Open-Source SARS-CoV-2 mRNA Vaccine

Gen 1 mRNA vaccine with conserved SARS-CoV-2 immunogen-modulated multivalence

Rapid Deployment Vaccine Collaborative (RaDVaC)

Preston Estep, Don Wang, Alex Hoekstra, Ranjan Ahuja, Brian Delaney, and over 100 others

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TERMS OF USE (updated 2022-05-27)

This document describes the rationale, design, formulation, and self-administration of an experimental vaccine candidate for SARS-CoV-2. By using this information you agree to the following: 1) you are a consenting adult (in the USA, at least 18 years of age); and 2) you take full responsibility for your use of RaDVaC information and vaccine or material--including redistribution, modification, vaccine formulation, production and administration.

A foundational principle of the vaccine development and deployment strategy of RaDVaC is rapid iteration and testing of vaccine designs, based on newly published information from the forefront of biomedical research. This agile approach has the potential to produce better vaccines much more quickly than traditional approaches. However, such rapid design improvements currently are not compatible with established clinical trial requirements of fixed vaccine design, nor with review and approval by ethics committees (due to shifting vaccine design and continually updating self-experimentation and testing protocols). Therefore, the information presented within has not been approved by an institutional review board or any other type of ethics committee, and you understand and agree that any implementation of this information constitutes self-experimentation.

The purpose of this open-source vaccine effort is to reduce risk of harm from SARS-CoV-2. In addition to providing the results of our own research and experimentation, we hope to motivate others to build on our work, and to pursue diverse evidence-based approaches. Given the immense complexity and variability of individual human biology, it is not possible to predict all potential physiological responses to any vaccine. But as is true for most or all vaccines used on a large scale, there is a tradeoff between a larger known risk from the disease the vaccine is designed to prevent or mitigate, and the smaller risk introduced by the vaccine itself. Any vaccine poses risks, and, if used in enough people, will cause some degree of harm. Furthermore, certain harm, such as allergic and possibly anaphylactic response, will be readily seen and measured, whereas benefit is more difficult and takes longer to assess. These vaccines are no different in this regard; and because quality of delivery is highly dependent on the meticulousness of individual end users, the vaccines might pose unique risks not posed by typical commercial vaccines.

Does Not Constitute or Substitute for Medical Advice

Information presented here is ongoing research, and is not intended as a substitute for medical advice. RaDVaC is not responsible for the decision to administer, or to receive administration of, any vaccine.

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No Promises or Guarantees of Efficacy

Vaccines are often received with the false hope of efficacy, without testing to determine the degree of individual immune response. For example, influenza killed about 100,000 people in the U.S. between late 2016 and early 2018. Yet, the influenza vaccines available in that period were substantially less than 50% effective against H3N2, the flu strain mainly responsible for the death toll. Many who died were vaccinated but not protected from the virus, and testing for vaccine-induced immunity was essentially non-existent. Vaccine-induced immunity can be more challenging to assess than immunity due to viral infection, and such is probably the case for the vaccine described here. Because this work is a research undertaking, no expectation is given regarding any degree of efficacy in granting protection against SARS-CoV-2. On the contrary, see possible risks and uncertain benefits below.

Preventive, Not Therapeutic

Even if this vaccine works as intended, it will not help someone who has been infected. It will only work as a preventive measure taken weeks in advance of virus exposure.

Not a Clinical Trial

Any use of the information in this document, or provided in correspondence with us does not constitute ‘partnership’ or ‘recruitment’ for an organized trial in any way. The supported conjecture here is given as a starting point for additional individual or organized, sponsored research. As of public release of this information, no organized clinical trials have been performed to test this vaccine.

Not Approved or Reviewed by the FDA

The information, procedures, and conclusions presented here have not been approved, or reviewed by the FDA, or any other regulatory body.

Possible Risks and Uncertain Benefits

- Immediate allergic or other serious reaction
- Unforeseen long-term effects
- Instillation/administration of the vaccine in an inappropriate way or in an infected area might increase the risk of infection by enhancing viral entry into the body
- Benefits are uncertain. There are extensive published histories of the materials and procedures described in this document, but every novel vaccine should be considered experimental, with the possibility there will be no benefit.

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- Even if there are signs of immune response there is no guarantee this response is indicative of protection from SARS-CoV-2 infection, or if protection is achieved, how long it will last.
- Even if the vaccine confers protection from the virus, certain methods for assessing protection--such as measuring antibodies due to previous infection--might not capture vaccine-induced immunity. In such cases, infection, and thus a positive test result, are unlikely to occur. Immunity passports and other privileges given to convalescents might be difficult to obtain for those with vaccine-induced immunity.
- Use of this vaccine may change the efficacy of any future vaccines you may take or that are administered to you, in unknown ways

No Offer of Service and No Access to Materials

All information in this document or via correspondence is made available as open science. We do not sell equipment or services, e.g. we will not prepare this formulation or any other on behalf of a requestor. All vaccine materials at our disposal are kept in a secured laboratory. We cannot and will not keep materials outside the lab for easy access or distribution. We will not provide laboratory or equipment access for vaccine production.

Probability and Coincidence

Vaccines have become associated with negative health events that do not result from the vaccine, but that happen to occur soon after administration. Negative health events will occur with baseline frequency even in the absence of vaccination. Increasing use of any vaccine will increase the probability of vaccine recipients experiencing a negative health event purely by chance, including shortly after vaccination. By using the information presented here, you acknowledge the increasing likelihood of such coincidences, and assume full responsibility for any use of the information herein and for real or perceived negative events, irrespective of the cause.

Self-Experimentation

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Additionally, the type of work described in this document requires certain equipment and level of skill with laboratory techniques. You agree to assume full responsibility for acquiring proper equipment, knowledge, and training, and for attempting to formulate or administer vaccine.

Licenses

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Overall RaDVaC project goal (updated 2022-05-09)

The Rapid Deployment Vaccine Collaborative (RaDVaC) was founded by members of the Mind First Foundation in March of 2020 in order to address the outbreak of the SARS-CoV-2 virus, which quickly became a global emergency. The SARS-CoV-2 pandemic produced many notable accomplishments, including the most rapid large-scale vaccine trialing and emergency deployments in history. Nevertheless, vaccines were only available to selected members of the public in high-income countries about a year after initial signs of the emerging pandemic. This year-long “vaccine access gap” allowed SARS-CoV-2 to spread largely unchecked throughout the world.

The goal of RaDVaC is to create a modular vaccine platform for extremely rapid deployment at the beginning of a serious outbreak of any pathogen for which no good vaccine is available. The foundational philosophy of RaDVaC is that the best way to accomplish this goal is to freely, openly, and publicly share all of its work on vaccines and trialing; and to advance a distributed, global network of researchers engaged in agile R&D. For many individual researchers, public health authorities, and others involved in vaccine development and deployment, this will include localized and decentralized vaccine production and trial design. While the SARS-CoV-2 pandemic was the catalyst for the initiation of the RaDVaC project, it will not be the last serious outbreak; it is critical to establish a reliable scientific and technological foundation for rapid, decentralized vaccine deployment in future scenarios. This long-term endeavor will necessitate paradigm shifts in vaccine R&D, testing, and production, as well as in data sharing and regulatory frameworks.

We intend for our work to comprise an evolving open-source toolkit (or Vaccine Developer Kit / VDK) to enable vaccine R&D, production, testing, approval, and deployment that is faster, less expensive, and more adaptable and agile to viral variants and regional needs. As proponents of citizen-science and deeper public engagement in scientific issues, we envision a scientific and medical culture that promotes greater access and participation, and which is more scientifically robust and resilient than the current norm. Ultimately, we seek to model and promulgate open-source practices of information- and expertise-sharing so that global challenges of all kinds can be addressed more effectively.

Vaccines and the COVID-19 global emergency (updated 2022-05-12)

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; a.k.a. 2019-nCoV; disease: COVID-19) is responsible for a worldwide pandemic far beyond the scope of any other public health crisis in over a century. As of May, 2022, the official worldwide COVID-19 death toll is over 6 million. According to analyses by the Institute for Health Metrics and Evaluation, and separately,

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by Samira Asma, Assistant Director-General of the WHO data and analytics division, the true number of deaths caused by COVID-19 is likely more than three times the official count[1],[2].

Higher-income countries now have excellent vaccine access, although substantial fractions of populations in almost all countries refuse to vaccinate. Vaccine access is far worse in low- and middle-income countries. These countries had only minimal access to commercial vaccines through 2021, and will likely not have sufficient access to these vaccines until well after 2022[3]. These significant delays not only lead to continued deaths and chronic illness, but also allow additional viral strains, including escape mutants to arise, compounding the scientific and public health challenges for both low-income and high-income nations, and threatening the fragile progress toward recovery as these new variants spread across the globe.

Because of these large, geographically distributed reservoirs, experts are forecasting that SARS-CoV-2 is transitioning toward being globally endemic. New variants are predicted to emerge regularly for the foreseeable future, causing case and mortality spikes similarly to influenza, though with higher absolute mortality. Only effective, broad-spectrum coronavirus vaccines have the potential to bring this deadly transition phase to an end. The unvaccinated might better benefit from such next-generation vaccines targeting newer variants, rather than settling for inferior protection from vaccines designed for variants no longer in circulation.

In a pandemic situation, ideally the first choice one must make in the decision to use a vaccine is which of the two possible ways one will first be exposed to immunogenic elements of a pathogen: by infection, or by vaccination. Vaccines are designed to stimulate an immune response that provides long-lasting protection against infection and/or disease by a pathogen. While infection by that pathogen and recovery from disease can sometimes also bestow such protection, pathogens, including viruses, possess many complex mechanisms for hijacking cellular and physiological functions and evading immune responses. Immunity gained from pathogen infection can therefore be suboptimal, or even dysfunctional due to these pathogen tactics. The same mechanisms can compromise health, and cause prolonged suffering and possibly death. We believe therefore that vaccine immunity is a far better choice; but in the current pandemic that option was available to almost no one for more than a year, and is still not available to much of the global population.

Starting in March, 2020, our group followed protocols for our first vaccine – a peptide-based intranasal vaccine,[4] continuing to improve the vaccine through multiple versions over the course

[1] https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(21)02796-3/fulltext
[2] Samira Asma, WHO press briefing, 2021-05-21. https://www.reuters.com/article/idUSL5N2N81LY
[3] https://www.theguardian.com/society/2021/jan/27/most-poor-nations-will-take-until-2024-[...]-immunisation
[4] https://radvac.org/white-papers/

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of nearly two years[5]. With the new RNA vaccine described in this paper, we continue to assess new research on SARS-CoV-2 in order to improve vaccine designs and efficacy, and continue to self-administer the vaccine versions, as before. We are also exploring additional vaccine platforms and technologies, including cell-free production of protein antigens/immunogens.

There is substantial published information on all aspects of vaccine production, testing, and delivery, an increasing amount of which is specific to the SARS-CoV-2 virus. In addition, thanks to generous grants from multiple organizations, including the Bakewell Foundation and the Balvi Filantropic Fund,[6] we will be able to extensively assess new versions of the vaccine with preclinical safety and immuno-efficacy studies of our own.

Vaccines are the safest of all therapeutic classes. Because of their safety and efficacy, they have by far the highest probability of success in the overall course of clinical trials (over 40%, which is about twice the rate of the next highest class).[7] Because of this and the impact of the pandemic, commercial trials have been greatly accelerated.

Nevertheless, commercial vaccines must be designed to enable large scale production and deployment, and they require regulatory approval for sale, both of which greatly constrain and slow progress. Critically, certain features of commercial vaccines aren't required for research-level production and testing; therefore, a much shorter timeline is possible for a smaller scale, self-administered vaccine. Herein we describe the formulation of a vaccine using proven and mostly off-the-shelf components, with a low per-dose cost.

This will be a versioned living document, embodying and enabling incremental improvements in vaccine design and testing.

VACCINE BACKGROUND AND DESIGN (updated 2022-05-16)

There are many advances in vaccine research and technology development that have not appeared in a commercial product, in part because commercial vaccine design and production is constrained by different factors than a small-scale research vaccine. Desirable features of a RaDVaC open-source vaccine are high safety and efficacy, low cost, and ease of production and administration. A key feature of the RaDVaC vaccine is rapid iteration in order to update designs as new discoveries about the biology and clinical manifestations of SARS-CoV-2 are published in

[5] Archived white papers on peptide designs and manufacture can be found at https://radvac.org/white-papers/. These designs may continue to be relevant in some contexts and under certain constraints of material and equipment availability.
[6] https://radvac.org/wp-content/uploads/2022/05/Balvi_RaDVaC-announcement.pdf
[7] https://projectalpha.mit.edu/pos/

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the biomedical literature and on preprint servers. This feature is critical to the RaDVaC mission of rapid development and deployment of effective vaccines. We address all of these features in this document, and consider subcategories, such as near-term and long-term safety.

Key issues and some differentiating factors between commercial vaccines and the RaDVaC research vaccine platform:

- **Rapidly responsive design.** A key feature of our rapid deployment strategy is the flexibility of the vaccine platform, allowing very fast and efficient updating of vaccine designs in response to recent research. On multiple occasions we have updated vaccine designs based on important information published in the biomedical literature only weeks or days earlier.

  One example is the important discovery showing that T-cell responses are found in patients almost two decades after recovery from SARS-CoV-1 (a very closely related coronavirus)[8]. The portions of the virus responsible for these very long-lived T-cell responses (CD4 and CD8) are in highly conserved parts of the virus. They are not present in the Spike protein, which is not highly conserved, and which is the primary target of leading vaccines in the current race to commercialization. A second publication reveals the identities of 29 experimentally verified, immunodominant CD8 T-cell epitopes of SARS-CoV-2[9]. This publication confirms several important findings of a prior preprint[10], and some of these epitopes are shared among the small group of long-lived epitopes from SARS-CoV-1, none of which resides in the Spike protein. Only 3 of 29 epitopes lie within the Spike protein, and only one lies within the RBD region of Spike. Another subsequent publication experimentally confirmed and extended these findings, also showing that Spike epitopes are not among the top 10 immunodominant CD8 T-cell epitopes[11]. These findings suggest that the Spike protein might not provide extensive and robust T-cell protection, and that RBD will provide even less protection for many people, especially the large numbers of people with MHC haplotypes not represented by these antigens.

  Consistent with this expectation, data on the leading commercial vaccines suggest that they do not confer sterilizing immunity (the immune system's ability to stop a pathogen from entering the body, or if it gains entry, from replicating within the body). Again, the RaDVaC strategy allows for frequent updating of vaccine designs based on ongoing

[8] https://www.nature.com/articles/s41586-020-2550-z
[9] https://www.medrxiv.org/content/10.1101/2020.07.24.20161653v2.article-info
[10] https://www.biorxiv.org/content/10.1101/2020.06.05.134551v1.full.pdf
[11] https://www.medrxiv.org/content/10.1101/2020.07.31.20165647v2.article-info

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research throughout the world, so that the level of protection keeps pace with new knowledge, and new variants.

- **Near-term safety.** Near-term safety of the vaccine described here should be excellent when prepared appropriately. RNA vaccines have demonstrated an extremely favorable risk profile.[12] The vaccine formulations described here can also be used for a number of delivery routes, including intranasal, inhalation, oral, or parenteral delivery (injection), but these, especially the latter, should be attempted only by skilled practitioners. In general, a vaccine is more safely used for inhalation into the lungs[13], or for oral or intranasal delivery, than for injection. This document is focused on mucosal delivery modes (intranasal, sublingual, etc.) and self-administration, but experts will know how to adapt the information herein to another delivery route. For any delivery mode, sourcing of high-quality materials and meticulous preparation are essential for maintaining vaccine safety.
- **Long-term safety.** The long-term safety of any new vaccine is currently difficult or impossible to predict. Even widely deployed commercial vaccines have resulted in serious and unforeseen complications. Vaccines that have shown serious side effects are injectable whole virus or subunit formulations. There are at least three potentially serious complications that might arise in the long term: tolerance, vaccine-enhanced disease (VED), and adjuvant-triggered immune or neurological complications.
    - **Immune tolerance** denotes diminished immunity resulting from exposure to an antigen. This attenuated immune response is commonly observed in food antigens, in “self” antigens of one’s own body, and in antigens from commensal organisms. In general, it is thought that extremely large and/or frequent exposures and oral doses lead to tolerance. While in the above examples, immune tolerance is desirable, in the case of vaccines it can cause the immune system not to mount an appropriate defensive response to infection.
    - **Vaccine-enhanced disease (VED)**. A small number of injected vaccines have led to enhancement of disease, meaning that infectivity is enhanced, or the disease is made more serious in people who have been vaccinated, relative to unvaccinated controls. Possible mechanisms underlying VED, such as antibody-dependent enhancement (ADE) are explored at length in the section below, together with various vaccine design features and strategies for reducing the likelihood of VED.
    - **Adjuvant hyperstimulation or toxicity.** Adjuvants help stimulate a robust immune response to a vaccine; however, certain adjuvants have caused hyperstimulation

[12] https://pssjournal.biomedcentral.com/articles/10.1186/s13037-021-00291-9
[13] https://www.sciencedirect.com/science/article/pii/S1549963415000313

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and other serious side effects. For example, alum produces a robust Th2 immune response, but an unbalanced ratio of Th2:Th1. A Th2 polarized response, and alum in particular, have been implicated in immunopathology, including ADE. Adjuvants can also be toxic. As one example, the intranasal use of a detoxified mutant form of *Escherichia coli* Heat Labile Toxin has resulted in transient Bell's palsy, or facial nerve paralysis.[14] Specific adjuvants have also been suggested to play a role in the development of autoimmunity. For example, the influenza vaccine Pandemrix appears to have increased the incidence of narcolepsy in Scandinavian countries [15]. The leading explanation is that the vaccine triggered a CD4-mediated autoimmune reaction against sleep-regulating neurons (although the vaccine-attributable risk is very low, with 1 case in 18,400 vaccinations, or about 0.005%). One suggested contributing factor is the strong squalene-based adjuvant used in Pandemrix. The primary reason for using strong adjuvants in commercial vaccines is to avoid the need for boosters, by triggering a robust immune response with a single dose.

