Vaccines and monoclonals to regain our freedom

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IBSA FOUNDATION for scientific research

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Presentation

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Introduction

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The emergence and rapid global spread of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has resulted in global morbidity and mortality along with widespread social and economic disruption.

The current COVID-19 pandemic has urged the international scientific community to find quick answers in terms of developing vaccines and monoclonal antibodies as a countermeasure to control SARS-CoV-2.

As the knowledge of SARS-CoV-2 pathogenesis and interactions with the immune system continues to be investigated, multipolicy drug candidates are under investigation and are currently undergoing clinical trials. Rapid progress has been made in the research of antibody response and in COVID-19 therapy, including isolation and characterization of a large panel of monoclonals neutralizing antibodies and early clinical testing, as well as the development of several COVID-19 vaccines.

A massive cooperation between public and private institutions allowed the development of several approved vaccines based on different technologies: adenovirus vectors (Oxford-AstraZeneca, Sputnik V and Johnson & Johnson), inactivated viruses (CoronaVac), spike protein subunit (EpiVacCorona authorized on 21st of April in Russia) and mRNAs encoding for the spike protein subunit (Pfizer/BioNTech and Moderna).

Specifically, mRNA vaccines by Pfizer/BioNTech and Moderna consist of lipid nanoparticle-encapsulated mRNA vaccines that encode a modified stabilized full-length spike protein. One of the main advantages of these vaccines is that they can be designed and manufactured within a short time to meet the need of COVID-19 variants outbreak. mRNA vaccines have demonstrated their efficacy to induce strong humoral and cellular immune response both in

preclinical and clinical studies and they have [already] been approved in several countries.

Monoclonal antibodies are laboratory-made proteins that mimic the immune system's ability to fight off harmful pathogens such as viruses. Monoclonal antibodies employed in COVID-19 treatment can block the interaction between the receptor binding domain RBD of Sars-CoV-2 S protein and its receptor ACE2, since they are able to identify the S1 fragment of SARS-CoV-2. Some monoclonal antibodies (bamlanivimab, casirivimab and imdevimab) received Emergency Use Authorization (EUA) from the FDA on November 2020 and other monoclonal antibodies against S protein are under development. The evidence of these studies is very promising and demonstrates the potential of this therapy as a therapeutic approach for COVID-19 infection.

After one year of pandemic mass immunization, the main goal is to reduce the global public health damage unleashed by this virus. The world's attention is on vaccines and monoclonals that, through active or passive immunization, will be the essential for the re-appropriation of our freedom.

Although the recent approval of anti-viral drugs and monoclonal antibodies, COVID-19 vaccines are essential not only to prevent virus spread, but also to restore social and economic activities via mass immunization.

Abstract

Vaccines and monoclonals to regain our freedom

Rino Rappuoli
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At the end of January 2020, the Chinese Centre for Disease Control and Prevention published the genomic sequence of the coronavirus disease 2019 (COVID-19) that is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and that first emerged in the Hubei province, China, in December 2019.

A year later the COVID-19 pandemic is registering a current world death toll of approximately 3 million and 117 million diagnosed cases and is hurling 21st century society into a deep socio-economic crisis.

In this scenario and in an unprecedented and exceptional short development timespan, active immunization, and passive immunization through vaccines and monoclonals, respectively, are proving to be the best weapons available to save lives, safeguard the economy, and regain our freedom.

Insights

Vaccinology in the post-COVID-19 era

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Abstract

The COVID-19 pandemic is a shocking reminder of how our world would look in the absence of vaccination. Fortunately, new technologies, the pace of understanding new and existing pathogens, and the increased knowledge of the immune system allow us today to develop vaccines at an unprecedented speed. Some of the vaccine technologies that are fast-tracked by the urgency of COVID-19 may also be the answer for other health priorities, such as antimicrobial resistance, chronic infections, and cancer, that the post-COVID-19 world will urgently need to face. This perspective analyzes the way COVID-19 is transforming vaccinology and the opportunities for vaccines to have an increasingly important role in health and well-being.

The path toward fully synthetic vaccines made using genomic information started in 2013. Sunday, March 31, 2013, was a nice Easter festivity when the World Health Organization (WHO) was notified about a new H7N9 avian influenza virus that had infected three people in China and killed two of them [1]. It was a new, potentially pandemic, virus for which the world was not prepared. The experience of the H2N2 in 1957, the H3N2 in 1968, and even of the H1N1 pandemic in 2009 had shown that vaccines had become available only after the pandemic peak, and therefore they were too late to be useful. On Monday, April 1, 2013, scientists at the J. Craig Venter Institute in San Diego, CA, accessed the sequence of the hemagglutinin and neuraminidase genes posted by the Chinese Center for Disease Control and Prevention on the Global Initiative for Sharing All Influenza Data system and used the enzymatic isothermal assembly method with self-error correction for the cell-free synthesis of the two genes [2]. The synthetic genes were then shipped overnight from California to Massachusetts. There, scientists from Novartis Vaccines used the synthetic genes to generate, in only 5 d, a synthetic influenza virus seed ready for vaccine manufacturing. In addition, they produced an RNA vaccine ready for animal immunization in the record time of 1 wk [3]. Fortunately, the H7N9 influenza virus did not transmit efficiently between humans, and, although

it caused a few hundred cases during the next few years, it did not cause a pandemic, limiting the use of these vaccines only to clinical trials.

Anticipated by the work of Craig Venter [4], teleportation of DNA code through great distances was not Star Trek's fiction anymore. For the first time, a fully synthetic viral vaccine was developed by *in vitro* cell-free synthesis of genes using the genomic sequence that had been teleported across the planet at the speed of light via the Internet. The process of teleporting the genomic sequence has the ambition to change forever the old – and dangerous – way we used to make viral vaccines by shipping viruses across the world. We use the term "Internet-based vaccines" to describe this new way of making vaccines using the Internet to share the genomic information, without the need to transport, access, and grow the real virus.

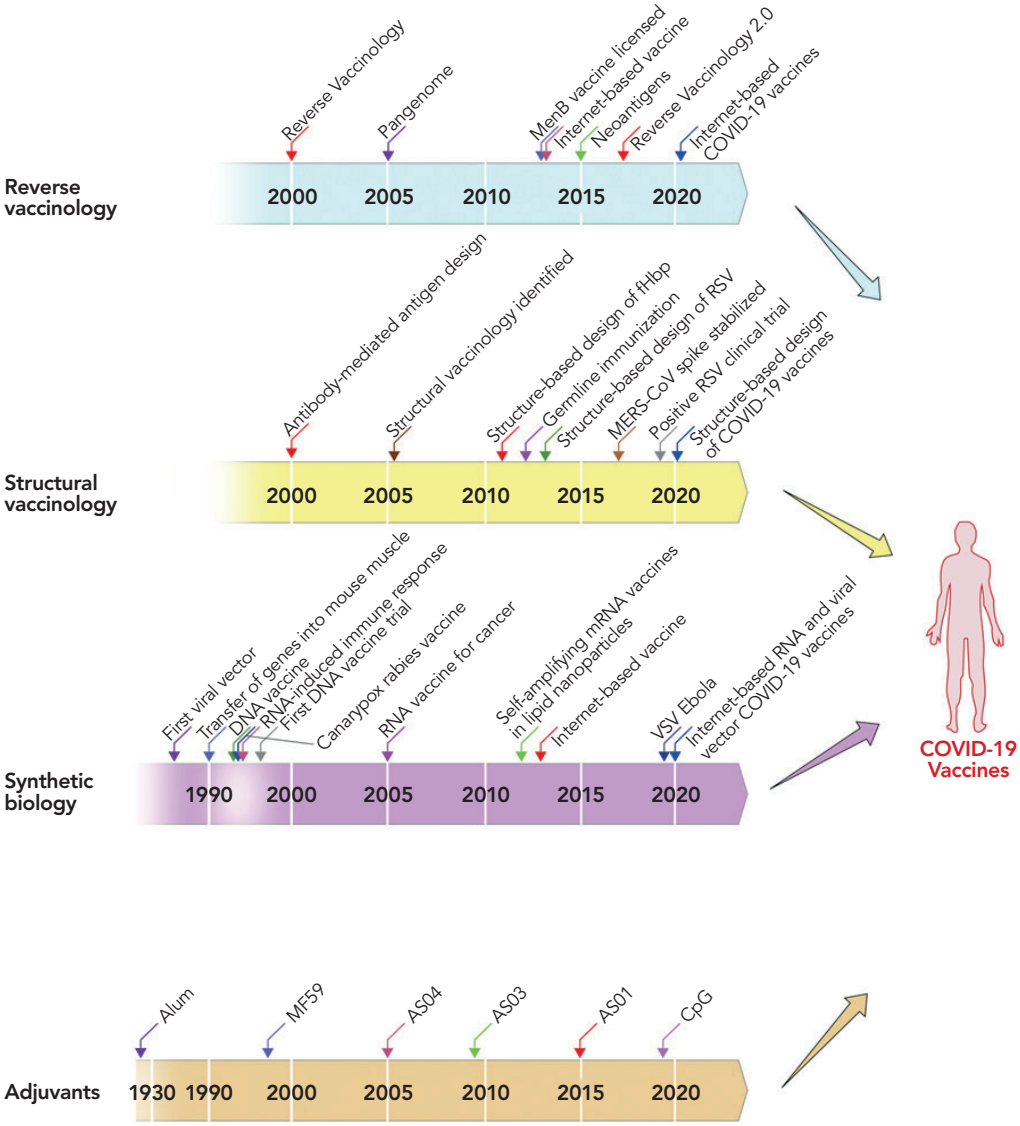
When, in January 2020, scientists from Fudan University and their collaborators posted on the Internet the genomic sequence of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), responsible for the current COVID-19 pandemic, most of the laboratories across the world were ready for the challenge. They not only had the technology to make vaccines starting from synthetic genes, but some of them could also use computer modeling of the atomic structure of the spike protein of similar coronaviruses to design, up front, an antigen stabilized in the prefusion conformation [5, 6]. Synthetic genes were used to rapidly start the development of more than 200 different vaccines. The remarkable quality and speed used for COVID-19 vaccine development was possible because the scientists combined, for the first time, three decades of scientific progress in independent fields: reverse vaccinology, structural vaccinology, synthetic biology, and vaccine adjuvants (• Figure 1). The advances in antigen selection and design (reverse and structural vaccinology) together with the use of innovative synthetic platforms such as nucleic acid vaccines (RNA and DNA based), viral vectors, and the availability of licensed adjuvants allowed for an unprecedented speed in the discovery of several COVID-19 vaccine candidates, many of which were already in clinical development stage.

Technologies used for COVID-19 vaccine development

Reverse vaccinology, structural vaccinology, synthetic biology, and vaccine adjuvants, that so far had been used independently to develop vaccines, were combined in an unprecedented worldwide effort to design and develop COVID-19 vaccines.

Reverse vaccinology, the science that identifies vaccine antigens from the genome of pathogens, was used for the first time in 2000 to identify novel

• Figure 1. Technological advances that merged to develop a COVID-19 vaccine



antigens for vaccine against meningococcus B, which, up to that moment, had been an impossible task for conventional technologies [7, 8]. The vaccine was licensed by the European Medicines Agency in 2013 and by Food and Drug Administration in 2015 and was recently shown to reduce by 74% the incidence of disease in United Kingdom and by 91% in Italy [9, 10]. During the last two decades, genomics has been used in the development of most vaccines, exploiting the pangenome of bacterial and viral species. Remarkable

progress in genome-based vaccines was made in 2013 when an RNA vaccine against a potentially pandemic H7N9 influenza virus was produced in 1 wk without culturing the virus but using the genome sequence available in public databases [3]. During the last few years, tumor immunologists used the genome of cancer cells to identify mutations coding for neoantigens to be incorporated in cancer vaccines [11].

