, especially for pandemic response. Lastly, the whole production system is a tested technology along with the pEAQexpress vector; thus, investing in this proposal doesn't bear a significant risk.

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