- **Stability.** Stability is a key determinant of a commercial vaccine. Formulations that are both safe and effective in a research setting, but with limited shelf life, are generally excluded from commercial products. We have discovered that there are formulations for vaccines that are extremely simple, safe, and effective, but have only short-term stability (on the order of weeks). For example, chitosan gel nanoparticles, used in our previous vaccine platform,[16] have been shown to be effective and extremely simple to formulate, but short shelf life has contributed to their limited use in commercial vaccines. RNA is extremely labile in aqueous solutions. Distribution of commercial RNA vaccines has relied on costly and extensive cold chains. Therefore, production at the point and time of use is highly preferred for liquid RNA formulations. Alternatively, dry RNA formulations might be prepared by freeze-drying or lyophilization of vaccine, although this currently remains challenging.
- **Ease of delivery.** Mucosa-targeted vaccines have advantages over other delivery approaches, including the most common delivery modality, parenteral/injection. Intranasal delivery, for example, has been demonstrated to be very safe, with mild side effects typically equal to those seen in placebo-treated subjects. Importantly, intranasal delivery can elicit not only systemic immunity, but also mucosal immunity at the point of infection for respiratory viruses.[17] Commercial intranasal influenza vaccine is available, and relative

[14] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2737308/
[15] https://pubmed.ncbi.nlm.nih.gov/28847694/
[16] https://radvac.org/white-papers/
[17] https://pdfs.semanticscholar.org/6e55/db027b08b367eaac55ec54a730f4c99061ec.pdf

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to parenteral (injected) administration shows equal efficacy for systemic immunity, but greater efficacy for mucosal immunity at sites of entry (nose, lungs) for respiratory viruses.[18] This is critically important for SARS-CoV-2 because early studies show that a majority of infections begin in the nasal cavity[19]. As of May 2022, most commercial SARS-CoV-2 vaccines in development are designed to be injected (parenteral), a route which is unlikely to provide mucosal immunity to infection. As of early 2022, we have also been exploring other methods of delivery that we expect to be easy to administer, safe, and effective, including sublingual and transdermal delivery systems.

- **Prime-Boost.** Alternative methods of delivery can be as effective as injection, but to achieve the same level of immune response and protection--especially with milder vaccines and adjuvants--multiple doses are often required. The initial dose is the prime and subsequent doses are given to boost or increase the immune response. Prior exposure to a pathogen or closely related one has a similar effect to a prime dose of vaccine. The only commonly used intranasal vaccine is for influenza, and since essentially everyone is naturally exposed to influenza, the nasal vaccination with attenuated virus is in essence a booster. Necessity of multiple boosters has limited the commercial production of intranasal (and other mildly/moderately immunostimulating) vaccines for which prior ubiquitous exposure is unlikely. An important corollary to a multiple-dose design is titration of adequate immune response for each individual. Whereas the immunogenicity-safety profile of commercial formulations must be standardized to an "average" level--overdosing some and underdosing others--with accompanying testing the RaDVaC approach should allow for more precise dosing.
- **Efficacy**. Intranasally delivered vaccines have shown mild side effects and high levels of efficacy of both mucosal and systemic immunity, when delivered in a prime-boost regimen (in both animal models and human trials; see one review by Smith et al.[20]). One of our research priorities is to determine when other non-parenteral/injection delivery modes will prove to be equally safe and efficacious.

Given the above key points, it is clear that a rapid-deployment vaccine that is both safe and effective is likely to have multiple attributes that differentiate it from a commercial vaccine. And it becomes clear why the formulation described here has not been used in a commercial product; it is primarily not lack of safety or efficacy, but other factors related to stability, scalability, patient compliance, profitability, and regulatory complexities of commercial vaccines. Commercial vaccines are designed to produce high levels of immunity with a single dose. A prime and

[18] https://www.tandfonline.com/doi/abs/10.1586/erv.12.31
[19] https://www.cell.com/cell/pdf/S0092-8674(20)30675-9.pdf
[20] https://www.tandfonline.com/doi/full/10.4161/hv.27449

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multi-boost schedule is used routinely in the research setting, but concerns about compliance (failure to boost) limit commercial deployment.

For multiple reasons, including those given above, non-parenteral/non-injection delivery is difficult to achieve for a commercial vaccine. However in the research context it has shown multiple advantages. To start, relative to injection, there is no risk of needle injury or transmission of blood-borne infection. Further, it is not only relatively easier to administer, but in certain cases and for some pathogen classes like respiratory viruses, intranasal or other mucosal delivery modes are in fact the preferred methods of delivery. Immunization by the intranasal route, for example, not only prevents viral infection through the nasal membranes, but also efficiently stimulates a strong mucosal immune response in the upper respiratory tract and lungs. Sublingual and other mucosal delivery modes are also likely to induce a strong mucosal immune response. As one example, Gai and colleagues have shown that a SARS vaccine delivered intranasally elicits a robust mucosal immune response, protecting against initial infection, whereas the same vaccine delivered by injection does not.[21] This difference is important because the area of the mucosal surfaces (nasal, lungs, gastrointestinal, urogenital, etc.) is very large, about 200 times the surface area of the skin, and about 70% of pathogens enter through these routes.[22] Compliance is also very high for a single dose because intranasal delivery doesn't involve needles or cause pain or significant discomfort. High safety and ease of administration are expected to contribute to high rates of immunization. For a recent review of research on nasal nanovaccines, see Bernocchi et al[23] (Table 1).

Both synthetic peptide-based vaccines, as well as nucleic acid vaccines permit a more curated and intentional immunogen design than do vaccine platforms which are based on attenuated whole viruses or even full-length Open Reading Frames (ORFs) of key epitope proteins. In commercial vaccines, there is a trend toward highly defined, minimal designs. According to Pompano and colleagues "[v]accine design is moving away from using whole pathogens in favor of selecting only the most protective antigens for immunization with a suitable adjuvant. The most tailored of these sub-unit vaccines utilize specific B-cell or CD8+ T-cell epitopes, such as the short peptides and carbohydrates that have been used to elicit antibody or killing responses against malaria pathogens, bacterial infections, or tumors. In addition to the desired target epitope, these vaccines also require one or more CD4+ T-cell epitopes to engage CD4+ helper T-cells."[24]

Such compact and efficient designs are not achieved easily, and require substantial data on the immunogenicity of all epitopes of a pathogen, but such designs have the advantage of limiting

[21] https://scholar.google.com/scholar?cluster=13722847195977601349&hl=en&as_sdt=0,22
[22] https://pdfs.semanticscholar.org/6e55/db027b08b367eaac55ec54a730f4c99061ec.pdf
[23] https://www.ncbi.nlm.nih.gov/pubmed/28698066
[24] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4227912/

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pathogenic effects of certain viral antigens. For instance, coronavirus Spike and Nucleocapsid full-length proteins have been associated with ADE in animals and human cellular studies. Yasui and colleagues showed that vaccination with Nucleocapsid protein does not provide protective immunity, yet enhances immunopathology.[25] Vaccination with certain epitopes of Spike protein does confer protection, but use of the full-length Spike might be problematic. For example, from Tais and colleagues: "...full-length S protein should be used with caution. Kam *et al*. reported that although a recombinant, trimeric SARS-CoV S protein vaccine elicited a protective immune response in mice the anti-S antibodies also mediated antibody-dependent enhancement of viral entry into human B-cells *in vitro*. In another study, ferrets vaccinated with SARS-CoV full-length S protein expressed by a recombinant modified vaccinia Ankara grown in BHK21 and Vero E6 cells show enhanced virulence of hepatitis induced by SARS-CoV. Furthermore, the use of a SARS S protein vaccine may lead to enhanced disease and immunopathology instead of protection as seen for feline coronavirus, feline infectious peritonitis virus. Given these concerns, the use of a SARS vaccine strategy in which the full-length S protein is used may not be optimal for humans. Hence, the best approach would probably be to use small S protein epitopes that are major neutralization determinants."[26] Vaccines against other pathogens that similarly use full-length constructs have also elicited enhancement of viral sensitivity or disease. Nevertheless, as of mid 2022, billions of people have been vaccinated with full-length Spike vaccines, and the frequency of serious side effects is minimal. Furthermore, as of Dec 2021, the combined seroprevalence of vaccination and/or infection reached about 95% of the adult population in the US.[27] Global exposure in 2022 approaches a similarly high frequency. Therefore, exposure to full-length Spike is the norm, and one can either be exposed through vaccination or through infection. We believe it is vastly preferable to be vaccinated than infected. The vaccine design described here is based on the full-length spike protein, in addition to predicted and experimentally validated T-cell epitopes. If you decide to modify the design detailed herein, please let us know and keep us informed of additional salient information.

[25] https://www.jimmunol.org/content/181/9/6337
[26] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2756483/
[27] https://jamanetwork.com/journals/jama/fullarticle/2793517

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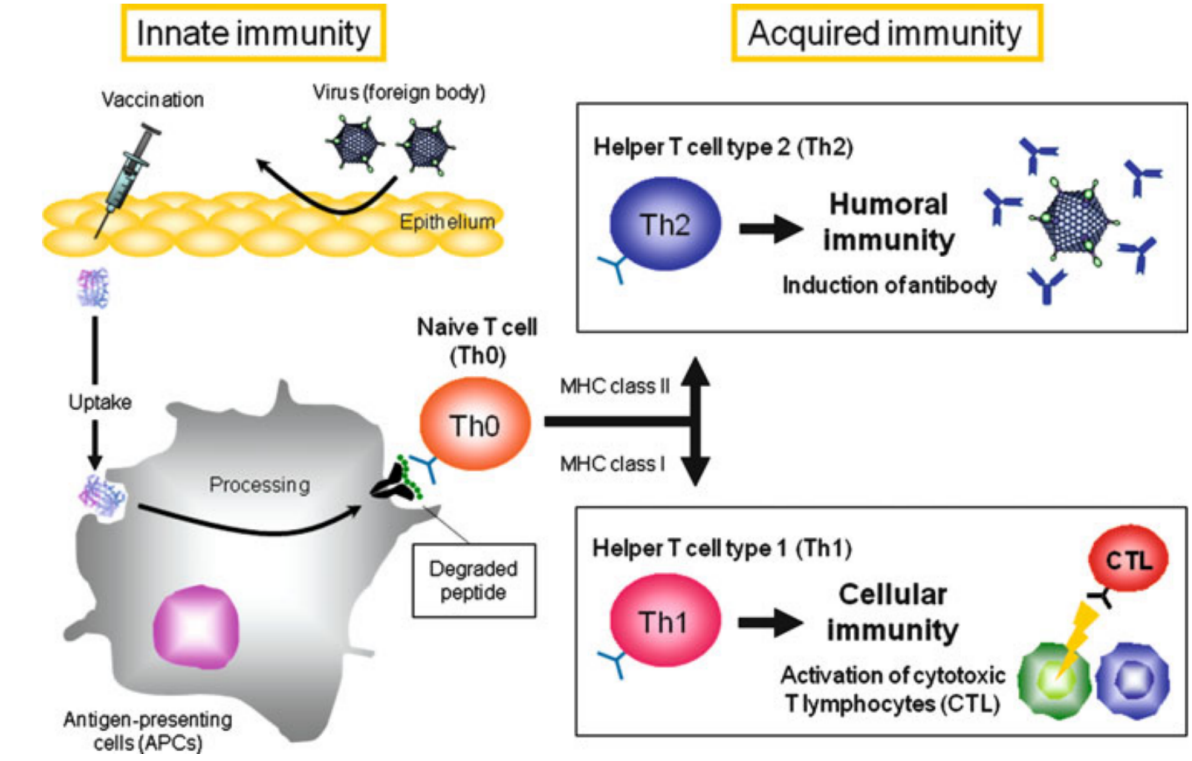
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Acquired immunity
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Epithelium
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Processing
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Degraded
peptide
Antigen-presenting
cells (APCs)
Helper T cell type 2 (Th2)
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Activation of cytotoxic
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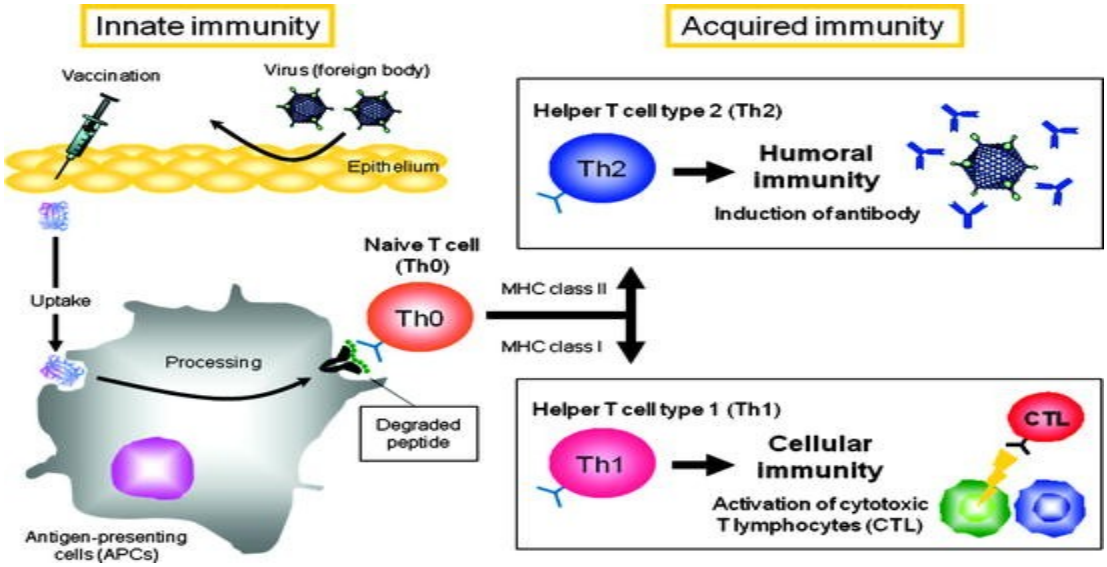
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Th2
Humoral
immunity
Induction of antibody
Helper T cell type 1 (Th1)
Th1
Cellular
immunity
Activation of cytotoxic
T lymphocytes (CTL)
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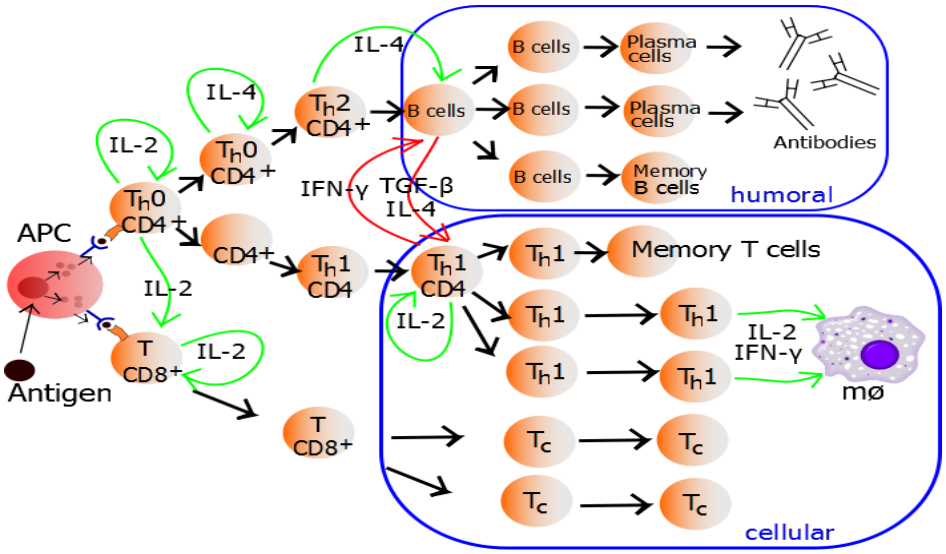
Schematic showing pathways for Th1 and Th2 acquired immunity (a.k.a. adaptive immunity), and roles of MHC class I and class II. Figure from Akagi, Baba and Akashi[28].



Th1/Th2 Model for helper T-cells. An antigen is ingested and processed by an Antigen Presenting Cell (APC). It presents fragments from the antigen to T-cells. The upper, Th0, is a T helper cell. The fragment is presented to it by MHC2. IFN-γ, interferon γ; TGF-β, transforming growth factor β; mø, macrophage; IL-2, interleukin 2; IL-4, interleukin 4. From Wikipedia[29].

Immunopathology isn't exclusively determined by immunogen, and can be influenced greatly by adjuvant choice. Antibody-dependent enhancement of disease and immunopathology appears to be mediated primarily by a Th2 (or Th17) response, and opposed by a Th1 response [30]. Extensive research on Respiratory Syncytial Virus, SARS, and other respiratory viruses shows that ADE immunopathology will be more likely with the use of alum or other Th2-boosting adjuvant.[31] From Honda-Okubo et al: although an anti-SARS vaccine "formulated with alum protected against mortality, these mice developed severe lung eosinophilia at day 6 post-challenge, reminiscent of the lung pathology induced by alum-adjuvanted RSV vaccines and indicating alum's general lack of suitability as a coronavirus vaccine adjuvant." These concerns make alum alone a poor adjuvant choice. However, seroconversion in response to vaccination becomes more difficult with age; thus, older people might benefit from the addition of additional potent adjuvants, including

[28] https://link.springer.com/chapter/10.1007/12_2011_150
[29] https://en.wikipedia.org/wiki/T_helper_cell
[30] https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0035421
[31] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4337527/

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those that promote some degree of Th2 response. This is an important consideration for SARS-CoV-2, since older people are most susceptible.