Structural vaccinology, or structure-based antigen design, was predicted as an emerging field in 2007 when it became clear that high-throughput structure determination was going to be possible in the near future [12]. However, it had already been anticipated in 2002 that the study of antibodies recognizing protective epitopes was going to inform vaccine design [13]. The first example, published in 2011, was the design of a single meningococcal antigen containing the epitopes of three antigenic variants of the same molecule [14]. In 2013, structure-based vaccine design was used for the first time to develop a vaccine that had been impossible for other technologies, when McLellan *et al*. [15, 16] described the stabilization of the Respiratory Syncytial virus (RSV) Fusion (F) protein in the prefusion conformation. In 2019, the prefusion stabilized F protein was shown to induce unprecedented levels of neutralizing antibodies and to be ready for phase III clinical trials [17]. In 2013, structure-based design was also used for germline immunization to generate broadly neutralizing antibodies against HIV [18]. Finally, in 2015, structural vaccinology was used to stabilize the spike protein of the Middle East respiratory syndrome-related coronavirus (MERS-CoV) in the prefusion conformation [5]. In 2017, a perspective in the Journal of Experimental Medicine predicted the merging of reverse and structural vaccinology and named it reverse vaccinology 2.0 [19].

Synthetic biology is the ability to use synthetic genes for vaccination or cancer therapy. It was pioneered in 1986 by the use of a cloned gene into a viral vector for gene therapy [20], and, in 1992, by the cloning of the glycoprotein gene of rabies virus into a canarypox viral vector for the development of a rabies vaccine [21]. In parallel, it was shown that protein expression could be achieved by the direct transfer of genes into mouse muscle cells [22]. This observation suggested the use of naked DNA [23, 24] and of RNA [25] for vaccination. DNA vaccination became very popular during the following decade, until it was realized that, while successful in most animal models, DNA vaccination has not been, until to date, successful in humans. The decline of DNA popularity led to the rediscovery of viral vectors and RNA at the end of the first decade of the 21st century. In this period, viral vectors became very popular and were extensively used for the rapid generation of vaccines to fight the Ebola epidemic of 2014, which led to the licensure

of the first viral vector vaccine in 2019. In the meantime, the technology to make, stabilize, and deliver RNA matured in the pharmaceutical industry for the development of antisense RNA therapeutics. This technology, which employed delivery of RNA using lipid nanoparticles, was transferred to vaccines and allowed the efficient delivery of RNA vaccines [26] and the rapid development of fully synthetic RNA vaccines in 1 wk against an emerging pathogen [3]. During the last few years, the production and clinical testing of RNA vaccines and viral vectors increased exponentially so that both technologies were ready to tackle the SARS-CoV-2 pandemic.

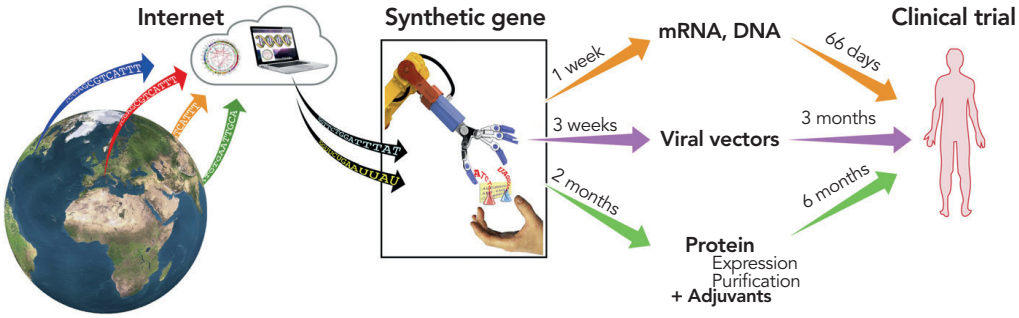
Adjuvants are substances added to vaccines to increase their potency. Aluminum phosphate or aluminum hydroxide has been used since the 1920s [27]. MF59, the first modern adjuvant, was licensed in 1997 to improve an influenza vaccine [28]. Since then, several novel adjuvants have been licensed and used in millions of people. The other adjuvants licensed today are AS03, AS04, AS01, and CpG oligonucleotides which are used for pandemic influenza, papillomavirus, herpes zoster, and hepatitis B, respectively [29]. In the clinical evaluation setting, alum, AS03, MF59, CpG, and Matrix-M are being used for COVID-19 vaccines.

In January 2020, these four technologies were used together for the development of a number of COVID-19 vaccines. A SARS-CoV-2 nucleotide sequence coding for the spike protein was derived from the genome sequence uploaded on public databases (reverse vaccinology); the synthetic gene was modified upfront to introduce the mutations previously identified to stabilize the coronavirus antigen in the prefusion form (structural vaccinology), and used for RNA and viral vector vaccines (synthetic biology). Finally, the protein-based vaccines (as stabilized recombinant trimers, viral-like particles, and nanoparticles) were combined with adjuvants. Although several SARS-CoV-2 vaccines in the clinic use other approaches such as inactivation or attenuation of the virus, the combination of these technologies and the coordinated global effort allowed for an unprecedented speed in the discovery of several COVID-19 vaccine candidates.

Vaccines for COVID-19

Several approaches are used to make COVID-19 vaccines, including nucleic acid-based vectors, inactivated or live attenuated viruses, recombinant proteins, and virus-like particles [30]. In this manuscript, we focus on the three main categories for which Internet-based vaccines are demonstrating massive developmental acceleration: synthetic RNA vaccines, viral vectors, and adjuvanted protein-based vaccines (• Figure 2).

• Figure 2. COVID-19 vaccines in development and their timeline to clinical testing in humans



Synthetic RNA vaccines are the fastest to develop. A fully synthetic gene is cloned in a plasmid vector, which is then used as template for the *in vitro* synthesis of the RNA vaccine [31]. There are two types of RNA vaccines: those encoding only the antigen and those encoding for both the antigen and the enzymatic machinery for RNA template replication following vaccination (self-amplifying RNA [26]). Given that RNA vaccines are fully synthetic and do not need a biological phase, they were able to reach clinical trials in the record time of 66 d, to move from phase I to phase II clinical trials in less than 5 mo [32], to produce promising immunogenicity and efficacy data in humans in 10 mo [33, 34]. Today, RNA vaccines are among the most promising vaccine technologies, and they will very likely be one of the most important platforms of the future. However, we need to be aware that, today, we do not have a licensed RNA vaccine yet; therefore, this type of vaccine still needs to go through the challenges of demonstrating safety, immunogenicity, and efficacy in a large human population. In addition, the manufacturing of RNA vaccines, despite being much simpler than conventional vaccines, has never been scaled up beyond the need of clinical trials, so that we have not yet developed the industrial capacity to make tens or hundreds of millions of doses. The urgency to cope with COVID-19 is providing an unprecedented opportunity to fast-track this technology and accelerate its maturation by several years.

In the case of viral vectors, the synthetic gene coding for the spike protein is inserted into one of many viruses that usually have been engineered so that they cannot replicate in the human host. The virus is then grown in culture and used to deliver the synthetic gene during vaccination. There are many viruses that can be used for this purpose. The most popular ones are adenoviruses (chimpanzee adenovirus, human adenoviruses 5 and 26),

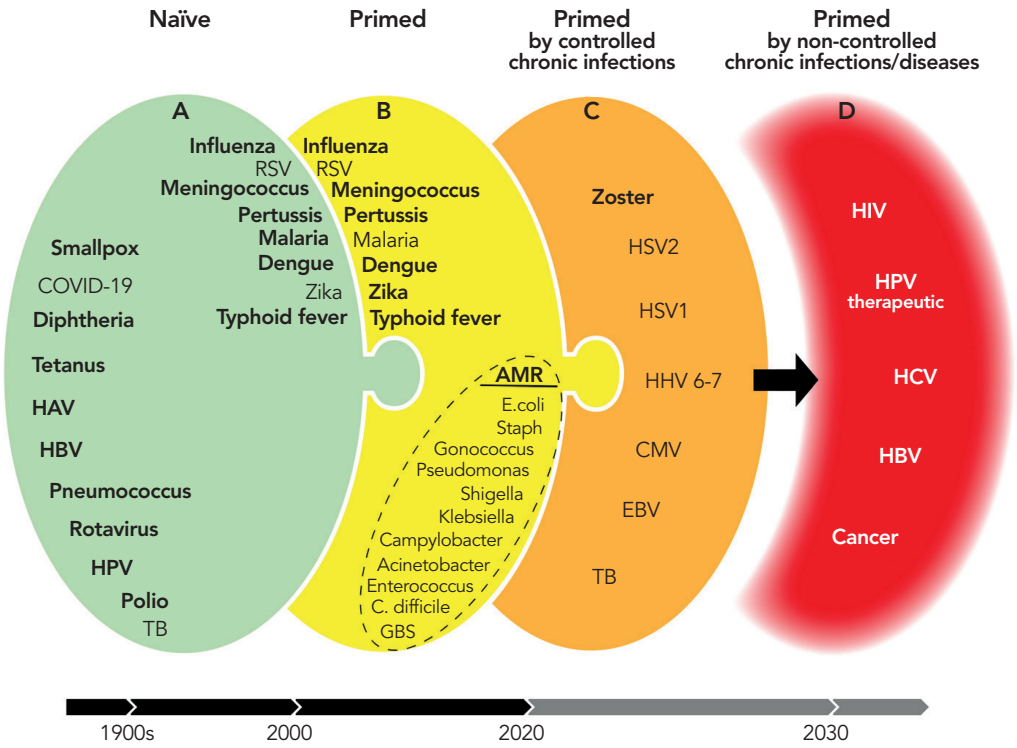
measles virus, modified vaccinia Ankara, vesicular stomatitis virus (VSV), cytomegalovirus (CMV), and others [35, 36]. So far, the only licensed vaccine based on viral vectors are the Ebola vaccines based on VSV and human adenovirus 26. Although we have a long experience of clinical trials with viral vector vaccines, these vaccines have never been used in millions of people, and therefore we still need to move forward cautiously. Large-scale manufacturing capacity to produce hundreds of millions of doses is not yet present in the industry, and it is being accelerated with unprecedented public and private investments to fast-track COVID-19 vaccines. It is also important to point out that vector immunity is a concern with viral and bacterial vectors. Boosting with the same vector has limitations, and this could impact using the same vector for a different pathogen, an issue that should be carefully considered.

Protein-based vaccines are the only ones for which we have large experience. In this case, the synthetic gene coding for the spike protein, prefusion stabilized or also receptor binding domain only, is used to engineer mammalian cells, baculovirus, or plant cells to produce the recombinant protein that then is purified, combined with adjuvants, and used as vaccine. The initial phase of these vaccines involving the generation of the cell line and the purification of the protein requires more time compared to RNA or viral vector vaccines, and therefore at least 6 mo were needed before the first protein-based COVID-19 vaccine started clinical trials [37]. Preliminary data on immunogenicity in humans show that these vaccines induce very high neutralizing titers which exceed those found in convalescent people. However, given the industrial and clinical experience accumulated with protein-based vaccines combined with licensed adjuvants, there is confidence that these vaccines will be well tolerated, effective, and available in large quantities.

Post-COVID-19 health priorities

Reverse vaccinology, structure-based design, synthetic biology, and adjuvants are the tools that we have today to design vaccines that can be delivered as purified antigens, or by RNA and viral vectors. The COVID-19 pandemic has accelerated the maturation of RNA and viral vectors by at least a decade and made these new platforms available not only for emerging infections but also for the other health priorities such as antimicrobial resistance (AMR), chronic infections, and cancer that our world will need to face with urgency as soon as the COVID-19 emergency is over. To analyze the new challenges for vaccines, in • Figure 3, we divided vaccines into four groups. On the opposite sides, there are vaccines that we already have or that can be made

• Figure 3. Vaccines developed addressing naïve, previously exposed, and chronic infections. Green (A) are vaccines available or doable with existing technologies. Bold, available vaccines. Yellow (B) and orange (C) are doable vaccines with increasing challenges for today's technologies. Red (D) are targets for which we do not yet have the scientific knowledge and technologies. HAV, hepatitis A virus; HBV, hepatitis B virus; HPV, human papillomavirus; TB, tuberculosis; RSV, respiratory syncytial virus; AMR, antimicrobial resistance; E. coli, *Escherichia coli*; Staph, *Staphylococcus aureus*; C. difficile, *Clostridium difficile*; GBS, group B Streptococcus; HSV1, herpes simplex virus 1; HSV2, herpes simplex virus 2; HHV, 6-7 human herpes viruses 6 and 7; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HIV, human immunodeficiency virus; HCV, hepatitis C virus.



with existing technologies (group A; • Figure 3A) and vaccines that we cannot yet approach with today's knowledge (group D; • Figure 3D). Vaccines in groups B and C (• Figures 3 B and C) are intermediate. A closer look at these groups shows that we can divide vaccination into two big categories, depending on whether we vaccinate a naïve immune system or vaccinate an immune system that has already encountered the antigen (primed immune system).

