Antibody mining in SARS-CoV-2 spike-immunized rhesus macaques

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Abstract

The SARS-CoV-2 pandemic has led to over 6 million deaths and derailed health care systems and economies. Despite the development of effective vaccines, the virus spread remains high and new variants keep developing. Additionally, the threat of different pathogens emerging from zoonotic events is ever-present, forcing the scientific community to be vigilant and to put a continued effort in developing techniques and strategies that enable rapid responses to new or changing pathogens[1]. Here, memory B-cells produced by rhesus macaques inoculated with prefusion stabilized SARS-CoV-2 (Wuhu-1) spike protein administered in the Novavax Matrix M adjuvant were selected for spike-specificity and sequenced. From the B-cells, 56 paired heavy and light chain V(D)J sequences were selected for cloning and characterization of the resulting antibody. This showed that of the 56 antibodies 44 were spike binders, 16 were receptor binding domain (RBD) binders and 13 displayed neutralizing activity. The 13 neutralizing antibodies were all RBD-binders. Competition ELISA suggested that 9 of the neutralizing antibodies may directly compete with human Ace2 receptor for binding, whereas 2 neutralizing antibodies bound to a different epitope on the RBD. Finally, a pseudotype-based neutralization assay showed that 4 antibodies neutralized Omicron BA.1 and BA.2 subvariants, whereas the BA.4/5 subvariant was only neutralized by monoclonal antibody 23. In addition to single cell sequencing, repertoire sequencing was performed at several time points before, between and after the inoculations. This provides a valuable dataset to determine the evolution and dissemination of the spike specific antibody responses in future studies. Overall, this project contributes to the knowledge about vaccine-induced SARS-CoV-2 immunity and cross-reactivity.

Layman summary

The outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) at the end of 2019 quickly developed into a pandemic. COVID-19, the disease caused by SARS-CoV-2, has led to over 6 million deaths since the end of 2019[2]. To prevent more deaths and to be prepared for next variants appearing, it is important to study the immune response against SARS-CoV-2. Antibodies are a crucial part of the immune response against SARS-CoV-2[3]. They can bind to the outside of the virus, thereby blocking the virus from entry into human cells. Therefore, it is essential to study what qualities influence the binding efficiency of these antibodies to the virus, as well as how the development of these antibodies by the body can be induced with vaccines.

Here, rhesus macaques were immunized with purified spike, a protein present on the outside of SARS-CoV-2. This protein is responsible for the entry of the virus into human cells, and is the primary target for blocking antibodies against SARS-CoV-2 infection. After immunizations, B-cells were isolated from the immunized rhesus macaques. Each of these B-cells produces a unique antibody. The RNA coding for an antibody was sequenced from single B-cells. Based on this RNA sequence, the antibodies were produced in the laboratory for further characterization. This way, 56 different antibodies were produced. 44 out of the 56 antibodies were determined to bind to the Spike protein. The specific part of the spike protein binding to the human cells is the receptor binding domain (RBD). It was determined that 16 out of the 44 spike binders could also bind to the RBD.

Although the antibodies bind to the virus, it does not necessarily mean that they can also prevent the virus from entering the cells. To find out if the virus can still enter the cells, a neutralization assay must be performed. From this assay, it became clear that 13 antibodies could prevent the virus from entering the cell. These antibodies are very interesting to study, because they might help to find antibody characteristics important for preventing or treating SARS-CoV-2 infection. Therefore, we wanted to see the specific site on the spike protein that the antibodies bound. We determined that there is a high probability that 9 of the antibodies have a similar binding site, whereas there are 2 antibodies that have a completely different binding site. Lastly, the appearance of new viral variants is a worldwide concern, therefore it is essential to find out which properties allow antibodies to bind to different variants. Thus, a neutralization assay was performed again, to determine if these antibodies could also prevent other SARS-CoV-2 variants from entering human cells. The Omicron subvariants BA.1 and BA.2 were still neutralized well by 4 out of the 5 tested antibodies, however Omicron subvariant BA.4/5 was only neutralized by 1 out of the 5 tested antibodies (antibody 23).