The amount of vaccine delivered is important. Too little antigen is unlikely to elicit a strong immune response, but too much might attenuate response. Delivery route is also important, with oral delivery being the route most associated with tolerance. Furthermore, high doses of mucosal antigens appear to be required in humans (50+mg) for inducing systemic tolerance, whereas low doses do not appear to induce systemic tolerance.[32]

One challenge for mucosal vaccines is verifying systemic efficacy. Many published reports show that nasally delivered vaccines have high efficacy for prevention of infection at the nasal mucosa, typically equivalent or superior to injected vaccines for this most important measure. However, efficacy is not as easily measured or predicted by traditional measures, such as anti-virus or anti-epitope antibody titer (e.g. as measured by ELISA) in serum. This is in part because blood is more quantifiable than mucosal secretions, and in part because the primary means of conferring immunity is through mucosal stimulation and response, which less reliably translates to systemic immunity. Measuring efficacy against SARS-CoV-2 is also difficult because the B-cell/antibody response to coronaviruses is highly variable between individuals, and provides uncertain immune protection. Sustained immune protection appears to be mediated in large part by the T-cell response, which is much more difficult to measure than the antibody response. In certain cases, nasal vaccination can impart mucosal immunity, but no measurable systemic immunity. In such cases, resistance to viral infection due to mucosal immunity will not be predicted accurately by a negative result for IgG systemic antibody. Antibody can be measured in nasal wash, however this is less quantitative and reliable than measurement in serum. Because of such issues, alternative approaches to predicting overall immunity have been developed and published. These include systems biology approaches of blood-based profiling of immune proteins and transcriptomics.

Summary of key general features of an open-source, citizen-science vaccine:

- **Safe ingredients.** Long history of published results.
- **Ease and robustness of production.** Simple to make but with the possible tradeoff of short stability.
- **Needle-free delivery.** Intranasal administration is a preferred delivery mode for vaccines against respiratory and nasal viruses, and offers one of the highest safety profiles. Potentially stimulates both protective mucosal immunity at the site of infection, and systemic immunity.

[32] https://academic.oup.com/intimm/article/26/9/517/2950826

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- **Immunogens: structurally accurate, immunogenic, and easily produced.** These can be mined from a rich literature, or produced de novo. DNA plasmid templates are fairly readily procured, and can then be adapted to generate mRNA constructs within cell- or cell-free expression systems..
- **Booster schedule.** Allows use of intranasal delivery and a lower dose or milder adjuvant, yet has the potential to produce immune responses equivalent to a single dose of hyper-stimulatory adjuvanted vaccines.

POSSIBLE MECHANISMS OF VACCINE-ENHANCED DISEASE, VACCINE-INDUCED AUTOIMMUNITY, AND MITIGATION STRATEGIES (updated 2020-10-24)

As mentioned briefly in the section above, a small number of injected vaccines have led to vaccine-enhanced disease (VED), meaning that disease is made more serious in people or experimental animals that have been vaccinated, relative to unvaccinated controls. This has occurred in response to vaccines for respiratory syncytial virus (RSV), dengue, Zika, and others. Some of these cases involve experimental vaccines, although RSV and dengue were deployed in healthy human populations. Although there isn't a single accepted or proven mechanism known to cause or contribute to VED, there are historical vaccine design features and adjuvant factors that are suspected contributors. The RSV vaccine that caused VED in the 1960s was formalin inactivated whole virus, adjuvanted with alum. This vaccine triggered a weak initial immune response, and theoretical explanations for how it caused VED have focused on the chemical alteration of antigenic structures caused by formalin and on the Th2 bias induced by alum. Recent studies have shown that, in addition to Th2 bias, RSV VED is driven by a few factors including deficiencies in Toll-like receptor activation and CD8 response, and hyperactivation of a distinct subset of CD4 T-cells.[33],[34],[35] Mitigation of any of these reduces or obviates VED. With dengue virus, a second infection with a distinct dengue serotype often causes more severe disease than the first infection. Use of the dengue vaccine Dengvaxia similarly results in more severe disease than in unvaccinated controls, suggesting that vaccination sets up a serious subsequent infection by similar mechanisms to a prior dengue infection. (It is important to note that although named "vaccine" enhanced disease, the same phenomenon of enhanced disease with subsequent infection has been described in natural infection with multiple pathogens, and could be due to any number of immune-evasive strategies viruses have evolved.)

[33] https://www.nature.com/articles/nm.1894
[34] https://pubmed.ncbi.nlm.nih.gov/25769044/
[35] https://pubmed.ncbi.nlm.nih.gov/17911628/

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One proposed mechanism for VED in dengue is antibody-dependent enhancement (ADE), in which antibodies of the systemic immune system act in “Trojan horse” fashion to facilitate viral entry into certain host immune cells. (For a review of ADE specific to SARS-CoV-2 see Eroshenko et al.[36]) Waning or weak antibody response, including a substantial non-neutralizing antibody component to this response, has been shown to facilitate viral entry into macrophages and monocytes. There are multiple possible ways this might occur. There are multiple dengue serotypes, which are distinguished by substantial protein sequence divergence. Amino acid differences in key epitopes can alter binding of antibodies, and the result is that antibodies that are neutralizing for one serotype bind antigens of another serotype, but in a manner that is weakly or non-neutralizing. One possible contributing factor in this process is a phenomenon referred to as “original antigenic sin,” or “antigenic imprinting.” Exposure to an initial antigen by infection by one serotype produces memory immune cells that recognize the amino acid sequence of this antigen, and upon subsequent exposure to a related but slightly different antigen, there is preferential expansion of memory cells that recognize the original antigen rather than cells that recognize and bind more tightly to the second antigen.[37] Thus, upon second infection, the antibody response is directed mostly at epitopes of the previous infection, and less robustly against the current infection. Antigenic imprinting is not limited to B-cell epitopes, and essentially the same phenomenon has been reported for T-cell epitopes and responses.[38]

Other aspects of the T-cell response also have been implicated in VED. In particular, a robust CD8 response is a key immune correlate of mild or asymptomatic infection for a number of respiratory viruses, including dengue, influenza, SARS-CoV-1, and SARS-CoV-2. Multivalent whole-virus dengue vaccines that provide robust and broad protection against dengue infection produce a robust CD8 response.[39] Notably, the CD8 epitopes that produce the strongest protection against all dengue serotypes are derived from conserved non-structural proteins, rather than the viral outer surface proteins, which have much greater amino acid sequence variation.[40] This appears to be a common feature of respiratory viruses, including influenza and SARS-CoV-2.[41],[42]

In contrast, Dengvaxia is based on a synthetic hybrid virus that uses the non-structural backbone of the related yellow fever virus, together with the outer structural proteins from dengue. Dengvaxia elicits a strong initial antibody response, but this strong response wanes relatively quickly. Since Dengvaxia lacks the immunodominant CD8 epitopes of the non-structural proteins,

[36] https://www.nature.com/articles/s41587-020-0577-1
[37] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7291596/
[38] https://pubmed.ncbi.nlm.nih.gov/9697771/
[39] https://jvi.asm.org/content/89/1/120.full
[40] https://www.frontiersin.org/articles/10.3389/fimmu.2019.02125/full
[41] https://pubmed.ncbi.nlm.nih.gov/18802496/
[42] https://www.nature.com/articles/s41590-020-0782-6

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the cytotoxic response is directed toward the weakly immunogenic dengue surface epitopes, and the immunodominant non-structural proteins of the yellow fever virus. Therefore, not only is the CD8 response specific to dengue epitopes relatively weak, but a possible exacerbating factor is that the primary CD8 response triggered by Dengvaxia is directed away from dengue by immunodominant yellow fever epitopes. To summarize one model of Dengvaxia-induced VED favored by some leading immunologists: upon waning antibody response (and possible ADE), compromised cellular immunity is weak, and upon subsequent infection T-cells mount a pathological cellular immune response, possibly exacerbated by antigenic imprinting, driven by the highly variable surface T-cell epitopes of the dengue serotype of the post-vaccination infection.

It is important to reiterate that immunopathological responses are not limited to vaccines, and are known to be mediated by viral exposures, as occurs in primary dengue virus infection followed by second infection by a distinct dengue serotype. However, as seen in RSV and dengue, vaccine designs can contribute to the likelihood of immunopathology. One early report on SARS-CoV-2 suggests that neutralizing IgG antibodies for the SARS-CoV-2 receptor binding domain (RBD) do not exhibit such enhancement; and as mentioned above, some experts suggest that ADE is not a clear concern in the development of vaccines against this virus[43],[44]. However, other experts are less confident that SARS-CoV-2 vaccine-induced ADE or other VED will not occur.

Given the immunopathology associated with Dengvaxia, we are especially concerned about the use of the same general design features that are suspected to have caused or contributed to VED. In particular, if SARS-CoV-2 immunodominant MHC class I/CD8 epitopes are concentrated on non-surface proteins, use of SARS-CoV-2 outer surface proteins alone might produce a weak CD8 response; and if this feature is combined with the backbone of another virus carrying potentially immunodominant CD8 epitopes, such a vaccine fits a general risk profile similar to Dengvaxia. As of late 2021, the most extensive quantitative T-cell epitope mapping data were published by Adaptive Biotechnologies and made public in the ImmuneCODE database[45]. The mapping of SARS-CoV-2 epitopes shows clear CD4 immunodominance, and also some degree of CD8 immunodominance. It is notable and concerning that the majority of immunodominant MHC class II/CD4 epitopes are found in Spike and other surface proteins (although these class II data do not include Orf1ab), while the majority of immunodominant MHC class I/CD8 epitopes (7 of the top 8; 12 of the top 15) of the entire proteome are found in non-structural proteins, analogous to the distribution found in dengue.

[43] https://www.biorxiv.org/content/10.1101/2020.04.10.036418v1
[44] https://www.nature.com/articles/s41587-020-0577-1
[45] https://www.researchsquare.com/article/rs-51964/v1

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Vaccine-induced autoimmunity (VIA) is another immunopathological complication of vaccination. It is a rare but worrisome side effect. Over the past decades, several vaccines have been implicated as autoimmune triggers[46]. Four possible factors have been proposed in explanations for mechanisms of VIA: molecular mimicry and cross-reactivity to host antigens; unbalanced or hyperstimulation of immunity by specific adjuvants; host genetics; and concurrent vaccination and infection. A combination of molecular mimicry of host antigens and certain potent adjuvants is especially concerning, and has been suggested as a leading explanation for VIA in narcolepsy triggered by Pandemrix, although specific Scandinavian host genetics appear to play a role. Other serious autoimmune diseases believed to be triggered by vaccines are Guillain-Barré syndrome, Multiple Sclerosis, and lupus. While these side effects are quite serious, they are also quite rare. The incidence of narcolepsy increased over 10-fold in those vaccinated with Pandemrix, but the vaccine-attributable rate was only 1 in 18,400[47]. In each case, molecular mimicry is a suspected primary contributor, and viral infection can trigger these same autoimmune reactions, sometimes even more frequently than vaccines. Therefore, as with other decisions regarding vaccination during a global pandemic, if certain antigens pose an increased risk of autoimmunity, one is likely to be exposed to those antigens eventually either by viral infection, or through vaccination. And in the case of vaccination, the overall adverse-outcome risk profile may be far lower.

In the list below, we summarize possible mechanisms of VED in precedent cases that might contribute to VED immunopathology in response to vaccination against SARS-CoV-2, along with some of the mitigation strategies we have used to address each of these in our vaccine designs.

1. Th2 and/or Th17 immune hyperpolarization, possibly accompanied by an attenuated Th1 response have been proposed to be leading risk factors for VED. It has been suggested that use of alum as an adjuvant creates such a Th2 bias and inhibition of Th1, while the use of other adjuvants, such as chitosan and toll-like receptor (TLR) agonists shift the balance in favor of Th1.
    a. In many published studies of mRNA packaged with lipid nanoparticles, our chosen delivery and adjuvant vehicle, vaccination elicits a balanced Th1/Th2 immune response.
2. ADE of viral infection is primarily associated with IgG and the Fcγ receptor, and much less often with IgA and IgM, and their Fc receptors.[48] Secretory IgA (sIgA) of mucosa has been

[46] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6078966/
[47] https://pubmed.ncbi.nlm.nih.gov/28847694/
[48] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7119964/

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shown to mediate ADE of bacterial infection but has not been demonstrated for viral infection.[49]

   a. Secretory IgA is the dominant antibody isotype at mucosal surfaces. It remains unknown if ADE of viral infection can occur through sIgA, but it has not been reported. Nevertheless, a robust mucosal immune response should greatly reduce or prevent IgG-mediated ADE in the systemic response by reducing or abrogating initial infection.

3. Certain B-cell epitopes appear to be more likely to give rise to ADE, including on the Spike protein of SARS-CoV-1, resulting in concern about the use of full-length Spike protein in SARS-CoV-2 vaccines.[50]
   a. As mentioned above, mRNA vaccination with full-length Spike has resulted in very low frequency of serious side-effects, and delivery with lipid nanoparticles produces a balanced Th1/Th2 response.
4. Waning or weak T-cell response, possibly driven by T-cell cross-reactivity to divergent epitopes derived from prior exposures to different viral serotypes. One model for how this might occur is through a T-cell original antigenic sin, analogous to the phenomenon described for antibodies.[51]
   a. Again, at the scale of a large population, it is impossible to completely avoid this risk since it is not restricted to vaccines, but also applies to viral infection. Nevertheless, selection of a few conserved epitopes should reduce the likelihood that a SARS-CoV-2 mutant will arise that results in this phenomenon. Following the same logic described for B-cell epitopes, if original antigenic sin occurs it can be limited to only those epitopes supplied in the vaccine. In the current vaccine design, less than 1% of the SARS-CoV-2 proteome (entire protein sequence) is represented as T-cell epitopes, and only about one-quarter to one-third of empirically verified immunodominant epitopes.
5. Similar to SARS-CoV-2, severe dengue infection is CD8 deficient, while mild infection is accompanied by a robust CD8 response. Multivalent dengue vaccines that elicit protective immunity include immunodominant MHC class I/CD8 epitopes from non-structural proteins. The Dengvaxia design employs a hybrid virus that uses the non-structural backbone of the related yellow fever virus, together with the outer structural proteins from dengue. Immunodominant MHC class I/CD8 epitopes are concentrated in the non-structural proteins of dengue, SARS-CoV-2, and other respiratory viruses, while immunodominant MHC class II/CD4 epitopes are concentrated in the outer

[49] https://pubmed.ncbi.nlm.nih.gov/12967664/
[50] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2756483/
[51] https://www.jimmunol.org/content/176/6/3821.long

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structural proteins. Dengvaxia elicits a strong initial, but rapidly waning antibody response, and a weaker and possibly immunopathological CD8 T-cell response in response to subsequent infection.

   a. RaDVaC vaccine designs incorporate a few immunodominant MHC class I/CD8 epitopes from non-surface and non-structural proteins. Some of these epitopes are highly conserved, and the most common HLA alleles are covered redundantly by at least 2 separate epitopes. We have not included all immunodominant epitopes for CD8 (or for B-cells or HLA class II/CD4), which should prevent original antigenic sin from compromising a future robust immune response to a cross-reactive serotype.

6. VED might arise through vaccine design-induced distortion of viral epitopes, as has been proposed to occur by formalin treatment of RSV.
   a. Our vaccine platform uses mRNA. After over one billion vaccinations with BioNtech/Pfizer and Moderna vaccines, there has yet to be a reported case of VED.

KEY TECHNICAL FEATURES AND SPECIFICATIONS (Updated 2022-03-06)

Certain technical features of the RaDVaC strategy follow from the above considerations, and are key to an open-source, citizen-science vaccine.

1. **Delivery.** Intranasal or sublingual delivery are probably among the safest choices of administration routes, though other delivery modes, especially other mucosal delivery modes, are likely also safe. For a respiratory virus, both intranasal and sublingual delivery have the advantage of eliciting a mucosal immune response at the most common sites of viral entry. These delivery modes can also elicit systemic immunity, although often not as robust as parenteral delivery after a single dose. Mucosal delivery is also less likely than other routes to trigger vaccine hesitancy.
2. **Lipid nanoparticle (LNP) packaged mRNA**. Successful vaccines depend on successful delivery and on immunogenic stimulation, often by the use of adjuvants that promote appropriate stimulation of the immune system. Many adjuvants are proven and available, with various strengths and weaknesses.
   a. The virus has an average size of about 100 nm to 150 nm.[52] Nanoparticle size ideally will be tuned to be between 100 nm and 200 nm. As described above, intranasal (or sublingual) delivery elicits both mucosal and systemic immune responses.

[52] https://www.nejm.org/doi/full/10.1056/NEJMoa2001017

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3. **Epitope selection** is critical. B-cell and T-cell epitopes have been selected and published by others. Multiple epitopes of both types should be selected, preferably in important functional and/or conserved regions of the virus. This gives a higher probability that at least one epitope will successfully stimulate immunity, especially given variability in individual immune responses, rather than relying on a single epitope.
    a. B-cell epitopes. Full-length Spike has been employed widely and successfully.
    b. T-cell epitopes. Empirical methods similar to but more complex than those used for B-cell epitope selection have been used for selecting superior T-cell epitopes.
4. **Testing**. Success of mucosal and systemic humoral immunogenic stimulation will be assessed by testing antibody titers of nasal wash, saliva, and serum. Tests of cellular immunity (T-cell) will also be performed, although as of mid-July 2022 there are no commercial options yet available for preferable kinds of tests, which require substantial laboratory infrastructure. Testing of immunity should be performed using standard assays and reagents, as well as the use of newer technologies, such as transcriptome or RT-qPCR profiling of peripheral blood mononuclear cells (PBMCs).

The importance and unique advantages of RNA-based vaccines

The SARS-CoV-2 pandemic triggered the most rapid vaccine deployments in history. The two vaccines first available in the US and many other countries were mRNA lipid nanoparticle vaccines. Not only was deployment rapid, but, as of March 2022, these vaccines have provided the highest level of protection of any of the dozens of vaccines produced thus far in the pandemic. In other words, mRNA was first, and, so far, mRNA is best. Nevertheless, when these vaccines were first produced and deployed there were many uncertainties about quality control and distribution, due to the need for these first generation vaccines to be stored at extremely low temperatures throughout the entire distribution network. There were also many other uncertainties and technical hurdles to overcome, but as billions of doses of these vaccines have been deployed, their successes have created substantial focus on each of these problems and substantial investments into solving them.