Vaccines for a Naïve Immune System. The vaccine against smallpox developed more than two centuries ago and the vaccines in development today against COVID-19 are based on a similar principle. They both introduce, into the body, antigens that had never been seen before by the immune system, aiming at stimulating a long-term protection for a future encounter with the virus. The large majority of the vaccines in use today are also based on antigens that had never been seen before by the naïve immune system (diphtheria toxin, tetanus toxin, measles, mumps, rubella, poliomyelitis, hepatitis B, papillomavirus, and infant vaccination against influenza, pneumococcus, and meningococcus) (• Figure 3A). When these vaccines are used, the antigens are taken up by professional antigen-presenting cells and presented to naïve B and T cells which mount an adaptive immune response. An important step in this process is the formation of germinal centers where follicular T helper cells and B cells cooperate to increase the potency of the B cells specific for the new antigen, via affinity maturation of antigen-reactive antibodies. This is the textbook vaccination for which we have both mechanistic and animal models, and is the vaccinology that we study when we inject animals (mostly mice) with a variety of antigens that are new for their immune system. In most cases, we have sufficient technologies and knowledge to develop vaccines against pathogens for which the immune system is naïve. There are cases, however, where we are not yet able to make vaccines. Examples are HIV, where the virus changes so rapidly that vaccines are not effective, or malaria, where the antigenic profile is very complex, and we struggle to make effective vaccines.

Vaccines for a Primed Immune System. Some of the vaccines described above, when delivered to adolescents, adults, or the elderly, may find an immune system that has already been exposed to the antigen, following natural infection or by other microorganisms carrying cross-reacting antigens (• Figure 3B). In this case, the immune system is not naïve any longer, and the vaccines are required to modify the preexisting immunity of antigen-experienced people. Seasonal influenza is probably the best example. In this case, we deliver a vaccine specific for a new influenza virus strain to an immune system that has already gone through the process of developing the response to the same antigen and has already generated specific memory B and T cells. The new vaccine quickly expands the preexisting memory B cells and, at the same time, triggers the expansion and affinity maturation of naïve B cells [38]. However, it is clear that the first exposure to the antigen has already shaped forever the way the immune system reacts to subsequent encounters with the same antigen. This phenomenon is known as "antigenic sin" [39]. Another recent example is vaccination against dengue virus. In this case,

a vector-based vaccine was effective in boosting a preexisting immunity in seropositive people, while it was unable to effectively prime the naïve immune system of naïve children where it induced antibody-dependent disease enhancement, which increased the risk of hospitalization [40]. Meningococcal and pneumococcal conjugate vaccines are another example [41]. When they are given to naïve infants, they prime the immune system to the new antigen, and it takes at least two immunizations to have a good immune response. However, when the same vaccine is given to adolescents or the elderly, who have already been exposed to these pathogens, one dose of vaccine is sufficient to get an excellent immune response. Although there are no definitive studies in humans describing the germinal center response in this context, it is likely that the single vaccination elicits an immediate antibody response – probably by an extrafollicular transformation of memory B cells into plasma cells – and then the immune system becomes refractory to any booster immunization for a long period (as long as 2 y). In this period, more affinity maturation happens, and new memory B cells are generated. Only after that, the immune system is ready to respond to a booster immunization with a massive level of antibodies which can be as high as 10 times the response to the first immunization [41]. Unfortunately, we do not have animal models able to reproduce what is described in the examples above, and we do not have a mechanistic understanding of what it takes to vaccinate an "experienced" immune system. The absence of animal models and the lack of knowledge are serious limitations for the development of new vaccines that target pathogens to which most people have already been exposed by natural infection.

A big and urgent example in this category is bacteria resistant to antibiotics and responsible for recurrent infections. AMR is a slowly evolving pandemic, with predicted catastrophic consequences for health and economy during the next 10 to 20 y [42]. Vaccines can help to tackle AMR [43]. We urgently need vaccines for pathogenic *Escherichia coli*, *Staphylococcus aureus*, *Clostridium difficile*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Salmonella typhi*, *Shigella*, *Acinetobacter baumannii*, *Enterococcus faecium*, and *Campylobacter* (• Figure 3B). Experimental vaccines against some of these pathogens are based on proteins or polysaccharides which induce normal or low response to the first vaccination when tested in naïve mice, followed by a better response to the second and third vaccinations. However, when adult volunteers were immunized with the same vaccines, a strong response was observed already after the first immunization, with no increased response to the second vaccination (at least in the short term). The main reason for this is that adult volunteers have already been colonized by these bacteria or by their relatives, and

they already have memory B and T cells that recognize them and respond to vaccination. In this setting, adjuvants failed to increase the antibody response. The consequence is that, during vaccine development, in most cases, we make the choice to make a one-dose vaccine without adjuvant [44]. However, we are not sure whether this is the right choice for long-term protection, and some of the vaccines failed even the primary efficacy endpoint [45]. While we do not yet fully understand the mechanistics of immunizing a primed immune system, or the lack of a protective immune response that allows reinfection, we have enough technologies and empirical knowledge to develop new vaccines for AMR. Similarly, we have enough knowledge to develop vaccines for some viral diseases such as respiratory syncytial virus, dengue, and Zika viruses even in adults and the elderly, where the immune system has been usually primed by natural infection.

Vaccines for an Immune System Primed by Controlled Chronic Infections. The difficulty of making vaccines increases when the immune system not only has already been primed by the exposure to the pathogen but somehow has already been defeated by it. The immune system has not been able to clear the pathogen, which has established a lifelong chronic infection. In some cases, once chronic infections are established, the immune system is still able to keep at bay the pathogen for most of the time. This is the case for herpes viruses (zoster, HSV1 and HSV2, EBV, and CMV) and for bacteria such as Mycobacterium tuberculosis (• Figure 3C). The pathogen establishes a latent infection and persists quietly in the body without causing disease. However, due to concomitant infections, immunosuppressive pharmacological treatments, or aging, the immune system becomes weak, and the pathogen takes over, causing disease.

Up to a few years ago, we had not a single example of a successful vaccine against chronic infections. It took us 20 y of research to start conquering some of them. The first step in this direction was the licensure of the live attenuated vaccine against herpes zoster in 2006 [46]. Although this vaccine was not able to eliminate the chronic infection, it was able to keep the chronic virus silent and avoid reactivation in 60% of the cases. Recently, a new vaccine composed of a protein antigen and the potent AS01 adjuvant (a liposome containing a TLR4 agonist and a saponin) showed an efficacy of 97% against herpes zoster [47]. This was followed by encouraging results against tuberculosis, where the combination of a protein antigen and the AS01 adjuvant was able to prevent reactivation and disease in 50% of the chronically infected people [48]. The successful vaccines against herpes zoster and the encouraging results against tuberculosis represent an incredible

milestone in the history of vaccination, because, for the first time, we have been able to make effective vaccines against chronic infections.

Vaccines for a Primed and Failed Immune System. There are cases in which the immune system has been exposed to pathogens and has been completely defeated. Examples are chronic infections, such as HIV, papillomavirus, hepatitis C virus (HCV), hepatitis B virus (HBV), and cancer, where the immune system is not able to control the pathogen or the cancer cells, which continue to replicate forever (• Figure 3D). So far, we have not been able to make successful vaccines against these diseases, and we do not have the scientific knowledge to make them. However, even this area is not without hope, because the progress made by immunotherapy in the area of cancer has shown that the defeated immune system is characterized by dormant regulatory T cells that can be activated using antibodies against the checkpoint inhibitors, removing the constrains imposed on the immune system [49]. The success of immunotherapy in the field of cancer and the increased understanding of mechanistic features of the defeated immune system suggest that, in the near future, vaccination may also be able to conquer cancer and chronic diseases.

Conclusions

The urgent need for COVID-19 vaccines has accelerated the time required to develop vaccines and the availability of powerful technologies. It is possible that evolution of the new technologies fast-tracked for COVID-19 (RNA vaccines, viral vectors, and protein-based vaccines with potent adjuvants) combined with the learning coming from immunotherapy will be the answer for some of the new challenges of modern society such as emerging infections, AMR, chronic infections, and cancer. For instance, RNA vaccines and viral vectors may be designed to encode not only antigens but also molecules able to reactivate the dormant immune system.

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Extremely potent human monoclonal antibodies from COVID-19 convalescent patients

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Summary

Human monoclonal antibodies are safe, preventive, and therapeutic tools that can be rapidly developed to help restore the massive health and economic disruption caused by the coronavirus disease 2019 (COVID-19) pandemic. By single-cell sorting 4,277 SARS-CoV-2 spike protein-specific memory B cells from 14 COVID-19 survivors, 453 neutralizing antibodies were identified. The most potent neutralizing antibodies recognized the spike protein receptor-binding domain, followed in potency by antibodies that recognize the S1 domain, the spike protein trimer, and the S2 subunit. Only 1.4% of them neutralized the authentic virus with a potency of 1-10 ng/mL. The most potent monoclonal antibody, engineered to reduce the risk of antibody-dependent enhancement and prolong half-life, neutralized the authentic wild-type virus and emerging variants containing D614G, E484K, and N501Y substitutions. Prophylactic and therapeutic efficacy in the hamster model was observed at 0.25 and 4 mg/kg respectively in absence of Fc functions.

Introduction

The impact of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, with more than 100 million cases, over 2 million deaths, an estimated cost of 16 trillion US dollars to the USA economy (Cutler and Summers, 2020), and 45 million people filing unemployment in the United States alone, is unprecedented (Aratani, 2020).

Vaccines and drugs against SARS-CoV-2 have recently received emergency use authorization (EUA) by the Food and Drug Administration (FDA) for prevention and treatment of coronavirus disease 2019 (COVID-19) (FDA, 2021, 2020).

In spite of this, it is predictable that waves of infection will continue to spread globally, and it is likely to be followed by additional waves over the next few years. This is supported by the emergence of new SARS-CoV-2 variants in the United Kingdom, South Africa, Brazil, and Japan (CDC, 2021).

It is therefore imperative to quickly develop, in parallel to vaccines, therapeutic tools against SARS-CoV-2 and its variants. Among the many therapeutic options available, human monoclonal antibodies (mAbs) can be developed in the shortest time frame. In fact, the extensive clinical experience with the safety of more than 50 commercially available mAbs approved to treat cancer, inflammatory, and autoimmune disorders provides high confidence of their safety (Wellcome and IAVI, 2020). These advantages, combined with the urgency of the SARS-CoV-2 pandemic, support and justify an accelerated regulatory pathway. In addition, the long industrial experience in developing and manufacturing mAbs decreases risks usually associated with technical development of investigational products. Finally, the incredible technical progress in this field allows shortening of conventional timelines and enables a

path from discovery to proof-of-concept trials within 5-6 months (Kelley, 2020). A key example is the Ebola case, where mAbs were developed faster than vaccines or other drugs (Kupferschmidt, 2019), becoming the first therapeutic intervention recommended by the World Health Organization (WHO) and approved by the FDA (Mullard, 2020).

During the first months of this pandemic, many groups have been active in isolating and characterizing human monoclonal antibodies from COVID-19 convalescent patients or from humanized mice, and some of them have been progressing quickly to clinical trials for the prevention and cure of SARS-CoV-2 infection (Shi *et al.*, 2020; Hansen *et al.*, 2020; Hsieh *et al.*, 2020; Pinto *et al.*, 2020; Zost *et al.*, 2020a, 2020b; Rogers *et al.*, 2020, Alsoussi *et al.*, 2020). Few of them are already in phase III clinical trials and reported promising preliminary results. Two of them received the EUA from the FDA (Lilly, 2020; Regeneron, 2020).