With this report, the description of several neutralizing antibodies induced by Spike-immunization can contribute to further understanding on what properties provide antibodies with the neutralizing capability. Additionally, this report resulted in the finding of an antibody capable of neutralizing several different variants that will be valuable to study in the future.

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Inhoud

Abbreviations

- BCR: B-cell receptor
- COVID-19: Coronavirus disease 2019
- IgH: heavy chain
- GC: germinal center
- mAb: monoclonal antibody
- Nb: nanobody
- PBMC: peripheral blood mononuclear cells
- RBD: receptor binding domain
- RBM: receptor binding motif
- RDRP: RNA-dependent RNA polymerase
- 3R: Replacement, Reduction and Refinement
- SARS-CoV-2: severe acute respiratory syndrome coronavirus 2
- SHM: somatic hypermutation
- V(D)J: variability, diversity, joining

Introduction

SARS-CoV-2

Background

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of coronavirus disease 2019 (COVID-19) that has led to 6 million deaths as of July 2022[2]. COVID-19 was first reported at the end of 2019, when a cluster of pneumonia patients was observed in Wuhan, China[4]. Typically, COVID-19 symptoms include coughing, high fever and fatigue[4]. Between 5 to 10% of the cases result in hospitalizations, with 1% developing acute respiratory and multiple organ failure[5]. In addition to hospitalizations, a proportion of COVID-19 patients develop long-term symptoms like fatigue, shortness of breath and memory loss[6]. SARS-CoV-2 is transmitted by aerosols and droplets with an average incubation period of 5 days. SARS-CoV-2 infections display lower mortality rates compared to previously reported coronaviruses, such as SARS-CoV-1 and MERS. A combination between the long incubation period, aerosolized transmission and the lower mortality rate led to a rapid spread of SARS-CoV-2, which derailed health care systems as well as economies all over the world[5]. To halt this spread and health crisis, great efforts have been made to generate clinical strategies to prevent and treat COVID-19[7,8].

Structure

SARS-CoV-2 is part of the beta genus from the *coronaviridae* family, with the common characteristic of a crown-like structure to which it owes its name[9,10]. The viral particle consists of a positive sense, single stranded RNA genome, which is packaged in nucleocapsid protein (N). The nucleocapsid is surrounded by a double membrane layer, the envelope (figure 1[11]). Within the envelope layer, the membrane (M) and envelope (E) proteins dictate the round shape of the particle. In addition, a viral spike protein is embedded in the viral envelope[12]. This metastable spike protein is comprised of two subunits. The S1 subunit contains the receptor binding domain (RBD), used to bind to human ACE2 receptor[13]. During the S-protein pre-fusion state, RBD can be in an “up” (open) or “down” (closed) conformation. The S2 subunit contains the fusion peptide.

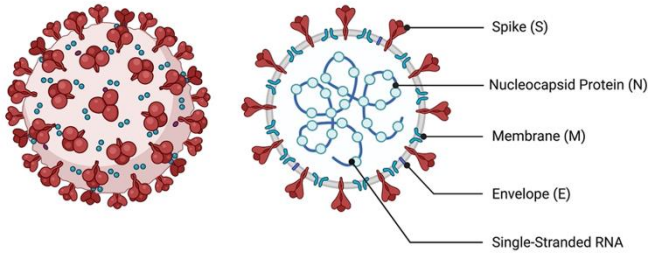

Figure 1: SARS-CoV-2 structure. Adapted from A comprehensive SARS-CoV-2 and COVID-19 review, Part 1: Intracellular overdrive for SARS-CoV-2 infection, Nature, 2022