RNA is extremely fragile and requires protection and special delivery vehicles, including lipid nanoparticles, polysaccharides and other polymers, or emulsions. BioNTech/Pfizer and Moderna created and released the first approved mRNA vaccines in history; both use proprietary lipid nanoparticle (LNP) formulations consisting of an ionizable cationic lipid, a neutral or “helper” lipid, cholesterol, and a lipid conjugated with polyethylene glycol (PEG) for stabilization in vivo. The ionizable lipid component is the key to delivery efficiency and safety, and a small number of companies associated with Canadian biochemist Pieter Cullis are at the forefront of R&D and own substantial IP in the area. BioNTech uses an ionizable lipid (ALC-0315) designed by Acuitas and

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licensed to BioNTech. Moderna uses lipid SM-102, which they developed in-house, but the technology is claimed to be covered by patents owned by Arbutus and Genevant, and these companies are engaged in legal disputes with Moderna over the technology. Ionizable lipid technology has improved immensely in the past decade, and there is increasing interest in improving the technology. At present, ALC-0315 and SM-102 are responsible for the highest performance available in LNP delivery. BioNTech/Pfizer is given as two 30 microgram doses and Moderna is given as two 100 microgram doses.

mRNA is the primary focus of this document, but it isn't the only RNA used in vaccines. RNA vaccines can also be made with self-amplifying RNA (saRNA). saRNA consists of a gene/antigen of interest and a separate replicase capable of amplifying the gene/antigen of interest. The self-amplifying feature of these vaccines enables them to achieve similar immune responses to mRNA vaccines with substantially lower doses, in many cases less than 10-fold lower than mRNA. However, saRNA vaccines often trigger an innate immune response, and their effectiveness in human pre-clinical and clinical trials is lower than that of mRNA. There is substantial R&D investment to overcome these issues, and many leading companies are pursuing saRNA vaccines.

There are few major advantages to RNA vaccines over other platforms, and some of these played key roles in their rapid design, production and deployment during the pandemic. Ultimately, mRNA vaccines were ready to deploy at scale many months or years before other platforms. All advantages of mRNA are shared with saRNA.

- **Safe and effective.** Over a billion doses of mRNA vaccines have been administered since the beginning of 2021. Most reported serious side-effects also occur at a much higher rate with viral infection. BioNTech/Pfizer and Moderna vaccines have shown about 95% effectiveness against the parental virus strain.
- **Non-infectious.** Unlike inactivated or attenuated whole virus vaccines, RNA vaccines have no potential for infection, including during vaccine development and production.
- **Clean and sterile.** RNA molecules for vaccines are produced by highly efficient *in vitro* transcription and are completely cell free, and therefore, risk of infectious or other biological contaminants is low.
- **Pure and conformationally correct immunogens.** RNA production of an immunogen within a human cell produces a structure essentially identical to the selected portion of the viral antigen. Many other platforms produce immunogens with uncertain or compromised conformations, leading to ineffective vaccines, or in some cases, immunopathology.
- **Rapid and simple design and production.** RNA vaccines are coded initially in DNA employing widely used, rapid and extremely robust production methods. DNA constructs can be designed and produced easily in less than a week.

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- **Simple and inexpensive distribution of production materials.** While RNA is very unstable, DNA is very stable, especially in dry form, making shipping simple and inexpensive.
- **Unlimited immunogen choices.** Immunogens encoded by RNA can be essentially any length, whereas certain other platforms have a somewhat low upper limit of immunogen sizes. The upper limit of synthetic peptides, for example, is typically substantially less than 100 amino acids, which greatly limits representation of conformationally correct immunogens. Plus, unlike inactivated or attenuated whole virus vaccines, immunogens can be selected to be free of known immunopathological sequences carried by viruses.
- **Highly effective systemic and mucosal vaccination.** RNA vaccines have worked on a global scale with extremely high levels of effectiveness when used in injectable form; but they also have shown excellent results in published experiments using multiple other delivery modes, including intranasal, inhalable, and pain-free microneedle delivery, and vaccination through these routes elicits strong systemic immunity, but also mucosal immune protection.

General workflow for mRNA/LNP vaccine (last updated 2022-03-15)

The general procedure for making mRNA is called in vitro transcription (IVT). IVT consists of a DNA template, a DNA-dependent RNA polymerase, and ribonucleotide triphosphates in a suitable buffer. The RNA polymerase copies the DNA template, producing the mRNA molecule. DNA templates for mRNA vaccines are typically plasmids grown in the bacterium *E. coli*, with specific antigen sequences from the pathogen cloned into the plasmid. Many companies synthesize genes at reasonable rates and turnaround times; some will aid in the design, and perform all necessary cloning steps. Contact RaDVaC for more information about the availability of DNA templates for SARS-CoV-2 mRNA vaccines. For those who are new to recombinant DNA, there are many resources for understanding basic principles for designing, creating, modifying and using synthetic DNA and plasmids. One good resource is the Plasmids 101 guide freely available from Addgene.[53] The workflow below describes a design with a poly(A) tail designed into the DNA template, rather than poly(A) tailing by use of poly(A) polymerase. The former is simpler, omitting a purification and poly(A) tailing. It also allows the creation of a poly(A) tail of defined length. However, long poly(A) tails of DNA templates are unstable in unmodified *E. coli*. Therefore, two adjustments are presented here to promote poly(A) stability: a segmented poly(A) tail, and propagation in a recA E. coli strain to reduce loss of poly(A) through recombination[54].

[53] https://info.addgene.org/download-addgenes-ebook-plasmids-101-3rd-edition
[54] https://rnajournal.cshlp.org/content/25/4/507.full.pdf

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Step-by-step workflow

1. Select immunogen(s) based on current biomedical literature
2. Design immunogen as a DNA construct for insertion into a common plasmid (a.k.a. vector, e.g. pUC57) to be grown in *E. coli*; see construct details in the section below.
3. Transform *E. coli* with plasmid and grow with appropriate antibiotic selection
4. Purify plasmid
5. Linearize DNA plasmid with specific type IIS restriction enzyme (cleavage at site designed into 3' end of construct) to create an "unmasked" or 3' terminal poly(A) tail
6. Produce mRNA by in vitro transcription (IVT). DNA is used as a template for IVT production of mRNA
    a. Use ribonucleotides A, C, G
    b. Use N1-methyl pseudouridine, instead of unmodified uridine.
    c. 5' cap the mRNA during the IVT reaction, using CleanCap reagent; the CleanCap reagent must be specific for the dinucleotide designed into the template.
7. [OPTIONAL BUT RECOMMENDED] Degrade the RNA polymerase and the DNA template with proteinase K and DNAse
8. Purify the in vitro transcribed and capped mRNA; remove double stranded RNA (dsRNA) using cellulose and 16% ethanol binding
9. Lipid nanoparticle encapsulate the purified mRNA, using a mixture consisting of ionizable cationic lipid, neutral lipid, cholesterol, and PEG-ylated lipid, suspended in ethanol
10. [OPTIONAL, depending on administration mode] Exchange the buffer and remove the ethanol by filtration, chromatography, dialysis, or other means

Detailed overview of DNA construct

- Design elements of expression construct. This process is done most easily by using gene/plasmid editing software. There are several options available for purchase, and few available for free. We use the free software A Plasmid Editor (ApE)[55], developed and maintained by the Jorgensen Lab at the University of Utah. ApE uses its own file format as well as standard formats, including .seq, .str, .dna and .gb (Genbank). Numerous tutorials for using ApE are available online[56]. Below is a detailed description of the components of the DNA construct, encoding the RaDVaC mRNA vaccine immunogen
    - Select coding sequence.
    - OPTIONAL: Perform codon optimization (many gene synthesis companies offer optimization tools on their websites).

[55] https://jorgensen.biology.utah.edu/wayned/ape/
[56] https://www.youtube.com/channel/UC_-pObWrnUZRhsO8YblX6gQ

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    - T7 promoter sequence, TAATACGACTCACTATAAG underlined A is first transcribed nucleotide; this sequence terminates in 5' capping AG dinucleotide sequence that works with CleanCap AG from TriLink Bio.[57]
    - 5' UTR
        - Kozak sequence
    - Start ATG to initiate translation.
    - Stop codon(s) to terminate translation (TAG, TAA, TGA in DNA encode UAG, UAA, UGA in RNA, respectively).
    - 3' UTR including terminator sequence. Commonly used mammalian terminators (SV40, hGH, BGH, and rbGlob) include the sequence motif AAUAAA which promotes both polyadenylation and termination.
        - Schematic: 5' GOI [stop] ... AAUAAA ... poly A
    - Poly A sequence; length or segmentation depends on *E. coli* strain for propagation; low recombination recA strain promotes stability. We have used a segmented poly(A) tail interrupted by a short linker (A30LA70, where L = GCAUAUGACU); this sequence further improves plasmid stability in *E. coli*.[58]
    - Poly A can be unmasked by cleavage at downstream Type IIS restriction site, e.g. SapI (NNNNGAAGAGC)[59]
- Perform DNA sequencing on the final construct to verify correctness.

MATERIALS AND METHODS (updated 2021-03-07)

Selection and purchase of the vaccine ingredients

All materials and ingredients are commercially available. However, some vendor deliveries might be interrupted by public health measures.

- IVT
    - RNA nucleotides A, C, G, U, and N1-methyl pseudouridine
    - T7 RNA polymerase, high-temp T7 RNAP (https://www.neb.com/products/m0658-hi-t7-rna-polymerase#Product%20Information)
- Buffers?
- Capping chemistry and enzyme
    - CleanCap AG kit, works with T7 pol promoter
- Purification column; Oligo dT column
- Other spin columns, NEB Monarch?

[57] https://www.neb.com/tools-and-resources/feature-articles/minding-your-caps-and-tails
[58] https://rnajournal.cshlp.org/content/25/4/507.full.pdf
[59] https://ashpublications.org/blood/article/108/13/4009/6595/Modification-of-antigen-[...]-RNA-increases

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Vectors and strains

- For mRNA vaccines, simple vectors for E. coli cloning can be used, e.g. pUC57, pBluescript, etc.
- For self-amplifying RNA; saRNA vector[60], e.g. T7-VEE-IRES-Puro (Addgene[61]); SP6 version used in this study by Chang et al[62]
- For trans-amplifying RNA; taRNA vectors will probably need to be constructed based on previously published designs[63],[64]; these are typically based on VEEV[65]

Reagents

- RNAse free water
- E. coli strain; when propagating a construct with a DNA-encoded poly(A) tail, use a recA minus strain to minimize recombination and loss of the poly(A) [66]
- E. coli media and antibiotics
    - Carbenecillin (more stable than Ampicillin)
    -
- Media petri dishes for growing E. coli
- dH_2O, deionized water. Widely available.
- OPTIONAL: Sodium bicarbonate $NaHCO_3$, or other mild base for adjusting the pH.

Preferred equipment and materials (updated 2020-11-04)

Certain equipment is required for efficient vaccine production and administration. Vaccine can be produced without specialized laboratory equipment but the process is more laborious and the results are likely to be more variable.

- Pipettes and filter tips
    - 200 - 1000 microliter
    - 20 - 200 microliter
    - 1 - 20 microliter or similar
- 37 degree C incubator for growing E. coli for producing DNA
- Sterile filtered pipette tips: 1000 microliter, 200 microliter
- Scale: gram scale accurate to 0.1 grams, or jeweler's scale for very small amounts
- Clean spatula for dry reagents
- Small beakers for mixing and stirring. 10 ml to 25 ml beaker for stirring

[60] https://www.sciencedirect.com/science/article/pii/S1567576921008675
[61] https://www.addgene.org/58970/#
[62] https://www.sciencedirect.com/science/article/pii/S0168365921004879
[63] https://www.sciencedirect.com/science/article/pii/S1525001619304125
[64] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3126202/pdf/zjv4739.pdf
[65] https://www.frontiersin.org/articles/10.3389/fmolb.2018.00071/full
[66] https://www.neb.com/products/c3040-neb-stable-competent-e-coli-high-efficiency#Product...

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- Syringes with small bore needles, 28 to 32 gauge; for injecting ethanol/lipids into stirring mRNA aqueous phase in solvent injection method
- Small bottles for stock solutions; 100 ml to 250 ml
- 15 ml conical tubes
- 50 ml conical tubes
- 1.5 to 2 ml microcentrifuge tubes
- Stirring apparatus: magnetic stir plate and small stir bar to fit in beaker
- OPTIONAL but preferable: pH strips, range at least 2.0 to 9.0
- Nasal spray apparatus. These are available through multiple vendors. We selected small nasal spray bottles (about 5 milliliter) with a pump-top apparatus. A tube stem about 4 cm long and approximately 1 mm inner diameter extends from the bottom of the apparatus, which can be placed into the bottom of a 2-ml tube containing vaccine. Our spray apparatus delivers about 100 microliters per pump.

Selection of epitopes for design of immunogen (updated 2022-05-19)

Epitopes are a critical element of a vaccine because they are specific to each pathogen. An epitope is the portion of an antigen (typically a protein) that interacts with the immune system, such as through binding of antibodies or T-cell receptors. Multiple data types can be used in the selection of epitopes. This section describes general guidelines and issues for consideration in epitope selection and design.

- **Epitopes for B-cell and T-cell responses can be quite different**, and there are critical differentiating features. Here are key considerations in the selection and design of B-cell and T-cell epitopes.
    - T-cell epitopes are presented as short linear peptide antigens, but B-cell epitopes (which can be linear or conformational) are presented in their native conformation.
    - The B-cell response is relatively easily measured by antibodies, but the T-cell response is more difficult to measure and more rarely performed. Antibody testing is a large-scale commercial activity (using lateral flow, ELISA, etc.) but T-cell testing is generally a research activity (using ELISpot, etc.). Therefore, T-cell epitopes are often based on computational prediction. This was true of early T-cell epitope selections in the biomedical literature and preprint servers. However, beginning in mid July, 2020, substantial empirical data have been published on T-cell epitopes.
    - B-cell or antibody binding epitopes are often mapped into two general classes: linear and conformational (or structural). Linear epitopes (single peptide chain of

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nearby amino acids) are typically mapped by binding antibody-containing serum from convalescent patients to large collections or arrays of short epitope peptides collectively representing viral proteins. Conformational epitopes often consist of amino acids that are far apart on a protein sequence but in close proximity in the folded protein. Linear epitopes are easier to map than conformational epitopes. However, conformational epitopes can be critically important.

- There are 2 classes of T-cell epitopes: MHC Class I and MHC Class II (for a thorough review, see Rock et al.[67]). Core motif binding of both Class I and Class II is about 9 amino acids but both are variable. The Class I binding site has closed ends, and epitopes range from 8 to 15 aa, but infrequently diverge from 9 aa. Class II binding is open-ended, and natural epitopes extend longer than the 9 aa core, and range from 13 to 25 aa[68]. Many computational predictive algorithms are available, and a large number of preprints provide Class I and Class II predictions for inclusion in a vaccine. If empirical data are available for protective T-cell epitopes (e.g. Le Bert et al, Adaptive Biotechnologies, Peng et al[69], and Zhang et al[70]), these data should guide T-cell epitope selection.
- Immune responses (both B and T-cell) can vary substantially with different ancestry/ethnicity. We have used predictions and empirical data for wide coverage of immune responses (our group is highly ethnically diverse). For specific geographies and ancestries, local immune response information should be taken into account.
- Although there can be differences between T and B-cell epitopes, they can overlap. If possible, immunogens that contain experimentally validated epitopes for both B and T-cells are ideal.

- **Multiple epitopes with low mutant escape potential.** It is important to note that most published neutralizing antibodies target Spike RBD, as do many vaccines in commercial development. However, given the high degree of mutability of the RBD portion of Spike, it is important to identify and select targets outside the RBD because of mutant escape potential. This strategy is summarized by Brouwer and colleagues: “In light of the rapid emergence of escape mutants in the RBD of SARS-CoV-1 and MERS, monoclonal NAbs targeting other epitopes than the RBD are a valuable component of any therapeutic antibody cocktail. Indeed, therapeutic antibody cocktails with a variety of specificities have been used successfully against Ebola virus disease and are being tested widely in

[67] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5159193/
[68] https://pubmed.ncbi.nlm.nih.gov/1380674/
[69] https://www.nature.com/articles/s41590-020-0782-6
[70] https://pubmed.ncbi.nlm.nih.gov/32668444/

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clinical trials for HIV-1. NAbs targeting non-RBD epitopes have been identified for SARS-CoV-1 and MERS, supporting the rationale…" for SARS-CoV-2[71]. Three neutralizing epitopes described in the following section, containing or immediately adjacent to the Spike protease recognition sites and heptad repeat 2 (HR2) fusogenic domain are highly conserved among these 3 coronaviruses, suggesting that they have very low mutant escape potential.

- Two of these sites are cleavage sites for host cell proteases. Cleavage of the S1/S2 site by furin is required for subsequent cleavage of the s2' site by TMPRSS2. Cleavage by both is required for efficient fusion of the virus to the host cell membrane, and these sequences are constrained by recognition by these host proteases. Another region of the virus (heptad repeat 2 or HR2) is also required for membrane fusion, and the homologous 50 amino acid region of the SARS-CoV-1 virus is both 100% conserved, and is bound by neutralizing antibodies.
- According to 3D structural model data, relative to the RBD, these epitopes are structurally simple, and the peptides can be short (under 25 amino acids) and are water soluble. These features make them ideal for a peptide vaccine. The two protease sites loop out from the Spike protein surface, which makes them accessible to the required host proteases, but also to antibodies. The HR2 domain is predicted to form a coiled-coil "tether" that links the Spike trimer to the viral membrane. The linear epitope peptide should adopt a coiled coil conformation very similar to that exhibited within the native Spike protein.
- Therefore, rather than focusing on ACE2-binding epitopes in the highly mutation prone RBD to inhibit virus binding to the ACE2 receptor, we targeted these B-cell epitopes in the highly conserved portions of the Spike protein to strategically neutralize proteolytic cleavage and membrane fusion. Furthermore, all three are bound by antibodies present in the sera of large fractions of convalescents, and they produce among the highest signals in linear epitope mapping studies, which are far higher than signals measured for binding to any linear epitope in the RBD.
- The Gen 7 and 8 vaccine formulations still contain epitope peptides from within the RBD, including 2 peptides in the ACE2-binding region. However, these 2 peptides are unlikely to be conformationally correct and elicit antibodies that bind the native Spike conformation. The reason we include these is that they are predicted to be effective CD4 T-cell epitopes, although empirical data from convalescent patients has not yet confirmed animal studies and computational predictions.