All these antibodies neutralize SARS-CoV-2 infection by binding to the spike glycoprotein (S protein), a trimeric class I viral fusion protein that mediates virus entry into host cells by engaging with the human angiotensin-converting enzyme 2 (hACE2) and cellular heparan sulfate as receptors (Clausen et al., 2020). The S protein exists in a metastable pre-fusion conformation and in a stable post-fusion form (Wang *et al.*, 2020; Walls *et al.*, 2020; Schäfer *et al.*, 2020). Each S protein monomer is composed of two distinct regions, the S1 and S2 subunits. The S1 subunit contains the receptor-binding domain (RBD), which is responsible for the interaction with hACE2 and heparan sulfate on host cell membranes triggering the destabilization of the prefusion state of the S protein and consequent transition into the post-fusion conformation. This event results in the entry of the virus particle into the host cell and the onset of infection (Wrapp *et al.*, 2020; Walls *et al.*, 2020; Tay *et al.*, 2020; Zou *et al.*, 2020).

As for other mAbs in the field of infectious diseases (Hooft van Huijsduijnen *et al.*, 2020; Sparrow *et al.*, 2017), the dose of mAbs so far used in clinical trials against SARS-CoV-2 is high, ranging from 500 to 8,000 mgs (NCT04411628; NCT04427501; NCT04441918; NCT04425629; NCT04426695; NCT04452318). The high dose poses two important limits to the application of mAbs in the infectious diseases field. First, the high dosage has cost-associated implications, and it only allows for intravenous delivery, making this therapeutic intervention extremely costly and therefore available almost exclusively in high-income countries. Indeed, the high price of this intervention has been a barrier to the global access of mAbs and their use to other fields such as infectious diseases. A solution would be the development of extremely potent mAbs that can be used at lower dosages leading to cost reductions and that can be delivered via intramuscular or subcutaneous injections. A first example is the

respiratory syncytial virus (RSV) case, where a potent mAb has recently shown its therapeutic effect in premature infants after only one intramuscular injection of 50 mg (Griffin *et al.*, 2020).

The second limit of mAbs in the field of infectious diseases is the risk of antibody-dependent enhancement (ADE) of disease, which is usually mediated by the binding of the fragment crystallizable (Fc) region portion of the antibody to Fc gamma receptors (FcγRs) expressed by immune cells (Lee et al., 2020). ADE has been clearly demonstrated in the case of SARS-CoV, RSV, and dengue viruses, and the theoretical risk has been raised in the case of SARS-CoV-2 (Lee *et al.*, 2020; Katzelnick *et al.*, 2017; Arvin *et al.*, 2020).

In this work, we pushed the limits of mAb application to fight infectious diseases by selecting extremely potent antibodies with the aim of using them at low dosage to make them affordable and conveniently delivered by intramuscular injection. In addition, we mitigated the risk of ADE by engineering their Fc region. Despite complete lack of Fc-receptor-binding and Fc-mediated cellular activities, engineered mAbs were able to prevent and treat SARS-CoV-2 infection in golden Syrian hamster at a concentration of 0.25 and 4 mg/kg respectively. These antibodies have the potential to globally extend the access and affordability of this important medical tool.

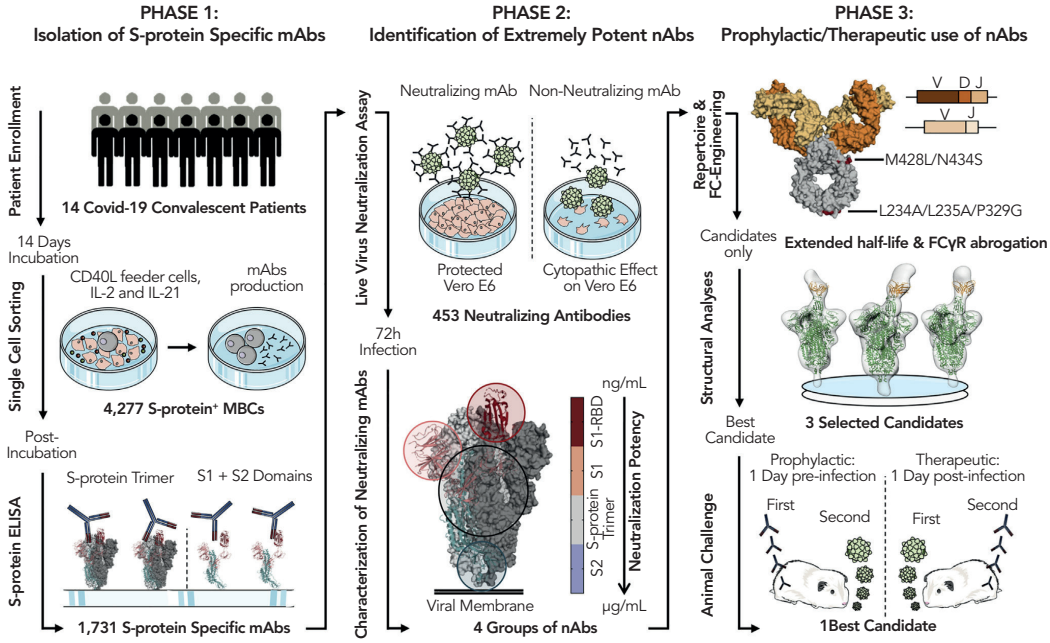
Results

Isolation and characterization of S protein-specific antibodies from SARS-CoV-2 convalescent patients

To retrieve mAbs specific for SARS-CoV-2 S protein, peripheral blood mononuclear cells (PBMCs) from fourteen COVID-19 convalescent patients enrolled in this study were collected and stained with fluorescently labeled S protein trimer to identify antigen-specific memory B cells (MBCs). • Figure 1 summarizes the overall experimental strategy. The gating strategy described in • Figure S1 A was used to single-cell sort, into 384-well plates, IgG^+ and IgA^+ MBCs binding to the SARS-CoV-2 S protein trimer in its prefusion conformation. The sorting strategy aimed to specifically identify class-switched MBCs ($CD19^+CD27^+IgD^-IgM^-$) to identify only memory B lymphocytes that underwent maturation processes. A total of 4,277 S protein-binding MBCs were successfully retrieved with frequencies ranging from 0.17% to 1.41% (• Table S1). Following the sorting procedure, S protein+ MBCs were incubated over a layer of 3T3-CD40L feeder cells in the presence of IL-2 and IL-21 stimuli for 2 weeks to allow natural production of immunoglobulins (Huang *et al.*, 2013). Subsequently, MBC supernatants containing IgG or IgA were tested for their ability to bind either the SARS-CoV-2 S protein trimer in its prefusion

conformation or the S protein S1 + S2 subunits (• Figure 2 A; • Figure S2 B) by enzyme linked immunosorbent assay (ELISA). A panel of 1,731 mAbs specific for the SARS-CoV-2 S protein were identified showing a broad range of signal intensities (• Figure 2A; • Table S1).

• Figure 1. **Workflow and timeline for SARS-CoV-2 neutralizing antibodies identification.** The overall scheme shows three different phases for the identification of SARS-CoV-2 neutralizing antibodies (nAbs). Phase 1 consisted in the enrolment of COVID-19 patients (n = 14) from which PBMCs were isolated. Memory B cells were single-cell sorted (n = 4,277), and after 2 weeks of incubation, antibodies were screened for their binding specificity against the S protein trimer and S1/S2 domains. Once S protein-specific monoclonal antibodies (mAbs) were identified (n =1,731) phase 2 started. All specific mAbs were tested *in vitro* to evaluate their neutralization activity against the authentic SARS-CoV-2 virus, and 453 nAbs were identified. nAbs showing different binding profiles on the S protein surface were selected for further functional characterization and to identify different neutralizing regions on the antigen. Phase 3 starts with the characterization of the heavy and light chain sequences of selected mAbs (n = 14) and the engineering of the Fc portion of three most promising candidates. The latter were also selected for structural analyses that allowed the identification of the neutralizing epitopes on the S protein. Finally, the most potent antibody was tested for its prophylactic and therapeutic effect in a golden Syrian hamster model of SARS-CoV-2 infection.

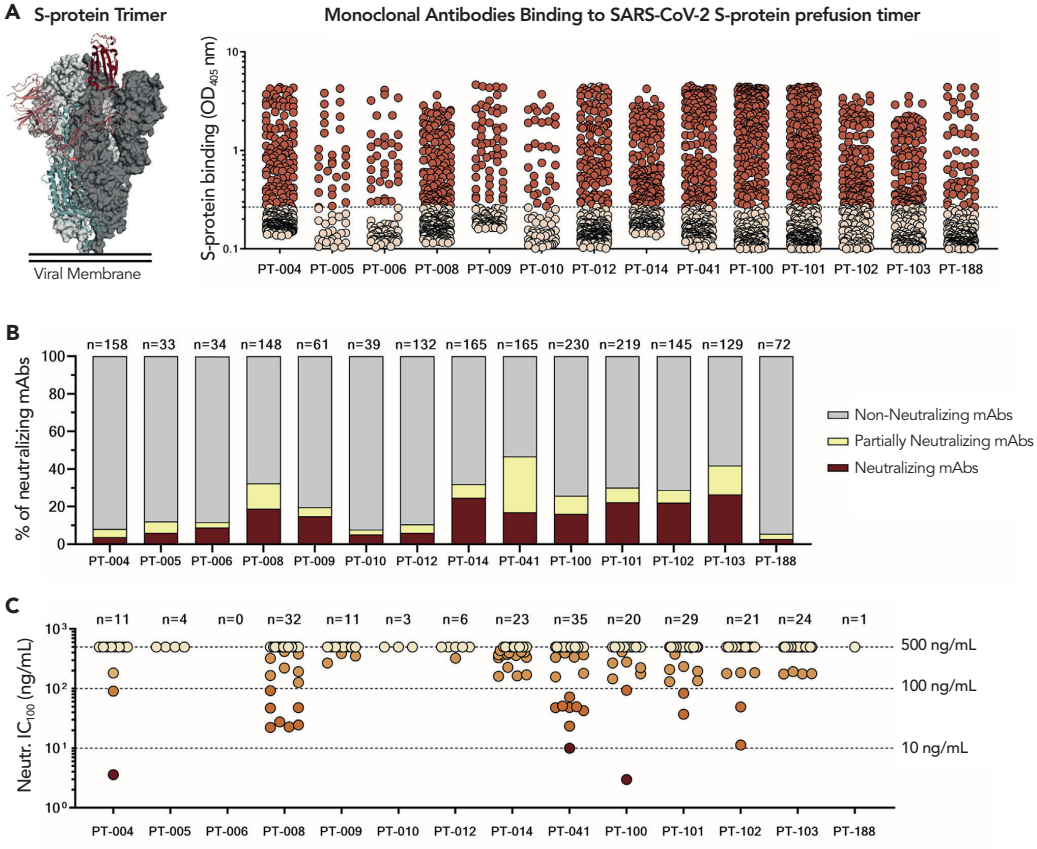


Identification of S protein-specific mAbs able to neutralize SARS-CoV-2

The 1,731 supernatants containing S protein-specific mAbs, were screened *in vitro* for their ability to block the binding of the streptavidin-labeled S protein to Vero E6 cell receptors and for their ability to neutralize authentic SARS-CoV-2 virus by *in vitro* microneutralization assay. In the neutralization of binding (NoB) assay, 339 of the 1,731 tested (19.6%) S protein-specific mAbs were able to neutralize the antigen/receptor binding, showing a broad array of neutralization potency ranging from 50% to 100% (• Figure S2C; • Table S1).