[71] https://science.sciencemag.org/content/early/2020/06/15/science.abc5902

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- **Immunodominance and limited effective epitope space**. Immunodominance is the phenomenon of antibody and/or T-cell responses to a small number of epitopes, despite a large number of possible epitopes within the virus. Findings to date suggest both B-cell and T-cell responses to SARS-CoV-2 display pronounced immunodominance. Some key findings about the immunodominant landscape of SARS-CoV-2:
    - A publication by Snyder and colleagues shows a few epitope peptides are responsible for over 50% of CD8 T-cell receptor binding events[72].
    - The immunodominance landscapes of CoV-1 and CoV-2 are:
        - Very different between CoV-1 and CoV-2 Spike proteins, possibly largely due to glycosylation differences.[73]
        - Highly similar for CoV-1 and CoV-2 Nucleocapsid proteins[74],[75].
    - One early but thorough preprint by Zhang and colleagues suggests immunodominance is a feature of the B-cell immune response to SARS-CoV-2[76].
    - Farrera and colleagues reported a set of immunodominant B-cell epitopes similar to those reported by Zhang, but in a study of Swiss convalescent patients[77].
    - Most of the epitopes reported by Zhang et al and Farrera et al were subsequently confirmed by Li and colleagues in the first large-scale linear B-cell epitope mapping of over 1,000 convalescent patients in Wuhan, China[78].
    - The emerging consensus suggests that there are about half a dozen dominant B-cell epitopes, which are the same in European and Chinese patients. The majority are found in key functional portions of the Spike protein, including the following (amino acid coordinates shown are approximate):
        - Spike 450-500; ACE2 binding residues of the RBD (Zhang et al); low degree of conservation; probably moderate to high mutant escape potential
        - Spike 655-685; immediately adjacent to the S1/S2 protease cleavage sites; high degree of conservation in the antibody-binding N terminal portion of the epitope; likely low to moderate mutant escape potential. Cleavage of this site greatly increases efficiency of host cell infection[79].

[72] https://www.medrxiv.org/content/10.1101/2020.07.31.20165647v2
[73] https://pubs.acs.org/doi/full/10.1021/acsinfecdis.6b00006
[74] https://jcm.asm.org/content/42/11/5309.full
[75] https://www.biorxiv.org/content/10.1101/2020.03.26.994756v1
[76] https://www.biorxiv.org/content/10.1101/2020.04.23.056853v2.article-info
[77] https://www.medrxiv.org/content/10.1101/2020.06.15.20131391v1
[78] https://www.medrxiv.org/content/10.1101/2020.07.13.20152587v1.full.pdf
[79] https://www.pnas.org/content/117/21/11727

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        - Spike 805-820; spanning the S2' protease cleavage site (815-816); high degree of conservation; likely low mutant escape potential.
        - Spike 1145-1160; adjacent to the heptad repeat 2 (HR2) region, which is critical for fusion of the viral and host cell membranes of the SARS virus[80]; very high degree of conservation; likely extremely low mutant escape potential.
    - These immunodominance findings are supportive of the peptide vaccine approach, since only a limited portion of the virus needs to be represented in the vaccine for it to be effective; plus, potentially counterproductive portions of the virus (such as those that might contribute to ADE) are not present in the vaccine.
- **The critical importance of MHC Class I/CD8 T-cell epitopes and immunity.** Antibodies get a lot of press attention (in large part because simple commercial antibody tests are widely available but T-cell tests are more complex and must be performed on isolated cells, and therefore are done in specialized research labs) but the most protective immune response consists of robust humoral (B-cell mediated) and cellular (T-cell mediated) responses. Early indications suggest T-cell responses might be key to both minimizing severity of illness and to longevity of immunity, but the specific type(s) of T-cell response that might be most protective is a critical yet still unresolved issue. Here are a few key findings about the importance of T-cell immunity to SARS-CoV-2 infection:
    - Mild COVID-19 is seen in convalescents with robust T-cell responses, including those who do not have detectable antibodies (are seronegative)[81]
    - Severe COVID-19 is accompanied by higher antibody levels than mild cases, but with unbalanced or impaired T-cell response, especially the CD8 response[82]. This study by Peng et al identified 6 immunodominant CD4 epitopes (3 of which are located on the Spike protein), a similarly small number of immunodominant CD8 epitopes, and they showed that a higher ratio of CD8/CD4 response is found in those with milder disease versus those with severe disease.
    - The effect of T-cell epitopes can vary depending on the protein of origin. Peng and colleagues also report that T-cell epitopes from Spike, Membrane and ORF3a proteins were associated with more severe disease. Higher frequencies of multi-cytokine producing Membrane and Nucleocapsid-specific CD8 T-cells (relative to Spike-specific CD8 T-cells) were observed in patients with mild cases. Because of these findings, Peng et al conclude that "The identification of T-cell specificity and functionality associated with milder disease, highlights the potential

[80] https://www.thelancet.com/journals/lancet/article/PIIS0140673604157887/fulltext
[81] https://www.biorxiv.org/content/10.1101/2020.06.29.174888v1
[82] https://www.biorxiv.org/content/10.1101/2020.06.05.134551v1.full.pdf

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importance of including non-spike proteins within future COVID-19 vaccine design." Many subsequent publications have further strengthened this conclusion. Nevertheless, the CD4 epitopes included in all RaDVaC designs to date do not include the 3 immunodominant CD4 Spike protein epitopes identified by Peng et al, and we have no plan to include these in future designs until this concern about CD4 hyperstimulation is resolved.

- Peng et al also used single cell analysis of bronchoalveolar lavage fluid from COVID-19 patients and found substantial clonal expansions of CD8 T-cells in mild but not severe patients, further reinforcing evidence that a robust CD8 response protects against severe disease.
- T-cell exhaustion or deficiency is correlated with disease severity (low T-cell response with more severe disease) [83],[84],[85],[86]
- Population MHC class I binding (and CD8 response) is predictive of mortality[87].
- Vaccine-enhanced disease involves a Th2-polarized response that is CD8 T-cell deficient[88]. A robust CD8 response is observed in people and animal models who do not develop vaccine-enhanced disease.
- Many reports have shown rapid declines of anti-SARS-CoV-2 antibodies in convalescents. It remains unclear how this decline impacts protection from reinfection or severity of disease, but initial results also show that T-cell immunity might be very long-lived. One report by Le Bert and colleagues shows that patients who recovered in 2003 from SARS-CoV-1 infection harbor SARS-CoV-2 cross-reactive T-cells 17 years later[89].
- Optimal T-cell epitopes vary by ethnicity and geography (MHC/HLA allele frequencies can be calculated using various tools[90]. (MHC in humans is also called Human Leukocyte Antigen, or HLA). Epitopes providing extensive MHC coverage have been reported for many pathogens, including SARS-CoV-2 (for example, as published by Ferretti et al[91] and by Liu and colleagues[92]). Such publications are useful for selection of T-cell epitope peptides for use in specific geographical regions and ethnicities. The publication by Ferretti and colleagues provides

[83] https://www.nature.com/articles/s41423-020-0401-3
[84] https://www.frontiersin.org/articles/10.3389/fimmu.2020.00827/full
[85] https://www.medrxiv.org/content/10.1101/2020.06.16.20130914v1
[86] https://www.cell.com/immunity/fulltext/S1074-7613(20)30333-2
[87] https://www.medrxiv.org/content/10.1101/2020.05.08.20095430v3.full
[88] https://link.springer.com/content/pdf/10.1007/s12026-007-0071-6.pdf
[89] https://pubmed.ncbi.nlm.nih.gov/32668444/
[90] http://allelefrequencies.net/
[91] https://www.medrxiv.org/content/10.1101/2020.07.24.20161653v2
[92] https://www.cell.com/cell-systems/fulltext/S2405-4712(20)30238-6

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empirical validation of CD8 T-cell epitopes, broad coverage of the most common MHC haplotypes, and largely confirms the empirical findings of Le Bert and of Snyder et al[93]. The study by Liu is computational and theoretically provides more complete haplotype coverage; however, only one of the SARS-CoV-1 cross-reactive T-cell epitopes described by Le Bert et al is present in this set of predicted epitopes, and it is predicted by Liu et al to be optimal for MHC1 and MHC2 haplotypes found mainly in Central Africa, a population not represented in the Singapore convalescents (mainly East Asian and European ancestry). And the top immunodominant MHC Class I / CD8 epitopes empirically verified by Snyder and colleagues are not represented in the computationally predicted set of Liu. Therefore, such computational predictions should be used with the caveat that they are not always consistent with empirical data.

- **Peptide epitopes** are preferred because they have the following advantages over other epitopes:
    - Well defined
    - Potentially pure
    - Enrichment of antigenic targets; not accompanied by substantial unnecessary and potentially counterproductive/non-neutralizing antigenic targets. Many epitopes are very short and well-represented by peptides (although uncertainty about epitope boundaries and addition of flanking amino acids to improve peptide solubility can warrant the selection of slightly longer sequences).
        - Some critical neutralizing B-cell epitopes can be very small, less than 20 amino acids.
        - The majority of CD4 T-cell epitope peptides are less than 20 amino acids
        - CD8 epitopes are often 9-11 aa.
    - Peptides allow specific mutations to be addressed in a modular fashion. The Gen 10+ vaccine designs include an epitope peptide with the increasingly common variant in the Spike RBD: N501Y. It is important to note that it is not necessary to include every mutated position of a variant of concern in order to neutralize the variant. For example, E484K is an increasingly common mutation in variants of concern, and appears to alter the interaction of Spike with the ACE2 receptor. Nevertheless, the widely studied neutralizing antibody REGN10987, and certain other neutralizing antibodies that bind the RBM, are not affected by mutations at position 484. Therefore, an epitope peptide that does not include position 484 of the receptor binding motif, but does include the portion of the RBM bound by

[93] https://www.medrxiv.org/content/10.1101/2020.07.31.20165647v2

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neutralizing antibodies should serve to elicit neutralizing antibodies, without regard to the identity of the amino acid at position 484.

- **Empirical evidence** should dominate selection criteria. Here are some best types of evidence:
    - Mapping of epitopes in blood and other samples collected from convalescent patients (ideally stratified by severity of illness). This can be accomplished by a few primary means:
        - 3D structural studies and modeling of neutralizing antibody binding to a viral antigen (e.g. Spike protein)
        - Mapping of linear B-cell epitopes by binding antibodies in convalescent sera to a library of peptides representing viral antigens. A strong signal in a linear epitope mapping study does not guarantee that the epitope peptide in the context of a vaccine will trigger the production of an antibody that binds to this epitope within the context of the virus. However, it is a good indicator that this is at least possible. Peptides can be constrained to approximate native conformation, making it more likely to bind the native epitope.
        - Mapping of T-cell epitopes by stimulating convalescent T-cells with epitope peptides, and measuring their response (e.g. cytokine secretion; ELISpot)
    - Epitope peptides from a peptide vaccine that has shown protection against infection
    - Successful use of epitope peptides in vaccines that elicit antibodies (or serum) effective in virus neutralization assays. B-cell epitopes that allow antibody binding to the virus but don't block viral function might increase risk of antibody-dependent enhancement.
    - Mapped epitopes that are effective in virus neutralization assays (e.g. peptides compete with viral sequences in cellular infection assays).
    - Successful use of epitope peptides in vaccines that elicit T-cell responses, or peptides shown to stimulate T-cells or cytokine production in ELISpot or other T-cell assay in cells from convalescents.
- **3D modeling of viral antigens** (especially Spike protein) is very helpful in B-cell epitope selection and peptide design.
    - Surface loop structures are often good antibody binding sites. Unstructured, hydrophilic loops that are stabilized by a minimal number of neighboring amino acid residues make good linear (rather than conformational) epitopes. One prime example in SARS-CoV-2 is Spike 804 to 818, the TMPRSS2 cleavage site (one of a few immunodominant epitopes reported by both Farrera et al and Li et al).

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- Certain amino acid residues are overrepresented and underrepresented in preferred antigens. According to structural studies, these amino acids are overrepresented (from most overrepresented): W, Q, P, K, S, E, N, H, C. And these are underrepresented (from most underrepresented): A, V, I, F, L, Y [94].
- There are multiple excellent 3D modeling packages and online servers. The NCBI supported iCn3d is very good and allows automatic access to Protein Database (PDB) files of viral protein structures[95]. Multiple 3D models of SARS-CoV-2 proteins are available, including Spike in various conformations, and complexed with antibodies and with the ACE2 receptor.
- Conformational B-cell epitopes can be difficult to produce with peptides, but potentially might be designed as single peptides that join discontinuous portions of virus proteins, or can be produced as multiple peptides that are predicted to physically interact into relatively stable conformations.

We have produced several iterations of our earlier, peptide-based vaccine candidates, and for each we selected multiple B-cell and T-cell epitopes. Which B-cell epitopes elicit antibody responses can be verified by immunoassay testing, and these epitope peptides can be carried forward in updated versions of vaccines. The use of multiple epitopes also ensures that, if vaccination is successful in eliciting immunity against, say, 2 or 3 epitopes, then a single mutation will not allow the virus to evade immunity. Mutating virus and epitope variants should be monitored (nextstrain.org, bioRxiv, etc) , and new epitope peptides incorporated into evolving designs as needed.[96]

Mapping of antibodies from people previously infected but recovered (convalescent) has shown that there are key regions for antibody binding. SARS and MERS are lethal coronaviruses that have been used for comparison and cross-reactivity of convalescent antibodies. Jiang et al used SARS-CoV-2 specific proteome microarrays to show that S and N proteins bind both IgG and IgM, and the C terminal portion of the N protein exhibits the highest convalescent serum antibody binding signal of all tested proteins.[97] Antibodies from convalescent patients have been mapped to short, linear epitope peptides by Poh and colleagues[98] (S protein only), Wang et al [99], and the previously mentioned Zhang et al, Farrera et al, and Li et al.

[94] https://www.jimmunol.org/content/181/9/6230.long
[95] https://www.ncbi.nlm.nih.gov/Structure/icn3d/full.html
[96] https://nextstrain.org/ncov
[97] https://www.medrxiv.org/content/10.1101/2020.03.20.20039495v1
[98] https://www.biorxiv.org/content/10.1101/2020.03.30.015461v1
[99] https://www.biorxiv.org/content/10.1101/2020.03.26.994756v1

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Neutralizing (or protective) antibodies are preferred to guide B-cell epitope selection. Antibodies to certain functional regions of the virus will neutralize its activity and infectivity. Many antibodies bind to the virus and some will inhibit function but only a subset of all antibodies will truly neutralize the ability of the virus to infect cells and replicate. Ongoing research is helping to identify which portions of the virus are key to neutralizing activity. Neutralizing activity is not observed in antibodies associated with antibody-dependent enhancement of disease (ADE).

High-resolution linear epitope mapping by Wang et al was done with arrays of peptides covering the entire length of ten SARS-CoV-2 proteins. Length of peptides is 15aa, and peptides overlap by 5aa. Convalescent sera IgG and IgM were applied separately to the arrays. Binding events to a given peptide but not to adjacent peptides in the array indicate tight binding primarily to the central 5aa portion. Table 3 shows the 61 bound epitopes. Only five of these span more than one peptide. One in the S protein spans 15aa or three peptides (start = 806). These stretches are highly suggestive of a long linear epitope, whereas shorter epitopes are potentially a portion of a structural epitope. Moreover, the longer sequence provides a larger target. The longer S protein epitope binds both IgG and IgM, and a largely overlapping epitope binds convalescent antibodies in the study by Poh and colleagues, and produces the highest signal in the later and larger-scale linear epitope mapping study by Li and colleagues. The epitope at this position (about Spike 805-820) spans a key proteolytic cleavage site required for viral entry into host cells. We included this epitope in Gen 3+ vaccine formulas, and as updated and progressively rich scientific information has become publicly available, we have added additional B-cell and T-cell epitopes to successive generations of vaccine. Each of these additions is detailed with references in the sections below.

Vaccine composition (last updated 2022-01-14)

General Guidelines

Spike protein, Wuhan parental strain[100]

Blue highlighting is the receptor binding domain (RBD). Red highlighted amino acids are ACE2 contact residues. Green and purple highlighting indicate protease cleavage sites, underline of the second cleavage site indicates the conserved fusion peptide. Yellow highlighting indicates a conserved stem-helix epitope that binds neutralizing antibodies.

```
1   mfvflvllpl vssqcvnltt rtqlppaytn sftrgvyypd kvfrssvlhs
51  tqdlflpffs nvtwfhaihv sgtngtkrfd npvlpfndgv yfasteksni
101 irgwifgttl dsktqslliv nnatnvvikv cefqfcndpf lgvyyhknnk
151 swmesefrvy ssannctfey vsqpflmdle gkqgnfknlr efvfknidgy
```

[100] https://www.ncbi.nlm.nih.gov/nuccore/NC_045512.2

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```
201 fkiyskhtpi nlvrdlpqgf saleplvdlp iginitrfqt llalhrsylt
251 pgdsssgwta gaaayyvgyl qprtfllkyn engtitdavd caldplsetk
301 ctlksftvek giyqtsnfrv qptesivrfp nitnlcpfge vfnatrfasv
351 yawnrkrisn cvadysvlyn sasfstfkcy gvsptklndl cftnvyadsf
401 virgdevrqi apgqtgkiad ynyklpddft gcviawnsnn ldskvggnyn
451 ylyrlfrksn lkpferdist eiyqagstpc ngvegfncyf plqsygfqpt
501 ngvgyqpyrv vvlsfellha patvcgpkks tnlvknkcvn fnfngltgtg
551 vltesnkkfl pfqqfgrdia dttdavrdpq tleilditpc sfggvsvitp
601 gtntsnqvav lyqdvnctev pvaihadqlt ptwrvystgs nvfqtragcl
651 igaehvnnsy ecdipigagi casyqtqtns prrarsvasq siiaytmslg
701 aensvaysnn siaiptnfti svtteilpvs mtktsvdctm yicgdstecs
751 nlllqygsfc tqlnraltgi aveqdkntqe vfaqvkqiyk tppikdfggf
801 nfsqilpdps kpskrsfied llfnkvtlad agfikqygdc lgdiaardli
851 caqkfngltv lpplltdemi aqytsallag titsgwtfga gaalqipfam
901 qmayrfngig vtqnvlyenq klianqfnsa igkiqdslss tasalgklqd
951 vvnqnaqaln tlvkqlssnf gaissvlndi lsrldkveae vqidrlitgr
1001 lqslqtyvtq qliraaeira sanlaatkms ecvlgqskrv dfcgkgyhlm
1051 sfpqsaphgv vflhvtyvpa qeknfttapa ichdgkahfp regvfvsngt
1101 hwfvtqrnfy epqiittdnt fvsgncdvvi givnntvydp lqpeldsfke
1151 eldkyfknht spdvdlgdis ginasvvniq keidrlneva knlneslidl
1201 qelgkyeqyi kwpwyiwlgf iagliaivmv timlccmtsc csclkgccsc
1251 gscckfdedd sepvlkgvkl hyt //
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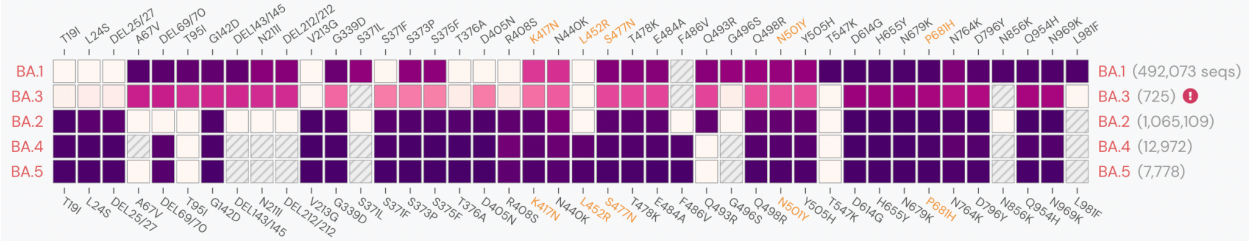
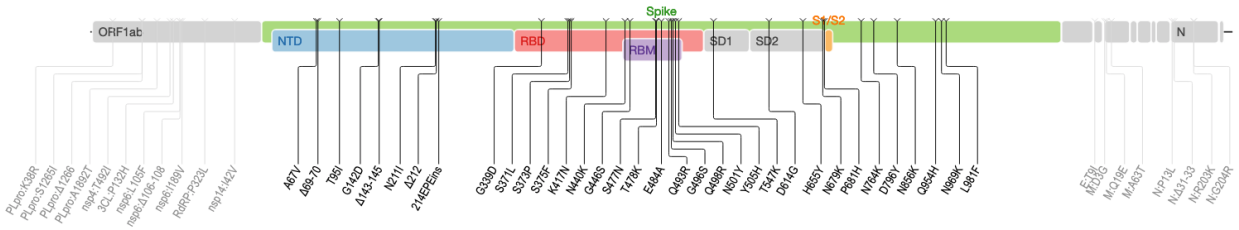
Omicron variants[101],[102],[103]



Omicron Spike mutations are shown in the above table for variants BA.1 through BA.5. BA.3 is a minor variant and is not shown in detail below.

Spike protein sequence, Omicron variant BA.1[104]



Spike mutations: A67V, Δ69-70, T95I, G142D, Δ143-145, Δ211, L212I, ins214EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, **Q493R,** G496S, **Q498R**, **N501Y**, **Y505H**, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F.

BA.1 Spike sequence

Pink highlights are omicron substitutions. Mutations above and below in bold type are substitutions of ACE2 contact residues. Numbering is the same as the Wuhan strain, ignoring the altered numbering due to insertions and deletions.

```
1   mfvflvllpl vssqcvnltt rtqlppaytn sftrgvyypd kvfrssvlhs
51  tqdlflpffs nvtwfhvihv sgtngtkrfd npvlpfndgv yfasieksni
101 irgwifgttl dsktqslliv nnatnvvikv cefqfcndpf ldvyyhknnk
151 swmesefrvy ssannctfey vsqpflmdle gkqgnfknlr efvfknidgy
201 fkiyskhtpi _ivepeрdlpqgf saleplvdlp iginitrfqt llalhrsylt
251 pgdsssgwta gaaayyvgyl qprtfllkyn engtitdavd caldplsetk
301 ctlksftvek giyqtsnfrv qptesivrfp nitnlcpfde vfnatrfasv
351 yawnrkrisn cvadysvlyn lapfftfkcy gvsptklndl cftnvyadsf
401 virgdevrqi apgqtgniad ynyklpddft gcviawnsnk ldskvsgnyn
```

[101] https://www.cdc.gov/coronavirus/2019-ncov/science/science-briefs/scientific-brief-omicron-variant.html
[102] https://www.ncbi.nlm.nih.gov/nuccore/OL672836
[103] https://outbreak.info/compare-lineages
[104] https://covdb.stanford.edu/page/mutation-viewer/#omicron

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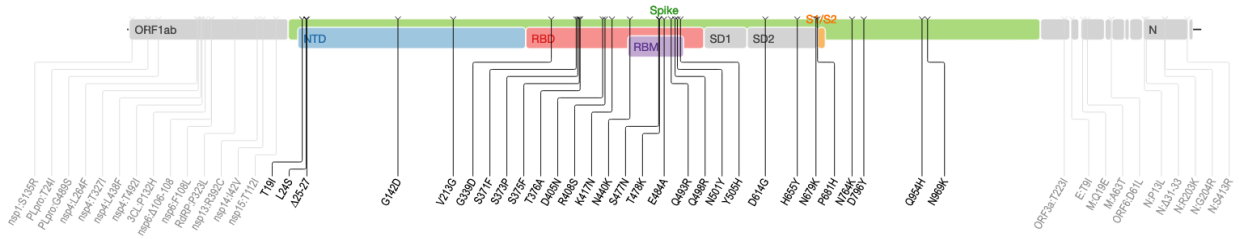
```
451 ylyrlfrksn lkpferdist eiyqagnkpc ngvagfncyf plRsysfRpt
501 YgvgHqpyrv vvlsfellha patvcgpkks tnlvknkcvn fnfnglkgtg
551 vltesnkkfl pfqqfgrdia dttdavrdpq tleilditpc sfggvsvitp
601 gtntsnqvav lyqgvnctev pvaihadqlt ptwrvystgs nvfqtragcl
651 igaeyvnnsy ecdipigagi casyqtqtks hrrarsvasq siiaytmslg
701 aensvaysnn siaiptnfti svtteilpvs mtktsvdctm yicgdstecs
751 nlllqygsfc tqlkraltgi aveqdkntqe vfaqvkqiyk tppikyfggf
801 nfsqilpdps kpskrsfied llfnkvtlad agfikqygdc lgdiaardli
851 caqkfkgltv lpplltdemi aqytsallag titsgwtfga gaalqipfam
901 qmayrfngig vtqnvlyenq klianqfnsa igkiqdslss tasalgklqd
951 vvnhnaqaln tlvkqlsskf gaissvlndi fsrldkveae vqidrlitgr
1001 lqslqtyvtq qliraaeira sanlaatkms ecvlgqskrv dfcgkgyhlm
1051 sfpqsaphgv vflhvtyvpa qeknfttapa ichdgkahfp regvfvsngt
1101 hwfvtqrnfy epqiittdnt fvsgncdvvi givnntvydp lqpeldsfke
1151 eldkyfknht spdvdlgdis ginasvvniq keidrlneva knlneslidl
1201 qelgkyeqyi kwpwyiwlgf iagliaivmv timlccmtsc csclkgccsc
1251 gscckfdedd sepvlkgvkl hyt //
```

BA.2



Spike mutations: T19I, L24S, Δ25-27, G142D, V213G, G339D, S371F, S373P, S375F, T376A, K417N, N440K, S477N, T478K, E484A, **Q493R, Q498R**, **N501Y**, **Y505H**, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K.

BA.4, BA.5

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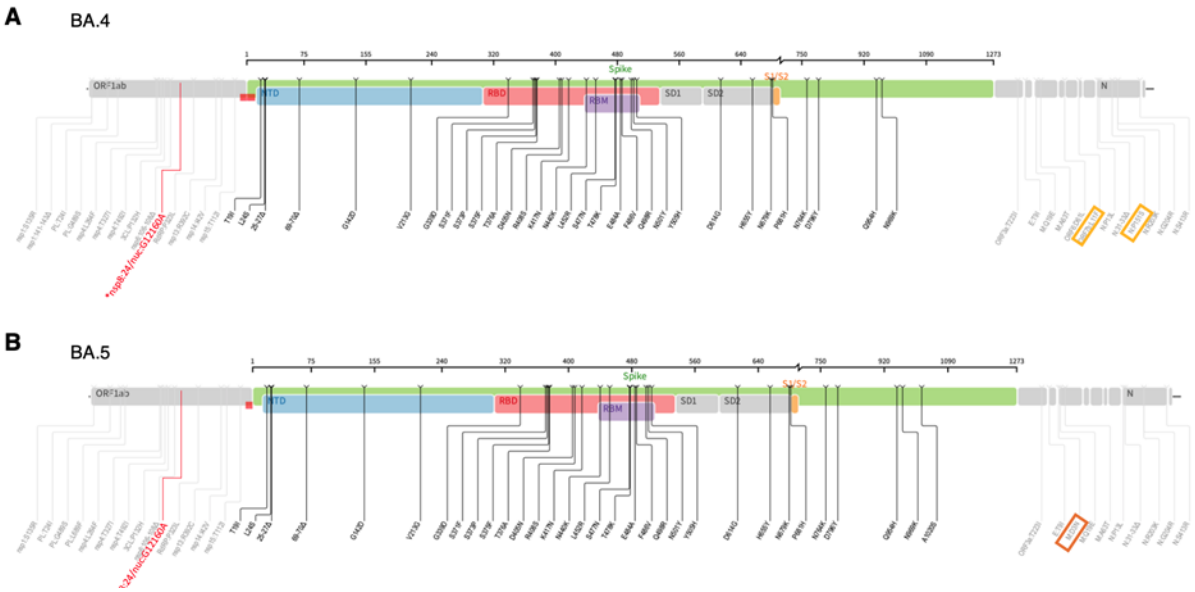

The sequences of BA.4 and BA.5 Spike proteins are identical in most sequenced samples. Spike mutations: T19I, L24S, Δ25-27, Δ69-70, G142D, V213G, G339D, S371F, S373P, S375F, T376A, K417N, N440K, L452R, S477N, T478K, E484A, F486V, **Q498R**, **N501Y**, **Y505H**, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K.

BA.4/5 Spike sequence

Pink highlights are omicron substitutions. Mutations above and below in bold type are substitutions of ACE2 contact residues. Numbering is the same as the Wuhan strain, ignoring the altered numbering due to insertions and deletions.

```
1   mfvflvllpl vssqcvnltt rtqlppaytn sftrgvyypd kvfrssvlhs
51  tqdlflpffs nvtwfhvihv sgtngtkrfd npvlpfndgv yfasieksni
101 irgwifgttl dsktqslliv nnatnvvikv cefqfcndpf ldvyyhknnk
151 swmesefrvy ssannctfey vsqpflmdle gkqgnfknlr efvfknidgy
201 fkiyskhtpi _ivepeprdlpqgf saleplvdlp iginitrfqt llalhrsylt
251 pgdsssgwta gaaayyvgyl qprtfllkyn engtitdavd caldplsetk
301 ctlksftvek giyqtsnfrv qptesivrfp nitnlcpfde vfnatrfasv
351 yawnrkrisn cvadysvlyn lapfftfkcy gvsptklndl cftnvyadsf
401 virgdevrqi apgqtgniad ynyklpddft gcviawnsnk ldskvsgnyn
451 ylyrlfrksn lkpferdist eiyqagnkpc ngvagfncyf plRsysfRpt
501 YgvgHqpyrv vvlsfellha patvcgpkks tnlvknkcvn fnfnglkgtg
551 vltesnkkfl pfqqfgrdia dttdavrdpq tleilditpc sfggvsvitp
601 gtntsnqvav lyqgvnctev pvaihadqlt ptwrvystgs nvfqtragcl
651 igaeyvnnsy ecdipigagi casyqtqtks hrrarsvasq siiaytmslg
701 aensvaysnn siaiptnfti svtteilpvs mtktsvdctm yicgdstecs
```

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```
751 nlllqygsfc tqlkraltgi aveqdkntqe vfaqvkqiyk tppikyfggf
801 nfsqilpdps kpskrsfied llfnkvtlad agfikqygdc lgdiaardli
851 caqkfkgltv lpplltdemi aqytsallag titsgwtfga gaalqipfam
901 qmayrfngig vtqnvlyenq klianqfnsa igkiqdslss tasalgklqd
951 vvnhnaqaln tlvkqlsskf gaissvlndi fsrldkveae vqidrlitgr
1001 lqslqtyvtq qliraaeira sanlaatkms ecvlgqskrv dfcgkgyhlm
1051 sfpqsaphgv vflhvtyvpa qeknfttapa ichdgkahfp regvfvsngt
1101 hwfvtqrnfy epqiittdnt fvsgncdvvi givnntvydp lqpeldsfke
1151 eldkyfknht spdvdlgdis ginasvvniq keidrlneva knlneslidl
1201 qelgkyeqyi kwpwyiwlgf iagliaivmv timlccmtsc csclkgccsc
1251 gscckfdedd sepvlkgvkl hyt //
```

Nucleocapsid or N protein, Omicron variants BA.5

Only T-cell epitope peptides were selected from the sequence of the Nucleocapsid protein. Underlined portions of sequence show coverage by epitope peptides. Omicron BA.5 mutations: P13L, Δ31-33, R203K, G204R, S413R shown in bright pink highlight. BA.4 has the same mutations, plus P151S (position shown in light pink). Numbering is the same as the Wuhan strain, ignoring the altered numbering due to deletion.

```
1   msdngpqnqr nalritfggp sdstgsnqng    garskqr rpqglpnnta swftaltqhg
61  kedlkfprgq gvpintnssp ddqigyyrra trrirggdgk mkdlsprwyf yylgtgpeag
121 lpygankdgi iwvategaln tpkdhigtrn pannaaivlq lpqgttlpkg fyaegsrggs
181 qassrsssrs rnssrnstpg sskrtsparm agnggdaala lllldrlnql eskmsgkgqq
241 qqgqtvtkks aaeaskkprq krtatkaynv tqafgrrgpe qtqgnfgdqe lirqgtdykh
301 wpqiaqfaps asaffgmsri gmevtpsgtw ltytgaikld dkdpnfkdqv illnkhiday
361 ktfpptepkk dkkkkadetq alpqrqkkqq tvtllpaadl ddfskqlqqs msradstqa
//
```

Primary immunogens

B-cell immunogens for Omicron BA.4, BA.5

The stabilized full-length Spike protein (amino acids 1 to 1273, Wuhan numbering), with mutations for stabilization and glycosylation unmasking is shown below. Part of the HR2 domain is deleted for structural stabilization of this immunogen, which is replaced by the linker amino acid sequence G4S.

```
701              siaiptnfti svtteilpvs mtktsvdctm yicgdstecs
751  nlllqygsfc tqlKraltgC aveqdkntqe vfaqvkqiyk tppikYfggf
```

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```
801  Qfsqilpdps kpskrsfied llfnkCtlad agfikqygdc lgdiaardli
851  caqkfngltv lpplltdemi aqytsallag titsgwtfga gaalqipfam
901  qmayrfngig vtqnvlyenq klianqfnsa igkiqdslss tasalgkCqd
951  vvnHnaqaln tlvkqlssKf gaissvlndi lsrldPPeae vqidrlitgr
1001 lqslqtyvtq qlirCaeira sanlaatkms ecvlgqskrv dfcgkgyhlm
1051 sfpqsaphgv vflhvtyvpa qeknfttapa ichdgkahfp regvfvsngt
1101 hwfvtqrnfy epqiittdnt fvsgncdvvi givnntvydp lqpeldsfke
1151 eldkyfkQht spdvdlgggg gs________ __________ ____eslidl
1201 qelgkyeqyi kwpwyiwlgf iagliaivmv timlccmtsc csclkgccsc
1251 gscckfdedd sepvlkgvkl hyt //
```

OPTIONAL:

Omicron BA.2, full-length Spike protein, with 2P mutations for stabilization. Similar immunogens can be produced as needed for new variants, e.g. BA.2.12.1, by adding/subtracting substitutions.

Pink highlights are omicron substitutions. Mutations above and below in bold type are substitutions of ACE2 binding residues. Numbering is the same as the Wuhan strain, ignoring the altered numbering due to insertions and deletions.