As for the authentic virus neutralization assay, supernatants containing naturally produced IgG or IgA were tested for their ability to protect the layer of Vero E6 cells from the cytopathic effect triggered by SARS-CoV-2 infection. To increase the throughput of our approach, supernatants were tested at a single-point dilution, and to increase the sensitivity of our first screening, a viral titer of 25 50% tissue culture infectious dose ($TCID_{50}$) was used. For this screening, mAbs were classified as neutralizing, partially neutralizing, and non-neutralizing based on their complete, partial, or absent ability to prevent the infection of Vero E6 cells, respectively. Out of 1,731 mAbs tested in this study, a panel of 453 (26.2%) mAbs neutralized the authentic virus and prevented infection of Vero E6 cells (• Table S1). The percentage of partially neutralizing antibodies and neutralizing antibodies (nAbs) identified in each donor was extremely variable ranging from 2.6%-29.7% and 2.8%-26.4% respectively (• Figure 2B; • Table S2). The majority of nAbs were able to specifically recognize the S protein S1 domain (57.5%; n = 244), while 7.3% (n = 53) of nAbs were specific for the S2 domain, and 35.2% (n = 156) did not recognize single domains but only the S protein in its trimeric conformation (• Figure S2A; • Table S3). From the panel of 453 nAbs, we recovered the heavy chain (HC) and light chain (LC) variable regions of 220 nAbs, which were expressed as full-length immunoglobulin G1 (IgG1) using the transcriptionally active PCR (TAP) approach to characterize their neutralization potency against the live virus at 100 $TCID_{50}$. The vast majority of nAbs identified (65.9%; n = 145) had a low neutralizing potency and required more than 500 ng/mL to achieve 100% inhibitory concentration (IC_{100}). A smaller fraction of the antibodies had an intermediate neutralizing potency (23.6%; n = 52) requiring between 100 and 500 ng/mL to achieve the IC_{100}, while 9.1% (n = 20) required between 10 and 100 ng/mL. Finally, only 1.4% (n = 3) of the expressed nAbs were classified as extremely potent nAbs, showing an IC_{100} lower than 10 ng/mL (• Figure 2C; • Figure S2B; • Table S4).

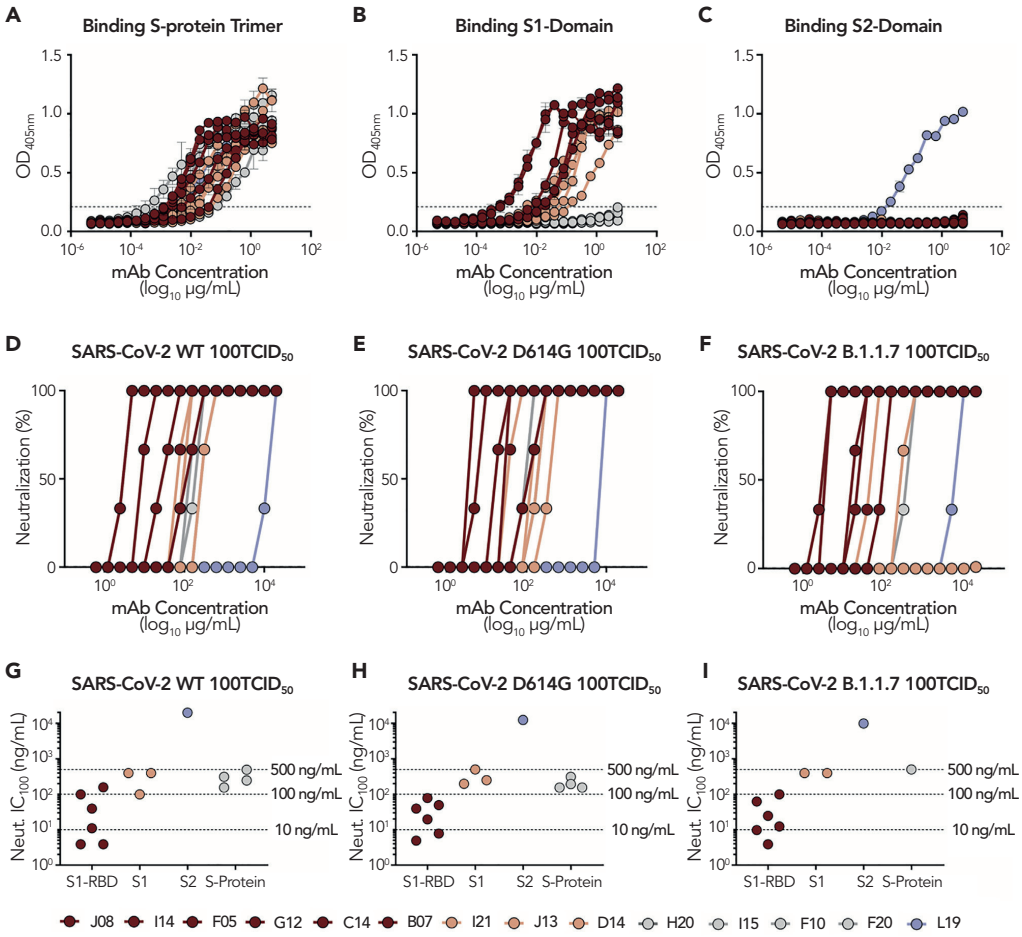
• Figure 2. Identification of SARS-CoV-2 S protein-specific nAbs. (A) The graph shows supernatants tested for binding to the SARS-CoV-2 S-protein stabilized in its prefusion conformation. Threshold of positivity has been set as two times the value of the blank (dotted line). Red dots represent mAbs that bind to the S protein, while pink dots represent mAbs that do not bind. (B) The bar graph shows the percentage of non-neutralizing (gray), partially neutralizing (pale yellow), and neutralizing antibodies (dark red) identified per each donor. The total number (n) of antibodies tested per individual is shown on top of each bar. (C) The graph shows the neutralization potency of each nAb tested once expressed as recombinant full-length IgG1. Dashed lines show different ranges of neutralization potency (500, 100, and 10 ng/mL). Dots were colored based on their neutralization potency and were classified as weakly neutralizing (>500 ng/mL; pale orange), medium neutralizing (100–500 ng/mL; orange), highly neutralizing (10–100 ng/mL; dark orange), and extremely neutralizing (1–10 ng/mL; dark red). The total number (n) of antibodies tested per individual is shown on top of each graph. A COVID-19 convalescent plasma and an unrelated plasma were used as positive and negative control, respectively, in all the assays.



SARS-CoV-2 neutralizing antibodies can be classified into four groups

Based on the first round of screening, 14 nAbs were selected for further characterization. All nAbs were able to bind the SARS-CoV-2 S protein in its trimeric conformation (• Figure 3 A). The mAbs named J08, I14, F05, G12, C14, B07, I21, J13, and D14 were also able to specifically bind the S1 domain (• Figure 3B). The nAbs named H20, I15, F10, and F20 were not able to bind single S1 or S2 domains but only the Sprotein in its trimeric state, while the nAb L19 bound only the S2 subunit (• Figures 3B and 3C). Among the group of S1-specific nAbs, only J08, I14, F05, G12, C14, and B07 were able to bind the S1 RBD and to strongly inhibit the interaction between the S protein and Vero E6 receptors, showing a half maximal effective concentration (EC_{50}) at the NoB assay of 78.6, 15.6, and 68.5 ng/mL for J08-MUT, I14-MUT, and F05-MUT, respectively (• Figures S3 A and S3B). On the other hand, I21, J13, and D14, despite showing S1 binding specificity, did not show any binding to the RBD and NoB activity (• Figure S3A). Based on this description, four different groups of nAbs against SARS-CoV-2 were identified. The first group (Group I) is composed of S1 RBD-specific nAbs (J08, I14, F05, G12, C14, and B07), which showed neutralization potency against the authentic wild type (WT), the D614G variant, and the emerging variant recently isolated in the UK B.1.1.7. S1 RBD-specific nAbs showing a neutralizing potency ranging from 3.9 to 157.5 ng/mL (• Figures 3D-3I; • Table S5) and picomolar affinity to the S protein with an equilibrium dissociation constant (KD) ranging from 0.2 to 4.6 E^{-10}M (• Figure S4). In addition to the D614G and the B.1.1.7 variants, the S1 RBD-specific nAb J08 showed also to neutralize SARS-CoV-2 variants containing the E484K mutation (Andreano *et al.*, 2020). The second group (Group II) included S1-specific nAbs that did not bind the RBD (I21, J13, and D14). These antibodies also showed good neutralization potency ranging from 99.2 to 500.0 ng/mL (• Figures 3D-3I; • Table S5) but inferior to that of S1 RBD-directed nAbs. One antibody from this group was not able to neutralize the B.1.1.7 variant (I21). The third group (Group III) is composed of antibodies able to bind the S-protein only in its whole trimeric conformation (H20, I15, F10, and F20). Antibodies belonging to this group showed lower affinity to the S protein trimer (KD 64.0 E^{-10}M-757.0 E^{-10}M) compared to Group I nAbs and medium neutralization potencies ranging from 155.0 to 492.2 ng/mL against the authentic WT and D614G (• Figures 3D-3I; • Figure S4; • Table S5). On the other hand, only one S protein-specific nAb (D21) showed moderate neutralization activity against the B.1.1.7 with an IC_{100} of 500.0 ng/mL. Three S protein-specific nAbs (I15, F10, and F20) did not show any functional activity against this latter

• Figure 3. Functional characterization of potent SARS-CoV-2 S protein-specific nAbs. (A-C) Graphs show binding curves to the S protein in its trimeric conformation, S1 domain, and S2 domain. Mean ± SD of technical triplicates are shown. Dashed lines represent the threshold of positivity. (D-F) Neutralization curves for selected antibodies were shown as percentage of viral neutralization against the authentic SARS-CoV-2 wild type (D), D614G variant (E), and the emerging variant B.1.1.7 (F). Data are representative of technical triplicates. A neutralizing COVID-19 convalescent plasma and an unrelated plasma were used as positive and negative control, respectively. (G-I) Neutralization potency of 14 selected antibodies against the authentic SARS-CoV-2 wild type (G), D614G variant (H), and the emerging variant B.1.1.7 (I). Dashed lines show different ranges of neutralization potency (500, 100, and 10 ng/mL). In all graphs, selected antibodies are shown in dark red, pink, gray, and light blue based on their ability to recognize the SARS-CoV-2 S1 RBD, S1 domain, S protein trimer only, and S2 domain, respectively.



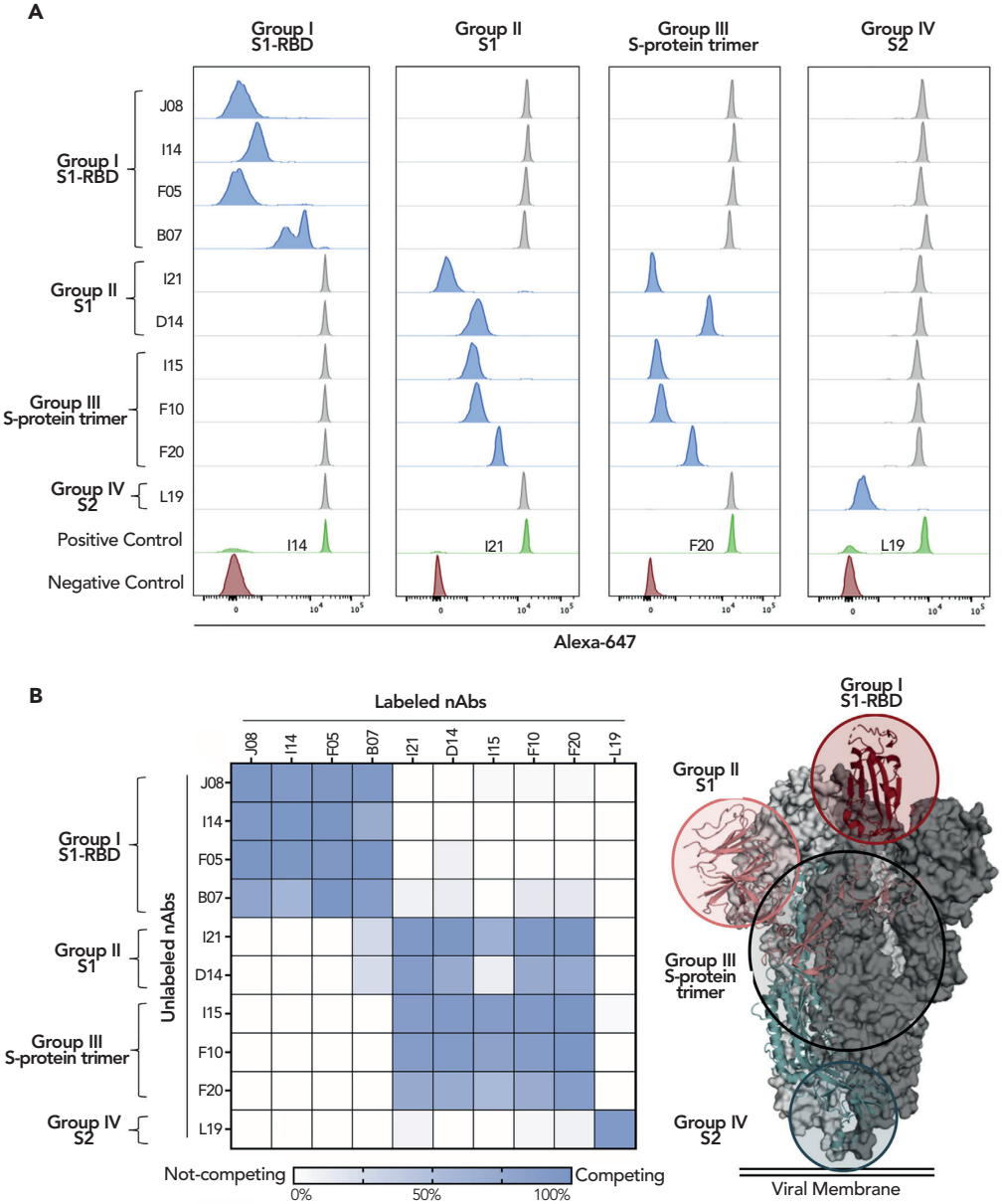
variant (• Figures 3D-3I; • Table S5). The fourth and final group (Group IV) is composed of antibodies that exclusively recognized the S2 domain. Different antibodies with similar properties were identified for Group IV, but only the nAb L19 is shown. The Group IV nAb L19 shows the lowest neutralization potency with 19.8 µg/mL for the authentic WT, 12.5 µg/mL against the D614G, and 9.9 µg/mL against the B.1.1.7 variant (• Figures 3D-3I; • Table S5).