Spike mutations: T19I, L24S, Δ25-27, G142D, V213G, G339D, S371F, S373P, S375F, T376A, K417N, N440K, S477N, T478K, E484A, **Q493R, Q498R**, **N501Y**, **Y505H**, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K.

```
1    mfvflvllpl vssqcvnlit rtqs___ytn sftrgvyypd kvfrssvlhs
51   tqdlflpffs nvtwfhaihv sgtngtkrfd npvlpfndgv yfasteksni
101  irgwifgttl dsktqslliv nnatnvvikv cefqfcndpf ldvyyhknnk
151  swmesefrvy ssannctfey vsqpflmdle gkqgnfknlr efvfknidgy
201  fkiyskhtpi nlgrdlpqgf saleplvdlp iginitrfqt llalhrsylt
251  pgdsssgwta gaaayyvgyl qprtfllkyn engtitdavd caldplsetk
301  ctlksftvek giyqtsnfrv qptesivrfp nitnlcpfde vfnatrfasv
351  yawnrkrisn cvadysvlyn fapfftfkcy gvsptklndl cftnvyadsf
401  virgdevrqi apgqtgniad ynyklpddft gcviawnsnk ldskvggnyn
451  ylyrlfrksn lkpferdist eiyqagnkpc ngvagfncyf plRsygfRpt
501  YgvgHqpyrv vvlsfellha patvcgpkks tnlvknkcvn fnfnglkgtg
551  vltesnkkfl pfqqfgrdia dttdavrdpq tleilditpc sfggvsvitp
601  gtntsnqvav lyqgvnctev pvaihadqlt ptwrvystgs nvfqtragcl
651  igaeyvnnsy ecdipigagi casyqtqtks hrrarsvasq siiaytmslg
701  aensvaysnn siaiptnfti svtteilpvs mtktsvdctm yicgdstecs
751  nlllqygsfc tqlkraltgi aveqdkntqe vfaqvkqiyk tppikyfggf
801  nfsqilpdps kpskrsfied llfnkvtlad agfikqygdc lgdiaardli
851  caqkfngltv lpplltdemi aqytsallag titsgwtfga gaalqipfam
```

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```
901 qmayrfngig vtqnvlyenq klianqfnsa igkiqdslss tasalgklqd
951 vvnhnaqaln tlvkqlsskf gaissvlndi lsrldPPeae vqidrlitgr
1001 lqslqtyvtq qliraaeira sanlaatkms ecvlgqskrv dfcgkgyhlm
1051 sfpqsaphgv vflhvtyvpa qeknfttapa ichdgkahfp regvfvsngt
1101 hwfvtqrnfy epqiittdnt fvsgncdvvi givnntvydp lqpeldsfke
1151 eldkyfknht spdvdlgdis ginasvvniq keidrlneva knlneslidl
1201 qelgkyeqyi kwpwyiwlgf iagliaivmv timlccmtsc csclkgccsc
1251 gscckfdedd sepvlkgvkl hyt //
```

T-cell epitopes

Mem 172T

172 TSRTLSYYKLGASQRVA

OPTIONAL Generation 9

Mem 172T

TSRTLSYYKLGASQRVA

IN TESTING, vaccine Generation 10, 11, 12

Combination MHC Class II/CD4 and MHC Class I/CD8 (A11:01, A33:03, B27:05, B39:01, C06:02, C07:01, C07:02).

The top ranking MHC Class II/CD4 epitope according to data published by Peng and colleagues, as well as the top-ranking MHC Class II/CD4 epitope among Adaptive Biotechnologies' data as of Sept 28, 2020. The peptide KEITVATSRTLSYYK and LSYYKLGASQRVAGD both are separately in the top 10 MHC Class II/CD4 epitopes identified by Tarke and colleagues in a whole proteome screen[105]. These sequences clearly are among the most potent MHC Class II epitopes in the SARS-CoV-2 proteome.

Nuc 100-120

100 KMKDLSPRWYFYYLGTGPEAG

IN TESTING, vaccine Generation 7, 8, 9

Nuc 102T

KDLSPRWYFYYLGTGPEAGL

OPTIONAL, vaccine Generation 10, 11, 12

Combination MHC Class II/CD4 and MHC Class I/CD8 (A01:01, A23:01, A26:01, B07:02, B08:01, B15, B58:01)

[105] https://www.sciencedirect.com/science/article/pii/S266637912100015X

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One of the cross-reactive SARS-CoV-2 epitopes to T-cells from SARS-CoV-1 convalescent patients tested by Le Bert and colleagues[106]. T-cells from these patients are cross-reactive to homologous epitopes from SARS-CoV-2, which are highly conserved between the two viruses. Also identified by Peng and colleagues as an immunodominant CD4/MHC class II epitope and CD8/MHC class I epitopes. The sequence SPRWYFYYL was also identified experimentally by Ferretti et al as a CD8 epitope with predicted HLA restriction of B07. The second highest ranked CD8 epitope identified by Snyder et al. in their proteome-wide screen, although in subsequent larger public data releases from Adaptive Biotechnologies, its rank dropped to ninth[107].

Nuc 263T

263 ATKAYNVTQAFGRRG

IN TESTING, vaccine Generation 8; OPTIONAL, Gen 9

Nuc 264T

ATKAYNVTQAFGRRG

OPTIONAL, vaccine Generation 10

Combination MHC Class II/CD4 and MHC Class I/CD8 (A23:01, A24:02, A33:03, B15:01, B35:01, B35:08, B53:01, C06:02, C07:01, C07:02)

The full-length epitope ATKAYNVTQAFGRRG was predicted by Fast and Chen as a broad-coverage epitope for MHC-I (74%) and for MHC-II (100%). This sequence largely overlaps a top MHC Class II/CD4 epitope in the public dataset of Adaptive Biotechnologies (PRQKRTATKAYNVTQAFGR), and of Tarke et al. (KRTATKAYNVTQAFG), and one of the cross-reactive SARS-CoV-2 epitopes to T-cells from SARS convalescent patients tested by Le Bert et al (KAYNVTQAFGRRGPE). The sequence KAYNVTQAF is the third highest ranked CD8 epitope identified by Snyder et al, although in subsequent larger public data releases from Adaptive Biotechnologies, its rank dropped to nineteenth. **CAUTION should be exercised in the use of the Nuc 264T peptide, since a similar sequence in SARS-CoV-1 has been implicated in suppression of cytokine signaling.**[108]

Nuc 321-345

321 GMEVTPSGTWLTYTGAIKLDDKDPN

IN TESTING, vaccine Generation 7, 8, 9

[106] https://pubmed.ncbi.nlm.nih.gov/32668444/
[107] https://www.medrxiv.org/content/10.1101/2020.07.31.20165647v2
[108] https://pubmed.ncbi.nlm.nih.gov/20976535/

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Nuc 322T

MEVTPSGTWLTYTGAIKLDD

OPTIONAL, vaccine Generation 10, 11, 12

Combination MHC Class II/CD4 and MHC Class I/CD8 (A01:01, A26:01, B18:01, B35:01, B35:08, B40:01, B44:02, B44:03, B53:01)

One of the cross-reactive SARS-CoV-2 epitopes to T-cells from SARS-CoV-1 convalescent patients tested by Le Bert and colleagues. T-cells from these patients are cross-reactive to homologous epitopes from SARS-CoV-2, which are highly conserved between the two viruses. This sequence contains a predicted dominant T-cell epitope of Grifoni et al (MEVTPSGTWL, HLA restriction B*40:01) [109]. MEVTPSGTWL was also verified experimentally by Peng and colleagues as a CD4 epitope and CD8 epitope likely covering HLA type B*40:01. MEVTPSGTWL is also among the top 20 ranked CD8 epitopes identified by Snyder et al. Mateus et al identified the peptide PSGTWLTYTGAIKLD as a CD4 epitope, with common coronavirus cross-reactivity[110].

Spike 1196

SLIDLQELGKYEQYIKWPWYI{-}ddddd

OPTIONAL Gen 11, 12

Combination MHC Class II/CD4 and MHC Class I/CD8 (A01:01, A03:01, A03:02, A11:01, A23:01, A24:02, B15:01, B18:01, B44:02, B44:03, C06:02, C07:01, C07:02)

The sequence of this portion of Spike protein lies at the boundary of the viral exterior and includes a few amino acids of the highly conserved transmembrane domain of the protein. It is bound by antibodies in serum from a small fraction of convalescent patients. However, Adaptive Biotechnologies data indicate that it is the most important MHC Class I epitope on the Spike protein, strongly binding a large number of common allelotypes. Given the broad HLA/MHC Class I coverage provided by this peptide it will be an attractive addition to vaccine designs; however, it is predicted to be very difficult to synthesize. According to Class II data published by Adaptive and by Tarke et al, this sequence is only a minor contributor to the MHC Class II response.

Nuc 359T

359 AYKTFPPTEPK

IN TESTING, vaccine Generation 8. 9 OPTIONAL Gen 9

Class I MHC/HLA restriction: A03:01, A03:02, and A11:01

The sequences KTFPPTEPK / AYKTFPPTEPK are among the top ten ranked CD8 epitopes identified by Snyder et al. The sequence KTFPPTEPK was also identified experimentally by Ferretti et al as a CD8 epitope with predicted HLA restriction of A*03 and A*11. Also identified

[109] https://www.sciencedirect.com/science/article/pii/S1931312820301669

[110] https://science.sciencemag.org/content/early/2020/08/04/science.abd3871

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experimentally by Peng and colleagues as a CD8/MHC class I epitopes likely covering HLA types A*03:01 and A*11:01.
AYKTFPPTEPKKDKKKKADETQALPQRQKKQ

Orf1 1636T

1636 HTTDPSFLGRY

IN TESTING, vaccine Generation 8. 9, 10, 11, OPTIONAL: Gen 12

Class I MHC/HLA restriction: A01:01, A03:02, A11:01, A26:01, A33:03

The sequence HTTDPSFLGRY is the top-ranked MHC class I/CD8 epitope identified by Snyder et al in a proteome-wide screen from Adaptive Biotechnologies, although in subsequent larger public data release from Adaptive, its rank dropped to second. The sequence TTDPSFLGRY was also identified experimentally by Ferretti et al as a CD8 epitope with predicted HLA restriction of A*01. Peng and colleagues didn't include Orf1 in their analysis, explaining the absence of this epitope in their preprint. This epitope sequence is part of the polyprotein Orf1ab; the sub-protein and start position numbering are Nsp3, amino acid 818 (Nsp3 818).

Orf1ab 4723T

4723 RKIFVDGVPFVVSTGYHFRE

IN TESTING, vaccine Generation 10, 11, 12

Class I MHC/HLA restriction: A02:01, A11:01, A23:01, A26:01, A33:03, B15:01, B35:01, B35:08, B53:01, C04:01, C05:01, C05:09

This peptide sequence RKIFVDGVPFVVSTGYHFRE is among the top five MHC Class I/CD8 epitopes in Adaptive Biotechnologies' data. The core peptide FVDGVPFVV is water insoluble but the longer peptide is water soluble, and covers a wide range of common Class I allelotypes. This epitope is highly conserved and in a January 2022 publication Kundu et al. showed that FVDGVPFVV is cross reactive with common coronaviruses in people uninfected with SARS-Cov-2.[111]

Orf1 5470T

5470 KLSYGIATVR

IN TESTING, vaccine Generation 9, 10

Class I MHC/HLA restriction: A02:01, A03:01, A03:02, A33:03

This peptide sequence KLSYGIATV is among the top ten MHC Class I/CD8 epitopes in Adaptive Biotechnologies' data and covers Class I MHC/HLA restriction A*02 (KLSYGIATV). With the addition of the flanking C terminal Arg, it is water soluble, and also covers A*03 (KLSYGIATVR)

[111] https://www.nature.com/articles/s41467-021-27674-x#Sec17

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and B*33. This epitope sequence is part of the polyprotein Orf1ab; the sub-protein and start position numbering are Nsp13, amino acid 146 (Nsp13 146).

Orf1ab 5528T

5528 DYGDAVVYRGTTTYKL

IN TESTING, vaccine Generation 12

OPTIONAL, vaccine Generation 10, 11

Class I MHC/HLA restriction: A01:01, A03:01, A03:02, A11:01, A23:01, A24:02, A26:01, A33:03, B15:01, B27:05, B35:01, B35:08, B39:01, C6:02, C07:01, C07:02

The peptide sequence DYGDAVVYRGTTTYKL is among the top 100 MHC Class I/CD8 epitopes in Adaptive Biotechnologies' data. It is highly conserved across coronaviruses.

Orf1ab 5246T

5246 LMIERFVSLAIDAYP

Part of the coding region of Nsp12. Combination MHC Class II/CD4 and MHC Class I/CD8 (A01:01, A02:01, B08:01, B35:01, C07:02). A top 20 CD4 epitope of SARS-CoV-2 from Tarke et al (Orf1 not included in Adaptive Biotechnologies' data). Very highly conserved across coronaviruses. This peptide is difficult to synthesize.

Orf8 107T

107 DFLEYHDVRVVL

IN TESTING, vaccine Generation 9; OPTIONAL, Gen 11, 12

Class I MHC/HLA restriction: A02:01, A23:01, A24:02, B39:01, B40:01, A33:03, C04:01, C05:01, C05:09, C06:02, C07:01, C07:02

This peptide sequence DFLEYHDVRVVL is among the top twenty MHC Class I/CD8 epitopes in Adaptive Biotechnologies' data. It is highly water soluble, and covers a wide range of common Class I allelotypes. **DELETED IN VARIANT OF CONCERN B.1.1.7**

Nsp7 21T

21 çAQCVQLH

IN TESTING, vaccine Generation 8; OPTIONAL, Gen 9

Class I MHC/HLA restriction: A02:01, B44:02, B44:03, A58:01

Placed into the list of optional peptides for Gen 9 due to absence from Adaptive Biotechnologies' MHC Class II/CD4 data release in late September 2020 (Orf1ab, which includes Nsp7 was not included in the generation of their data set). It is also absent from the MHC Class II data of Tarke et al., which does include Orf1ab and Nsp7[112] One of the cross-reactive SARS-CoV-2 epitopes to T-cells from SARS-CoV-1 convalescent patients tested by Le Bert and colleagues. The sequence

[112] https://www.sciencedirect.com/science/article/pii/S266637912100015X

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KLWAQCVQL was also identified experimentally by Ferretti et al as the top-ranked CD8 epitope with predicted HLA restriction of A*02. The same sequence is among the top 20 ranked CD8 epitopes identified by Snyder et al.

Other possible epitopes and peptides, including high solubility synthetic peptides are listed at the end of this document in the Supplemental Materials section.

SUMMARY OF THE PROTECTIVE STRATEGY OF THE GEN 1 mRNA VACCINE DESIGN (updated 2022-05-17)

This section describes the evolution of the design strategy of the RaDVaC vaccine to achieve the goal of sterilizing immunity. The B-cell-based protective strategy of the first few generations of vaccine was focused on the Spike RBD interaction with the host ACE2 receptor. However, there are three points that make this strategy suboptimal.

1. The ACE2 contact portion of Spike RBD (the receptor binding motif, RBM) has a low degree of conservation. Structural models show that CoV-1 contacts ACE2 slightly differently from CoV-2, and the related MERS virus interaction is even less conserved. These data suggest that this portion of Spike has higher mutant escape potential than more highly conserved portions of the virus.
2. Mutants with higher transmissibility and potentially higher pathogenicity have arisen in multiple amino acids in and around the RBM. Many mutations appear to alter the interaction of Spike with ACE2, and the binding of certain neutralizing antibodies.
3. The full RBM is very difficult to produce as synthetic peptides. Even if produced, conformational correctness is a top concern with such a structurally complex portion of Spike, which is likely to be critical for eliciting an appropriate antibody response to this antigen.

Given these concerns, and the fact that early attempts at synthesis of RBD epitope peptides was expensive, inefficient, and complex, RBM peptides were retired in Gen 9. However, we continued to work on solving the problem of creating peptides to represent the RBM, and emerging variants of concern show that mutations in the RBD are important for transmission and pathogenicity. Therefore, for the Gen 10 and forward designs, we include a specially designed single peptide that represents key residues on the RBM. We use this simplified approach to represent a portion of the RBM shown to be bound by neutralizing antibodies, such as REGN 10987, while incorporating key mutations. There are many such mutations in this portion of the omicron RBM, and we have incorporated many in the omicron-specific RBM peptide RBMO-sc. But this is not the only or even primary focus of the Gen 12 design. As with multiple prior generations, a major focus

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remains B-cell targets on the Spike protein known to be involved in fusion of the virus and host membranes; in particular, the two targets at about Spike 815 and 1145, with a lesser focus on the less conserved protease cleavage site at about 685. Here is a summary of key reasons for these foci:

1. These portions of the Spike protein are highly to extremely highly conserved. The 50 amino acid Heptad Repeat 2 (HR2) domain is 100% conserved between CoV-1 and Cov-2, as is the immediately flanking sequence at 1145 to 1160. The S2' protease cleavage site is also highly conserved, and mutant escape potential is constrained because this site must be recognized and cleaved by the host protease TMPRSS2.
2. These 3 targets are required for membrane fusion. Even if one mutates to escape antibody binding, a remaining antibody bound site should inhibit efficient fusion.
3. The two primary target sequences form simple structures that are easily synthesized with relatively short peptides.
4. These 3 targets are strongly bound by antibodies in large fractions of convalescent patients, as described in the linear epitope mapping studies cited in the section above.
5. These 3 targets bind neutralizing antibodies, as described in the studies cited in the section above.

It is important to note a few points about this strategy. As linear peptides, Li et al. demonstrated that these individual epitopes do not produce strong neutralizing activity.[113] However, their fusion peptide epitope mapping created a division between two peptides directly in the center of the best peptide candidate. Their mapping of epitope SFKEELDKYFKNH did not have this problem. Nevertheless, they did not measure the combined neutralizing effects of these epitopes, which are expected to be synergistic, since they both target membrane fusion. However, it is being reported that the omicron variant of concern is largely resistant to current commercial vaccines and to previous infection, and omicron is heavily mutated in the N terminal domain and the RBD but not the C terminal domain or epitopes involved in fusion. Convalescents have a mix of antibody responses to fusion-related sights: about 45% have antibodies to the fusion peptide PSKRSFIEDLLF, and about 69% have antibodies to SFKEELDKYFKNH.

In addition to refocusing the B-cell targeting of the Gen 10 and Gen 11 vaccine designs, we maintain our focus on empirically validated T-cell epitopes. Similar to our strategy for B-cell epitope redundancy, we have included multiple T-cell epitopes for common alleles.

PREPARATION OF NANOPARTICLE VACCINE (updated 2022-05-17)

[113] https://www.sciencedirect.com/science/article/pii/S2211124721002291

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Delivery of RNA has been accomplished using a range of ingredients and concentrations. Many published protocols exist for the robust production of nanoparticle RNA vaccines.

ADMINISTRATION OF THE VACCINE (updated 2021-03-07)

Dosage amount. Human studies have used as little as 7.5 micrograms inactivated virus or other replication incompetent antigen.

Booster schedule. The vaccine described here is designed to be only moderately immunogenic. Therefore, our standard regimen has been at least four doses. Booster doses have been taken as soon as a few days after the prior dose, or as long as a few weeks. Ideally, doses are spaced by 1 week or more. Given the seriousness of the pandemic and individual circumstances, even shorter intervals between boosters might be used. This is done rarely even in a research setting, but most research is conducted in support of eventual commercialization, and reflects those constraints. Considering the burden and level of stimulation that a few weeks of CoV-2 infection exerts chronically on the immune system, more and frequent booster doses is not an unreasonable strategy. Nevertheless, there is some evidence of greater efficacy for longer intervals between doses. Testing for immune response throughout the booster schedule and a few weeks after is ideal for monitoring building immunity; however, this is not practical for some people. Some have reported after-effects that increase with later booster doses. This is a positive qualitative sign of immune response, but even positive antibody and other test results should not be relied upon as correlates of protection (a perfect proxy for sterilizing immunity).

Pre-administration. To assess the pre-vaccination state of the immune system, one or more saliva samples, nasal wash or swab, and blood draws are ideal prior to vaccination.

Administration. Vaccination is achieved by nasal administration with the use of small (5ml to 20ml) commercial nasal sprayers. We have tried a few sources of spray bottles. Depression of the sprayer top of those we have tested delivers approximately 100 to 150 microliters of fine mist. This should be tested empirically by spraying into a small tube or beaker, and measuring the volume using a pipette. Dose is adjusted accordingly. Spray should be directed into each nostril. The sprayers we obtained come as a top sprayer unit, and either opaque white plastic, amber, or clear bottle. The sprayer unit stem stretches to the bottom of the bottle. The delivered volumes of vaccine will be too small to be delivered reliably using the bottle. Therefore, we do not use the bottle for vaccination, and instead place the sprayer stem directly into the smaller vaccine vial. The stem of our tested sprayer units are slightly longer than a 2 ml microcentrifuge tube. We

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pipette an aliquot of vaccine into the tube, then place the stem of the sprayer into the vaccine for spray administration.

Protocol for vaccine administration

To administer vaccine, it is ideal to wear gloves and have a spray bottle of 70% alcohol (ethanol or isopropanol) to sterilize your gloved hands, vials, and equipment. Select a well-lit work surface that can be sterilized with alcohol.

Required materials:

- Clean and well-lit work surface
- Gloves (nitrile, latex, etc)
- 70% alcohol (ethanol or isopropanol), preferably in a spray bottle
- Small (5ml to 20ml) commercial nasal sprayer bottle
- Vial of vaccine containing 1 dose plus extra for priming the sprayer (total, 800 microliter)
- Clean and preferably sterile paper towels or tissues
- OPTIONAL: saline wash solution and collection tube(s)
- OPTIONAL: rack or holder for vaccine vial

1. Sterilize the work surface with alcohol. Place a paper towel or tissue on the clean work surface, and spray with alcohol. You can place items on the surface and use the towel or tissue to dab or wipe items (e.g. the sprayer stem) while maintaining sterility.
2. Wipe the vaccine vial with an alcohol wipe. Loosen the cap so that it can be removed by lifting it off, but leave the cap in place, and set the vial upright on the work surface. If you choose to use a tube holder or rack for the vial, make sure it is sterilized.
3. Blow your nose thoroughly with a tissue or paper towel to clear your nasal passages. Use an alcohol soaked wipe or tissue to clean the outer area of the nose and just inside each nostril. This will help prevent inadvertent contamination of the sprayer tip with infectious virus that might be present on or just inside the nose. We do not recommend cleaning deep into the nasal passage; this is likely to do more harm than good.
4. Sterilize your spray bottle by placing 2 to 4 ml of 70% alcohol into the spray bottle, screw on the cap, and deploy the sprayer until a fine mist is sprayed.
5. Remove the spray top, and spray the residual alcohol. While you are doing this **DO NOT TOUCH THE SPRAYER TIP OR STEM** to maintain sterility. After sterilization or the following optional nasal wash, do not return the spray top to the bottle prior to using the vaccine.

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6. **OPTIONAL**. You can use the sterilized spray top apparatus at this point to spray 100 mM NaCl into nostrils for collection of nasal wash samples. Spray saline into nostrils, inhale slightly, and then evacuate nasal wash into a collection tube. Collect at least 500 microliters.
7. While holding the spray top in one hand, uncap the vaccine vial with the other hand, and place the clean and sterile stem of the sprayer directly into the vaccine vial so that the stem touches the bottom of the vial.
8. Grasp the vaccine vial with one hand, and the spray top with the other hand so that your index finger and middle finger are on the side handles of the sprayer.
9. Deploy the sprayer into the air (not up your nose) until you see a fine mist, in order to clear residual alcohol and/or saline from the sprayer.
10. Insert the spray tip into a nostril. While inhaling through the nose, depress the sprayer (ideally, a total of about 100 to 150 microliters per spray). Sniff in the vaccine. Repeat the dosing, for a total of 200 to 300 microliters into each nostril. The entire procedure can take seconds or minutes but don't let the vaccine drip out of the nose, and don't blow your nose for at least an hour or two.
11. **OPTIONAL**. In later booster doses you can collect nasal washes or saliva after vaccination to assess immune response. At 4 hours post vaccination, collect the sample as described in the previous OPTIONAL nasal wash. Cytokine levels in the sample can be measured as indicators of response, if you have the means to do this.

ASSESSMENTS OF IMMUNE RESPONSE (updated 2021-03-07)

There are multiple important immune responses to an effective vaccine, and, therefore, multiple measures of potential effectiveness. These are not measures of effectiveness, but measurable immune response correlates of protection.

- B-cell/Antibody response
    - Neutralizing vs non-neutralizing
    - Neutralizing titer
    - Avidity/affinity of antibodies
- T-cell response (often measured by cytokine secretion)
    - Cell mediated: Th1/Cytotoxic T Lymphocytes (CTL)/CD8+
    - Humoral: Th2/CD4+

Neutralizing antibodies provide protection against viral infection and replication. They bind to key functional parts of the virus. Non-neutralizing antibodies can be beneficial by marking a virus for destruction, but they do not interfere with virus activities such as infection and replication, and

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they are also associated with ADE. High antibody titer is generally required for optimal protection. Avidity or affinity of the antibody binding to an antigen is another important measure of an effective antibody. An ideal B-cell response involves high-avidity neutralizing antibodies that bind to multiple viral epitopes. The sections below address details of measuring each of these key metrics. T-cell responses are often measured by cytokine secretion, or by counting cytokine secreting cells.

Samples to be collected and tested

- Whole blood and serum
- Nasal wash
- Saliva

Baseline samples will be collected, and then post-vaccination samples will be taken at booster timepoints, and also at later dates. Each of the above sample types has been used successfully to detect viral RNA and antibodies.

Collection of samples for immune testing. Ideally, all samples will be collected immediately prior to vaccination, and at various time points after initial and booster vaccinations.

Blood is ideally collected by venipuncture and vacutainer, but can be collected by lancet (finger) and deposited into sterile microcentrifuge tubes, without preservative or clotting chemistry. Antibody assays such as ELISA, typically start at dilutions of 1:100 or 1:200 and thus can be run on a few microliters of serum. About 50% of whole blood is recoverable as serum. Therefore, a few drops of blood (30+ microliters) is sufficient for each individual at each time point, for each test. It is ideal to have enough serum for several tests. Blood and serum can be stored for relatively short periods (weeks) at —20°C until assayed. Whole blood or buffy coat are required for certain assays, such as ELISpot, that use peripheral blood mononuclear cells (PBMCs).

Nasal wash. Method adapted from de Fijter et al [114]. Use nasal sprayer to deposit 200+ microliters of sterile 100mM saline into one nostril. Collect nasal wash effluent in a clean, labeled tube (a small funnel that fits into the collection tube is very helpful). Collection tube should be free of preservative, or contain non-denaturing preservative such as EDTA. Repeat the procedure with the other nostril. Repeat until you have collected a total of at least 0.5 ml. Don't collect more than 2 ml. Store at —20° C until further processed or assayed. Once all aliquots are collected, vortex with glass beads, then centrifuge the samples at 1000 g for 10 minutes at 4°C to clarify. Pipette the supernatants into fresh microcentrifuge tubes, and either refreeze at —20° C or process immediately.

[114] https://pubmed.ncbi.nlm.nih.gov/8872971

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Saliva. At least 0.5 ml should be deposited into sterile tubes, free of preservative, or containing a non-denaturing preservative such as EDTA. Store at —20°C until assayed. Depending on dilution factor (typically starting at 10 fold or greater), assays can be run on a few microliters of saliva.

Antibodies and B-cell immune response

Methods developed for assessing immunity to other respiratory viruses, such as influenza, can be adopted for use. Immunity will be assessed using standard indirect ELISA antibody assays (shown below) of serum, nasal swab or wash, and possibly saliva.

Variations of ELISA allow testing of the following:

- Presence/absence of antibodies that bind a viral specific antigenic target
- Antibody titer (by dilution series)
- Antibody avidity (e.g. by pulse of chaotrope, such as thiocyanate or urea)

While neutralizing antibodies can't be identified by ELISA, this information can be inferred from published information on which epitopes are bound by antibodies that show neutralizing activity. Thus, if a specific epitope peptide used in a vaccine is bound by an antibody, we can infer that it should be a neutralizing antibody without directly testing it in a neutralization assay.

Initial ELISAs will be performed using recombinant Spike RBD as the target antigen. A few of our B-cell epitope peptides lie within the RBD, and this region is highly represented in immunodominant neutralizing antibodies[115]. Followup ELISAs will be performed on various antibody fractions with individual epitope peptides in 96 well plates. Serum will be diluted 1/200, and plated in a standard 2X dilution series. Nasal washes are performed by use of a nasal sprayer and 100 mM saline, and the samples are analyzed by ELISA essentially as by Barria et al.[116] For a review of assessing the effectiveness of nasal vaccines for influenza, see Gianchecchi et al[117].

[115] https://immunology.sciencemag.org/content/5/48/eabc8413
[116] https://academic.oup.com/jid/article/207/1/115/874878
[117] https://onlinelibrary.wiley.com/doi/full/10.1111/irv.12664

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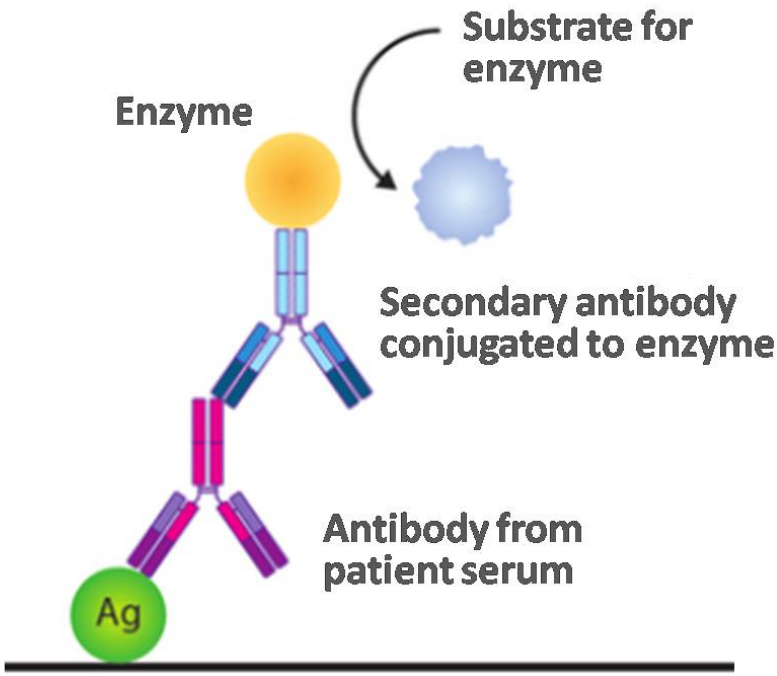

Schematic of an indirect ELISA assay. Viral antigen (Ag) is attached to a surface (plate well). Patient serum is added to the plate well, washed and blocked. An enzyme-conjugated secondary antibody that binds to human antibodies is added to the plate well. A substrate for the enzyme is added to the plate well, producing a signal if the antigen is bound by antibodies in the patient's serum.

Standard protocols and reagents for testing for SARS-CoV-2 virus exposure have been published.[118],[119] These protocols and reagents allow testing for immunity either elicited from our vaccine or from virus exposure. Slight modification of the protocols to use our peptides in ELISAs, and for testing of nasal washes, will allow us to test for immunity specific to the epitopes of our vaccine, and for nasal mucosal immunity.

Antibody avidity can be measured by multiple methods, as reviewed by Klasse[120]. In our vaccine schema, individual epitope peptides are used to elicit antibodies, and each of these can be used independently to assess avidity of binding antibodies.

B-cell epitopes and ELISA antigen targets

Spike protein epitopes / antigen targets

[118] https://labs.icahn.mssm.edu/krammerlab/wp-content/[...]expression-and-serology-protocol-3_24_20.pdf
[119] https://labs.icahn.mssm.edu/krammerlab/covid-19/
[120] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4766047/

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Purified recombinant Spike protein and Spike receptor binding domain (RBD) used as target antigen will allow for testing of viral exposure, and for immunity elicited by our vaccine, but will not be specific for immunity elicited by any specific epitope of our vaccine. Stadlbauer and colleagues use the recombinant RBD protein for testing rather than the full-length Spike protein, due to low expression and purification of full-length Spike. We will use RBD as a primary target antigen.

Epitope peptides used in vaccine production can be used in ELISA and other tests for immune response. ELISA testing with recombinant RBD and separately with epitope peptides might help determine if immunity is due to the vaccine, or to virus exposure. ELISA with a protein other than Spike will be more definitive in distinguishing between immunity from the vaccine, and immune response to virus exposure.

Nucleocapsid protein B-cell epitopes / antigen targets

Nucleocapsid is widely used as a highly sensitive antigen target for antibody testing. We ordered a peptide of one of the most antigenic sequences of the highly antigenic Nucleocapsid protein (NP produces large signals in ELISA of convalescent sera). Because of concerns about ADE, we have not yet included Nucleocapsid B-cell epitopes in our vaccine designs. It is possible to use them as negative controls for ELISA. If concerns about ADE risk are resolved, then these selections might be considered for use in a vaccine. We have designed longer peptides, and shorter portions of this peptide.

Nuc 371-399
Longer candidate: `DKKKKADETQALPQRQKKQQTVTLPAAD`
Long single linear epitope near the C terminus of the Nucleocapsid protein. Substitution of Lys 379 to reduce the positive charge near the N terminus: Lys>Gly. This region has the longest linear epitope discovered by protein microarrays in SARS-CoV-2 (Wang) and SARS-CoV (Zhu) [121]. This epitope is split into two overlapping peptides, **Nuc 376-399** and **Nuc 357-382.**

Nuc 357-382: `IDAYKTFPPTEPKKDKKKKADETQAL`
Overlaps with **Nuc 376-399**.

Nuc 371-394: `DKKKKADETQALPQRQKKQQT`
SYNTHESIZED and ready for use as a negative control in testing
Nucleocapsid antibodies have been implicated in Antibody-directed enhancement. Take notice before selection for use in a vaccine. Useful as a negative control.

[121] https://www.pnas.org/content/103/11/4011.long

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Cys1, Cys24 disulfide,
2958 g/mol. 2.96g/ml = 1M. 2.96mg/ml = 1mM 1mg/ml = 338uM

Use of the recombinant RBD as a standard for ELISA will not allow high confidence that an immune response is due to the vaccine rather than virus exposure. Use of envelope or other non-Spike SARS-CoV-2 proteins in ELISA will be more definitive. A positive result in either or both Spike-RBD and Spike1, but negative for non-Spike proteins is suggestive of immune response due to the vaccine.

Positive controls for infection should be obtained. Blood and other samples should be obtained from people who are or have been infected. They should have systemic IgG antibodies to not only Spike protein, but to other proteins as well. They might also have sIgA antibodies in nasal wash and saliva.

T-cell immune response

There are many approaches to testing T-cell immunity specific to the virus. We are considering the following possibilities, and we welcome collaborations with scientists who can contribute to these and other analyses of T-cell responses.

- ELISpot[122]
- Transcriptomic profiling of PBMC[123,124]
- Immunome assessment
- RT-PCR of T-cell transcripts

SUPPLEMENTAL MATERIALS

ADDITIONAL SEQUENCES

Wuhan strain Spike protein sequence

without breaks or spaces, for peptide search:

```
mfvflvllplvssqcvnlttrtqlppaytnsftrgvyypdkvfrssvlhstqdlflpffsnvtwfhaih
vsgtngtkrfdnpvlpfndgvyfasteksniirgwifgttldsktqsllivnnatnvvikvcefqfcnd
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tpinlvrdlpqgfsaleplvdlpiginitrfqtllalhrsyltpgdsssgwtagaaayyvgylqprtfl
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tgkiadynyklpddftgcviawnsnnldskvggnynylyrlfrksnlkpferdisteiyqagstpcngv
```

[122] https://en.wikipedia.org/wiki/ELISpot
[123] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3946932/
[124] https://www.ncbi.nlm.nih.gov/pubmed/?term=30205979

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egfncyfplqsygfqptngvgyqpyrvvvlsfellhapatvcgpkkstnlvknkcvnfnfngltgtgvl
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clkgccscgscckfdeddsepvlkgvklhyt//

Nucleocapsid protein sequence

without breaks or spaces, for peptide search:

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paadlddfskqlqqsmssadstqa

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