All the antibodies described above were also tested for their ability to cross-neutralize other human coronavirus strains. nAbs were tested against lentiviral pseudotypes expressing the SARS-CoV-2, SARS-CoV-2 D614G, SARS-CoV, and Middle East respiratory syndrome (MERS)-CoV S protein on their viral membrane surface. Neutralization activity was shown against SARS-CoV-2 and D614G pseudotypes, therefore confirming previous data. None of the antibodies reported here were able to cross-neutralize other coronavirus species (• Figure S5).

Different pathogen vulnerability regions identified on the S protein

The fourteen selected nAbs were further characterized by a competition assay that allowed speculation on the S protein regions recognized by these antibodies. Briefly, beads were coated with SARS-CoV-2 trimeric S protein and incubated with a primary unlabeled antibody in order to saturate the binding site on the antigen surface. Following the first incubation step, a secondary Alexa-647-labeled antibody was incubated with the antigen/unlabeled-mAb complex. If the secondary labeled antibody did not recognize the same epitope as the primary unlabeled mAb, a fluorescent signal would be detected when tested by flow cytometry. Through this assay, we observed that all Group I nAbs competed among themselves for binding to the S protein RBD, indicating that these antibodies possibly clash against each other and recognize a similar epitope region. All Group II nAbs showed different competition profiles and competed with Group II and Group III nAbs. These results confirmed that Group III antibodies can recognize various regions on the S protein surface as they compete with themselves as well as with antibodies belonging to Group II. Interestingly, nAbs belonging to Group II also competed with the B07 RBD-directed antibody, thereby suggesting that this latter nAb may have a different binding orientation compared to other nAbs included in the Group I. Finally, the Group IV nAb L19 did not compete with any of the other groups identified in this study, suggesting that this class of nAbs recognizes a distant epitope region as compared to Group I, II, and III nAbs (• Figures 4A and 4B).

• Figure 4. Identification of four different sites of pathogen vulnerability on the S protein surface (A) Representative cytometer peaks per each of the four antibody groups are shown. Positive (beads conjugated with only primary labeled antibody) and negative (un-conjugated beads) controls are shown as green and red peaks, respectively. Competing and not-competing nAbs are shown in blue and gray peaks, respectively. (B) The heatmap shows the competition matrix observed among the 14 nAbs tested. Threshold of competition was set at 50% of fluorescent signal reduction. A speculative representation of the vulnerability sites is shown on the S protein surface.



Genetic characterization of SARS-CoV-2 nAbs

The genes encoding the HCs and LCs of the 14 selected nAbs were sequenced, and their IGHV and IGKV genes were compared with publicly available SARS-CoV-2 neutralizing antibody sequences (• Figures 5A and 5B). Four nAbs used one of the most predominant HC V genes for SARS-CoV-2 nAbs (IGHV1-69), while three nAbs used one of the least representative HCV genes (IGHV1-24). Two other nAbs employed the most common germline observed for SARS-CoV-2 nAbs, which is IGHV3-53 (• Figure 5A) (Yuan *et al.*, 2020).

Interestingly, while IGHV1-69 and IGHV1-24 accommodate IGHJ diversity, nAbs belonging to the IGHV3-53 gene family only showed recombination with the IGHJ6 gene (• Table S6). The HC V genes somatic hypermutation level and complementary determining region 3 (H-CDR3) length were also evaluated.

Our selected nAbs displayed a low level of somatic mutations when compared to the inferred germlines with sequence identities ranging from 95.6% to 99.3% (• Figure 5C left panel; • Table S6), confirming what was observed in previous publications (Pinto *et al.*, 2020; Zost *et al.*, 2020b; Rogers *et al.*, 2020; Griffin *et al.*, 2020). The H-CDR3 length spanned from 7 to 21 amino acids (aa) with the majority of the antibodies (n = 6; 42.0%) having a length of 14 to 16 aa that is slightly bigger than previously observed (• Figure 5C right panel; • Table S6).

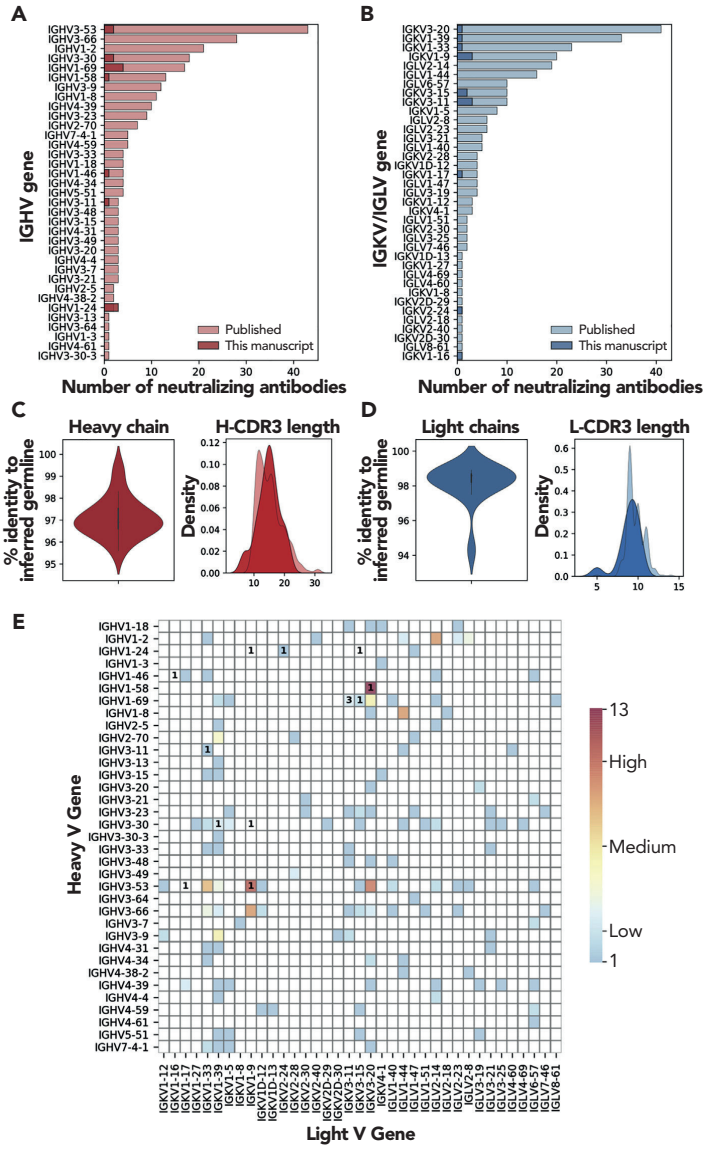
All of our nAbs used the κ chain, and the majority of them used the common genes IGKV1-9 and IGKV3-11 (n = 6; 42.0%) (• Figure 5B; • Table S6).

The level of IGKV somatic hypermutation was extremely low for LCs showing a percentage of sequence identities ranging from 94.3% to 98.9% (• Figure 5D left panel; • Table S6). The LC CDR3 (L-CDR3) lengths were ranging from 5 to 10 aa, which is in line with what was previously observed for SARS-CoV-2 nAbs (• Figure 5D right panel; • Table S6).

When paired HC and LC gene analysis was performed, IGHV1-69-derived nAbs were found to rearrange exclusively with IGKV3 gene family, whereas IGHV1-24-derived nAbs accommodate LC diversity (• Table S6).

Of note, some of our candidates showed unique HC and LC pairing when compared to the public SARS-CoV-2 nAb repertoire. Particularly, five different HC and LC rearrangements not previously described for nAbs against SARS-CoV-2 were identified. These included the IGHV1-24;IGKV1-9, IGHV1-24;IGKV3-15, IGHV1-46;IGKV1-16, IGHV3-30;IGKV1-9, and IGHV3-53;IGKV1-17 (• Figure 5E).

• Figure 5. Heavy and light chain analyses of selected nAbs. (A and B) Bar graphs show the heavy and light chains usage for neutralizing antibodies against SARS-CoV-2 in the public repertoire compared to the antibodies identified in this study. Our and public antibodies are shown in dark and light colors, respectively. (C and D) The heavy and light chain percentage of identity to the inferred germline and amino acidic CDR3 length are shown as violin and distribution plot, respectively. (E) The heatmap shows the frequency of heavy and light chain pairing for SARS-CoV-2 neutralizing human mAbs already published. The number within the heatmap cells represent the amount of nAbs described in this manuscript showing already published (colored cells) or novel heavy and light chain rearrangements (blank cells).



Fc engineering of candidate nAbs to abrogate Fc receptor binding and extend half-life

ADE of disease is a potential clinical risk following coronavirus infection (Lee *et al.*, 2020). Therefore, to optimize the suitability for clinical development and reduce the risk of ADE, five different point mutations were introduced in the constant region (Fc) of the three most potent nAbs (J08, I14, and F05), which were renamed J08-MUT, I14-MUT, and F05-MUT.

The first two point mutations (M428L and N434S) were introduced to enhance antibody half-life and to increase tissue distribution and persistence (Zalevsky *et al.*, 2010; Gaudinski *et al.*, 2018; Pegu *et al.*, 2017). The remaining three point mutations (L234A, L235A, and P329G) were introduced to reduce antibody dependent functions such as binding to FcγRs and cell-based activities (Schlothauer *et al.*, 2016).

To confirm the lack of FcγR binding as well as the extended half-life, a beads-based Luminex assay was performed. Briefly the beads were coated with SARS-CoV-2 S protein RBD. Antibodies were tested at eight-point dilutions, and the binding was detected with FcγR2A and neonatal Fc receptor (FcRn) at pH6.2 and 7.4.

The FcγR2A was selected as it is predominantly expressed on the surface of phagocytic cells (such as monocytes, macrophages, and neutrophils) and is associated with phagocytosis of immune complexes and antibody-opsonized targets (Ackerman *et al.*, 2013).

On the other hand, FcRn, which is highly expressed on endothelial cells and circulating monocytes, was selected as it is responsible for the recycling and serum half-life of IgG in the circulation (Mackness *et al.*, 2019).

This latter receptor was shown to possess a tighter binding at lower pH (e.g., pH 6.2) compared to a physiological pH (e.g., pH 7.4) (Booth *et al.*, 2018).

Results shown in • Figure S6 demonstrate that binding to the FcγR2A was completely abrogated for the mutated version of candidate nAbs (J08-MUT, I14-MUT, and F05-MUT) compared to their respective WT versions (J08, I14, and F05) and controls (CR3022 and unrelated protein) (• Figure S6A).

Furthermore, Fc-engineered antibodies showed increased binding activity to the FcRn at both pH 6.2 and 7.4 compared to their WT counterpart (• Figures S6B and S6C).

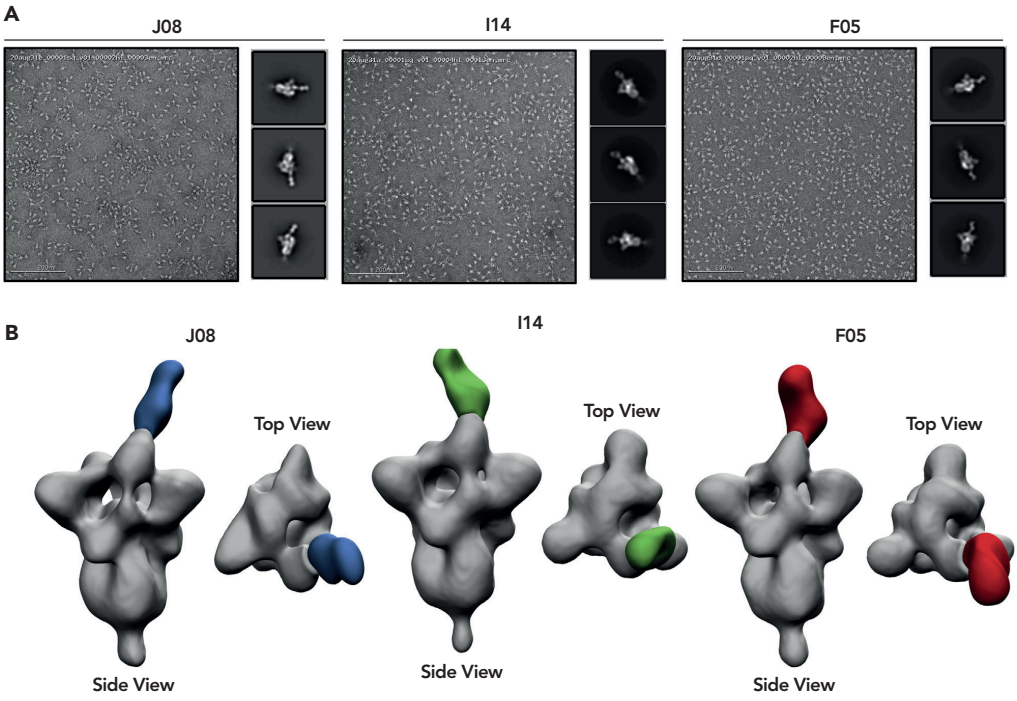
Finally, to evaluate the lack of Fc-mediated cellular activities by our three candidate nAbs, the antibody-dependent neutrophil phagocytosis (ADNP)

and antibody-dependent natural killer (ADNK) cell activations were evaluated (Butler *et al.*, 2019; Ackerman *et al.*, 2016; Karsten *et al.*, 2019; Boudreau *et al.*, 2020).

For the ADNP assay, primary human neutrophils were used to detect antibody binding to SARS-CoV-2 S protein RBD-coated beads, while ADNK activity was evaluated by using primary human NK cells and detecting the release of the proinflammatory cytokine interferon gamma (IFN-γ).

Complete abrogation of both ADNP and ADNK was observed for all three Fc-engineered candidate nAbs compared to their WT versions and control antibody (CR3022), thus confirming the lack of Fc-mediated cellular activities (• Figures S6D and S6E).

• Figure 6. EM epitope mapping of RBD mAbs- (A) Negative stain for J08, I14, and F05 in complex with the S protein. 200 nm scale bar is shown. (B) Figures show the binding of J08 (blue), I14 (green), and F05 (red) to the SARS-CoV-2 S protein RBD.



Potency and autoreactivity evaluation of Fc-engineered candidates

The three engineered antibodies were tested to confirm their binding specificity and neutralization potency against both the WT, the widespread SARS-CoV-2 D614G mutant and the emerging variant B.1.1.7 (Korber *et al.*, 2020, CDC, 2021) to evaluate their cross-neutralization ability.

The three engineered nAbs maintained their S1 domain binding specificity and extremely high neutralization potency with J08-MUT and F05-MUT being able to neutralize both the WT and the D614G variant with an IC_{100} lower than 10 ng/mL (both at 3.9 ng/mL for the WT and the D614G strains) (• Figure S6F-K; • Table S5).

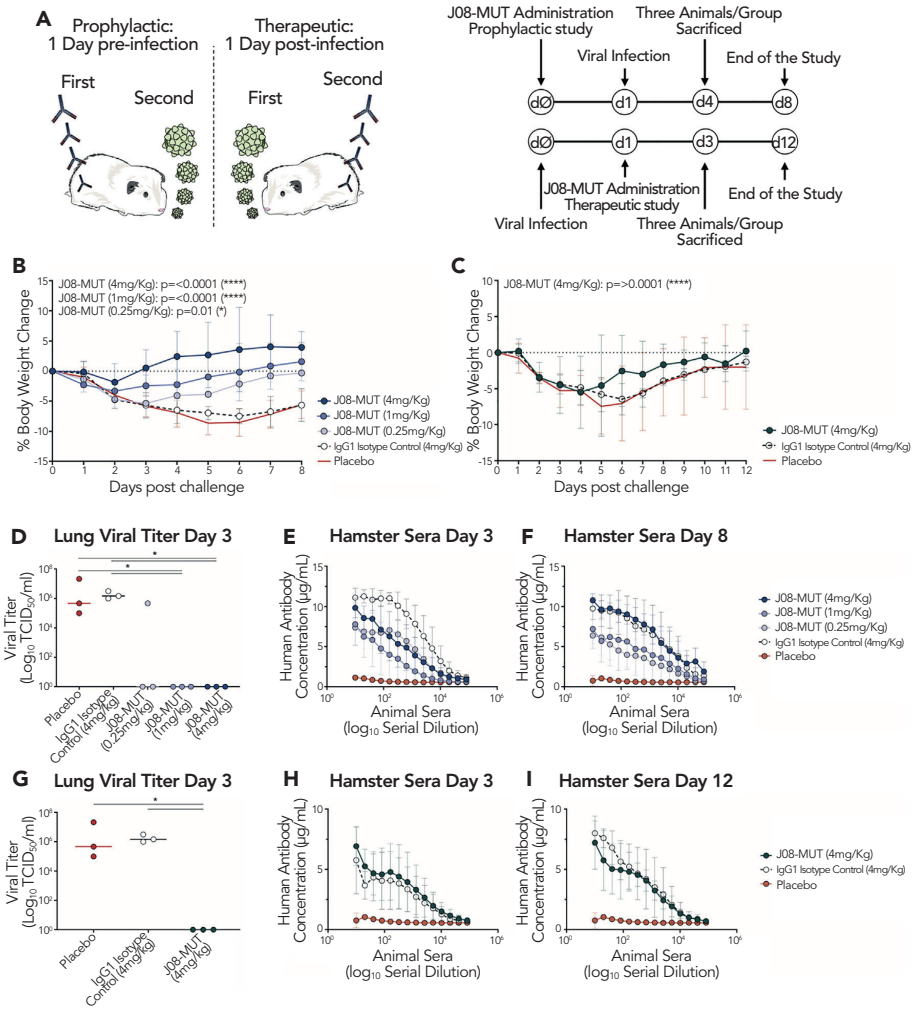
The antibody J08-MUT also showed extreme neutralization potency against emerging variants as it was able to neutralize the B.1.1.7 with an identical IC_{100} compared to the WT virus (• Figure S6K; • Table S5) and has also showed to neutralize variants that include the E484K mutation (Andreano *et al.*, 2020).

Since it has been reported that SARS-CoV-2 elicited antibodies that can cross-react with human tissues, cytokines, phospholipids, and phospholipid-binding proteins (Zuo *et al.*, 2020; Bastard *et al.*, 2020; Kreer *et al.*, 2020), the three candidate mAbs in both their WT and MUT versions were tested through an indirect immunofluorescent assay against human epithelial type 2 (HEp-2) cells, which expose clinically relevant proteins to detect autoantibody activities (• Figure S7 A).

As reported in • Figure S7B, the positive control presents a different range of detectable signals based on the initial dilution steps (from bright green at 1:1 to very dim green at 1:100).

Among all samples tested, only F05 showed moderate level of autoreactivity to human cells, while no signal could be measured for the other antibodies (• Figure S7B).

• Figure 7. Prophylactic and therapeutic efficacy of J08-MUT in the golden Syrian hamster model of SARS-CoV-2 infection. (A) Schematic representation and timelines of prophylactic and therapeutic studies performed in golden Syrian hamster. (B and C) The figure shows the prophylactic impact of J08-MUT at three different concentrations (4, 1, and 0.25 mg/kg) (B) on body weight loss change (C). The figure shows the therapeutic impact of J08-MUT at 4 mg/kg on body weight loss change. Mean ± SD are denoted in the graphs. (D-F) The figures show the lung viral titer at day 3 (D) and the detection of human antibodies in hamster sera at day 3 (E) and day 8 (F) in the prophylactic study. Mean ± SD of technical triplicates are shown. (G-I) The figures show the lung viral titer at day 3 (G) and the detection of human antibodies in hamster sera at day 3 (H) and day 12 (I) in the therapeutic study. Mean ± SD of technical triplicates are shown. Statistical differences were calculated with two-way analysis of variance (ANOVA) for body weight change and with a nonparametric Mann-Whitney t test for the lung viral titer. Significances are shown as *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.



Structural analyses of candidate nAbs

Single-particle negative-stain electron microscopy (nsEM) was used to visualize a stabilized SARS-2-CoV-6P-Mut7 S protein in complex with three separate Fabs: J08, I14, and F05. This recombinant, soluble S protein primarily exhibits 3 RBD's "down" but can switch to RBD "up" conformation with antibody bound. Inspection of the 2D class averages revealed a mixed stoichiometry of unbound S protein, one Fab bound, and two Fab bound classes, which allowed for 3D refinements of each (• Figure 6 A).

The three different Fabs bind to the RBD in the "up" conformation, although at different angles and rotations, likely due to the flexibility of the RBD. Model docking of PDB 7BYR (one RBD "up" bound to antibody) shows that the fabs overlap with the receptor-binding motif (RBM) and therefore are positioned to sterically block receptor hACE2 engagement (• Figure 6B).

To determine the epitope, HC and LC sequences of Fabs J08, I14, and F05 were used to create synthetic models for docking into the nsEM maps. Based on the docking, we predicted that a loop containing residues 477 to 489 (STPCNGVEGFNCY) appeared to be involved in the binding specifically with residue F486 extending into a cavity that is in the middle of the HC and LC of each antibody.

J08-MUT prevents SARS-CoV-2 infection in the golden Syrian hamster

The golden Syrian hamster model has been widely used to assess monoclonal antibody prophylactic and therapeutic activities against SARS-CoV-2 infection. This model has shown to manifest severe forms of SARS-CoV-2 infection mimicking more closely the clinical disease observed in humans (Baum *et al.*, 2020; Imai *et al.*, 2020; Rogers *et al.*, 2020; Sia *et al.*, 2020). We designed a prophylactic study in golden Syrian hamster to evaluate the efficacy of J08-MUT in preventing SARS-CoV-2 infection. For this study, 30 hamsters were divided into five arms (six animals each), which received, J08-MUT at 4, 1, and 0.25 mg/kg via intraperitoneal injection. Placebo and IgG1 isotype control groups were included in the study, which received a saline solution and an anti-influenza antibody at the concentration of 4 mg/kg, respectively. The J08-MUT at 4 mg/kg group and the 1 and 0.25 mg/kg groups were tested in two independent experiments. The IgG1 isotype control group was tested in parallel with the J08-MUT 4 mg/kg group, whereas the placebo is an average of the two experiments. Animals were challenged with 100 µL of SARS-CoV-2 solution (5×10^5 plaque-forming units [PFU]) via intranasal distillation 24 h post-administration of the antibody. Three hamsters per group were sacrificed at 3 days post infection, while the remaining animals were culled at day 8 (• Figure 7 A).

Body weight change was evaluated daily and considered as a proxy for disease severity. Animals in the control group and those that received the IgG1 isotype antibody lost more than 5% of their original body weight from day 1 to day 6 and then stabilized. These data are in line with previously published data of SARS-CoV-2 infection in a golden Syrian hamster model (Kreye *et al.*, 2020; Liu *et al.*, 2020). In marked contrast, in the prophylactic study, all animals that received J08-MUT were significantly protected from weight loss. Protection was present at all J08-MUT concentrations and was dose dependent (• Figure 7B). When J08-MUT was administered at 4 mg/kg, we observed protection from SARS-CoV-2 infection and only a minimal weight loss (average −1.8% of body weight) was noticed 1 day post viral challenge. A higher body weight loss was observed 1 day post infection in hamsters that received J08-MUT at 1 mg/kg (from −1.8% to −3.3%) and 0.25 mg/kg (from −1.8% to −4.7%). In the J08-MUT 4 mg/kg group, all animals quickly recovered and reached their initial weight by day 3. From day 4 on all hamsters gained weight increasing up to 5% from their initial body weight. Hamsters that received the 1 and 0.25 mg/kg dosages completely recovered their initial body weight at day 6 and 8, respectively. Hamsters in the control groups did not recover their initial body weight and at day 8, still showed around 5% of weight loss (• Figure 7B). The prophylactic activity of J08-MUT was also reflected in the complete absence of viral titer in the lung tissue at 3 days post infection in all hamsters that received J08-MUT at 4 and 1 mg/kg and also in two out of three hamsters that received J08-MUT at 0.25 mg/kg. On the other hand, hamsters that received the IgG1 isotype control or in the placebo group showed a significantly higher viral titer (• Figure 7D).

Finally, we performed an ELISA assay to detect the presence of human IgG in hamster sera. All samples that received J08-MUT or the IgG1 isotype control showed detectable human IgGs in the sera in a dose-dependent fashion, while no human IgGs were detected in the placebo group (• Figures 7E and 7F). Human IgGs were detected at 3 and up to 7 days post infection (• Figures 7E and 7F).

J08-MUT therapy of SARS-CoV-2 infection in the golden Syrian hamster

For the therapeutic study, three groups of six animals each were used to evaluate the ability of J08-MUT to treat SARS-CoV-2 infection in the golden Syrian hamster model. One group received J08-MUT via intraperitoneal injection at 4 mg/kg, and the other two groups received placebo and 4mg/kg IgG1 isotype control, respectively. The experiment was performed in parallel with the initial prophylactic study where J08-MUT was administered at 4 mg/kg and the two control groups. Animals were challenged with 100 µL of

SARS-CoV-2 solution (5×10^5 PFU) via intranasal distillation 24 h prior to the administration of the antibody. Three hamsters per group were sacrificed at 3 days post infection while the remaining animals were culled at day 12 (• Figure 7A). Despite J08-MUT and control groups showed a similar trend in weight loss in the first 4 days post infection, the treatment group showed a significantly quicker weight recovery (• Figure 7C). At day 12, only hamsters that received J08-MUT recovered the initial body weight (• Figure 7C). When we analyzed the viral titer in lung tissues, we observed complete absence of the virus at day 3 in all the hamsters treated with J08-MUT at 4 mg/kg, while animals that received the IgG1 isotype control or in the placebo group showed a significantly higher viral titer (• Figure 7G). To evaluate the presence of human antibodies in hamster sera, we performed an ELISA assay. All samples that received J08-MUT or the IgG1 isotype control showed detectable human IgGs in the sera in a dose-dependent fashion, while no human IgGs were detected in the placebo group (• Figures 7H and 7I). Human IgGs were detected at 3 and up to 11 days post infection (• Figures 7H and 7I).

Discussion

This work describes a systematic screening of memory B cells from SARS-CoV-2 convalescent patients to identify extremely potent mAbs against SARS-CoV-2 and their engineering to extend half-life and eliminate the potential risk of ADE. The best antibody neutralized the authentic WT virus and emerging variants at pico molar concentration *in vitro* and showed prophylactic and therapeutic efficacy in a SARS-CoV-2 hamsters model of infection when used at 0.25 and 4 mg/kg, respectively. The antibody described is a promising candidate for the development of a broadly affordable tool for prevention and therapy of COVID-19.

In the search for potent antibodies, we found that approximately 10% of the total B cells against the S protein isolated produce neutralizing antibodies, and these can be divided into four different groups recognizing the S1 RBD, S1 domain, S2 domain, and the S protein trimer. Most potently neutralizing antibodies are extremely rare and recognize the RBD, followed in potency by antibodies recognizing the S1 domain, the trimeric structure and the S2 subunit. From these data we can conclude that in COVID-19 convalescent patients, most of the observed neutralization titers are likely mediated by antibodies with medium-high neutralizing potency. Indeed, the extremely potent antibodies and the antibodies against the S2 subunit are unlikely to contribute to the overall neutralizing titers because they are respectively too rare and too poor neutralizers to be able to make a difference. We and others found that the antibody repertoire of convalescent patients is mostly

germline-like. This may be a consequence of the loss of Bcl-6-expressing follicular helper T cells and the loss of germinal centers in COVID-19 patients, which may limit and constrain the B cell affinity maturation (Kaneko *et al.*, 2020). It will be therefore important to perform similar studies following vaccination as it is likely that the repertoire of neutralizing antibodies induced by vaccination may be different from the one described here.

Out of the 453 neutralizing antibodies that were tested and characterized, one antibody (J08) showed extremely high neutralization potency against both the WT SARS-CoV-2 virus isolated in Wuhan and emerging variants containing the D614G, E484K, and N501Y variants. During the last few months, several groups reported the identification, 3D structure and passive protection in animal models of neutralizing antibodies against SARS-CoV-2. Most of these studies, with few exceptions, reported antibodies that require from 20 to several hundred ng/mL to neutralize 50% of the virus *in vitro*. While these antibodies are potentially good for therapy, they will require a high dosage, which is associated with elevated cost of goods, low production capacity, and delivery by intravenous infusion.

The extremely potent mAb described in our study is likely to allow the use of lower quantities of antibodies to reach prophylactic and therapeutic efficacy and as a consequence, decrease the cost of goods and enable sustainable development and manufacturability. This solution may increase the number of doses produced annually and therefore increase antibodies availability in high-income countries as well as low-and middle-income countries. Therefore, our antibodies have the potential to meet the expectations of the call to action to expand access to mAb-based products, recently published by the Wellcome Trust and supported by the WHO and the Coalition for Epidemic Preparedness Innovations (Wellcome and IAVI, 2020).

A potential issue associated with the use of human mAbs against viral pathogens is the potential selection of escape mutants. This is usually addressed by using a combination of antibodies directed against non-overlapping epitopes. While this is an ultimate clear solution, it increases the complexity of development, costs of production, drug availability, and affordability. In our case, we believe that selection of escape mutants upon treatment with a single mAb may be quite difficult as the SARS-CoV-2 RNA-dependent polymerase possesses a proofreading machinery (Romano *et al.*, 2020), and the epitope recognized by the antibodies herein described overlaps with the region necessary to bind the hACE2 receptor. In this regard, it took more than 70 days of continuous co-culture of the virus in presence of the antibodies before we were able to detect the first emergence of escape mutants of the WT SARS-CoV-2 (data not shown).

Finally, a peculiar part of our approach consisted in depleting possible antibody Fc-mediated functions of the antibodies to avoid the risk of ADE. While there is no evidence of ADE in SARS-CoV-2, and most vaccines and mAbs tested so far seem to be safe, it is too early to make definitive conclusions. In addition, two recently published reports suggested that we need to continue to monitor the potential risk of ADE. The first report showed that severe SARS-CoV-2 patients are characterized by an increased proinflammatory signature mediated by the Fcγ receptors triggered by afucosylated IgG1 antibodies (Chakraborty *et al.*, 2020). The second report described that one antibody was associated with worse clinical outcomes when administered to hospitalized patients requiring high-flow oxygen or mechanical ventilation (Lilly, 2020). Therefore, we believe it is important to develop and test antibodies where Fc-mediated functions have been eliminated in the clinical practice. Since the Fc portion contributes significantly to the in vivo potency of the antibodies (Schäfer *et al.*, 2020), removing Fc functions may be a problem for mAbs with low neutralization potency because they may no longer be effective when tested in clinical settings, as already described in other contexts (DiLillo *et al.*, 2014). The extremely high potency shown by our antibodies allowed us to remove Fc functions while maintaining in vivo potency at minimal dosage.

Limitations of the study

While we believe that our antibodies are extremely potent when compared to most of those described in literature, we acknowledge that in most cases, direct comparison was not performed, and we rely on published data.

The second limitation of the study is that *in vitro* neutralization and in vivo protection in the SARS-CoV-2 hamster model of infection cannot be fully predictive of the behavior of the same antibody in humans, and therefore the real benefit of described antibodies can only be assessed in clinical studies.

Methods

Detailed methods are provided in the online version of this paper and include the following:

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  - Prophylactic and therapeutic passive transfer studies in golden Syrian hamsters
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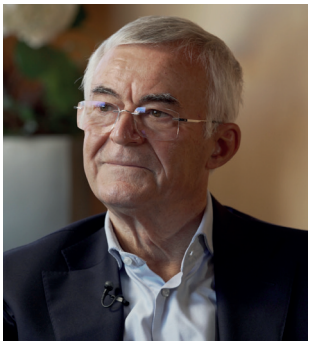
Biographies

Andrea Alimonti

Andrea Alimonti, graduated at Sapienza University of Rome, has completed his studies in clinical oncology at the Regina Elena National Cancer Institute, in Rome. He continued his education in the United States, first at the Memorial Sloan-Kettering Cancer Center in New York and then at the Harvard Medical School in Boston, where he worked at the Pandolfi Laboratory, a cutting edge cancer research institute. He is currently Head of Molecular Oncology at Institute of Oncology Research (IOR), a research division of the Oncology Institute of Southern Switzerland (IOSI) in Bellinzona and Professor of Biomedical Science at Università della Svizzera italiana (USI) in Lugano. His research is focused on the characterization of a novel type of cellular senescence response which is elicited by complete loss of the tumour suppressor PTEN, and on the identification of novel compounds with pro-senescence activity. His final aim is to develop the concept of pro-senescence therapy for cancer, from experimental evidences to clinic, investigating the efficacy of "pro-senescence" compounds in phase I clinical trials. This will also allow for the identification of senescence markers in human tumour samples to be used in clinic.

Silvia Misiti

Silvia Misiti, MD Ph.D. In 2001 she started working for Sapienza University of Rome as a researcher in Endocrinology. In 2012 she moved to Lugano, where she lives and directs the IBSA Foundation for scientific research, a non profit organization founded by the pharmaceutical company IBSA, Institut Biochimique SA. Her mission is to combine her great passion in scientific research with the promotion of different activities focused on innovation, education and dissemination, by collaborating with cultural and academic institutions. She is also Head of Corporate Communication & CSR for IBSA.



Rino Rappuoli

Rino Rappuoli is Chief Scientist and Head External R&D at GSK Vaccines, based in Siena, Italy and Professor of Vaccines Research, Imperial College, London. Prior positions were head of Vaccine R&D at Novartis, CSO of Chiron Corporation, and head for R&D at Sclavo. He earned his PhD in Biological Sciences at the University of Siena, Italy, and was visiting scientist at Rockefeller University and Harvard Medical School. He is elected member of US National Academy of Sciences (NAS), the European Molecular Biology Organization (EMBO), and the Royal Society of London. Awards received include: the Paul Ehrlich and Ludwig Darmstaedter Prize, the Gold Medal by the Italian President, the Albert B Sabin Gold Medal, the Canada Gairdner International Award, and the European Inventor Award for Lifetime Achievement. He was nominated third most influential person worldwide in the field of vaccines (Terrapin). He has published more than 690 works in peer-reviewed journals. He introduced novel scientific concepts: genetic detoxification; cellular microbiology; reverse vaccinology; pangenome. Developed licensed vaccines: acellular pertussis containing a non-toxic mutant of pertussis toxin; the first conjugate vaccine against meningococcus C; MF59, the first vaccine adjuvant after aluminium salts; meningococcus B; CRM197 that is used as carrier in many conjugate vaccines. Rino Rappuoli is among the world scientific leaders dedicated to the sustainability of global health.

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The current COVID-19 pandemic has urged the international scientific community to find quick answers in terms of vaccines and antibody development as counter measures to control SARS-CoV-2.

In this scenario and in an unprecedented and exceptional short time, active immunization, and passive immunization, respectively, are proving to be the best weapons available to save lives, safeguard the economy, and regain our freedom. Thanks to vaccines and monoclonals.