Replication

SARS-CoV-2 infects human cells by binding to the ACE2 receptor with the S-protein, after which the viral genome is translated to assemble new virions[12]. When the virus enters the human airway, S2 is primed by several host proteases such as TMPRSS2, Furin and to a lesser extent by Cathepsin L and B[12,14,15]. While the virus is in the extracellular stage, the RBDs present on the S-protein continuously alternate between the “up” and “down” conformation. A likely explanation for the alternation between “up” and “down” conformations by the RBD is to keep a balance between “up” conformation to enter cells and “down” conformation to minimize antibody neutralization. The “up” conformation allows RBD to bind ACE2, initiating infection[13,16,17]. ACE2 binding by spike opens a cleavage site in the

S-protein, that will subsequently be cleaved[18]. Following activation by ACE2 binding and S cleavage, conformational change in the S-protein exposes the fusion peptide. Subsequently, the viral envelope and cell membrane are brought in close proximity in preparation of membrane fusion, which can take place at the cell- or endosomal membrane.

Fusion of the cellular and viral membrane results in the release of the viral capsid and genome in the cytosol. The positive sense, single-stranded genome is directly translated after entry to the cell. In addition to the structural proteins including Spike (S), the viral genome also encodes for polyproteins. Following cleavage, these polyproteins generate non-structural proteins such as RNA-dependent RNA polymerase (RDRP) and proteases[19,10]. In double membrane vesicles originating from the ER, the genome is translated by host machinery and replicated by RDRP[20]. The resulting negative sense RNA can be used for transcription of sub-genomic mRNAs or for replication of the genome[10]. A new virion is assembled with the structural proteins and the newly replicated genome and is released as a new replication-competent virus at the cell membrane.

Vaccines

Large-scale efforts within the research community resulted in the development of several highly effective vaccines and monoclonal antibodies. Although vaccine inequity and hesitancy issues remain, by July 2022, 11 billion vaccine doses have been administered globally[2]. The implementation of these interventions lead to a drastic drop in mortality caused by COVID-19[21]. Frequently used platforms for COVID-19 vaccines include mRNA (BNT162b2, mRNA-1273), viral vector (AZD1222, Ad26.COV2-S, Sputnik V) and inactivated (CoronaVac, BBIBP-CorV) vaccines[21]. In phase III trials, the mRNA vaccines showed a higher efficacy (~90%) than viral vector vaccines (~60-90%)[22]. However, these trials were performed in different time periods and in different countries where new variants were appearing, which could account for some of the variability. Subunit protein vaccines such as the NVX-CoV2373 had a similar efficacy (90%) to the mRNA vaccines, however they were reported to have at least an order of magnitude higher levels of neutralizing antibodies[23]. Although all vaccines have a high efficacy against the development of severe disease following infection with the original SARS-CoV-2 strain, waning immunity and new variants with the ability to escape vaccine-induced response decrease vaccine effectiveness[22].

Variants

Despite the global rollout of several vaccines, the virus spread remains high and new, more infectious viral variants are increasingly capable of evading the vaccine-induced immune response. For example, at the start of 2021, the Delta (B.1.617.2) variant became the most prominent circulating variant, containing amongst others a P681R mutation located near the furin cleavage site, likely contributing to the higher level of transmission of variants that carry this mutation[24,25]. Currently, the majority of cases are caused by various sub-lineages of Omicron, which contains several immune escape mutations and appears to be less pathogenic[26,27]. The initial Omicron variant is now being outcompeted by subvariants such as BA. 4/5, which are reported to even more efficiently escape immunological memory generated by infection with previous Omicron variants[28].

Immune response

B-cell development

Both the innate and adaptive branch of the immune system are involved in the immune response against viruses. One component of the adaptive immune system that is essential for protection against SARS-CoV-2 is the B-cell[29]. B-cells originate from progenitor cells in the bone marrow, where they undergo stepwise Variable, Diversity and Juntion (V(D)J) recombination, thereby defining the genes that will translate to the variable part of the B-cell receptor (BCR)[30]. Gene arrangements first take place

in the heavy chain (IgH) locus, starting with the D and J gene segments being joined, after which the Variable (V) genes are joined with DJ[31]. The newly recombined heavy chain locus is expressed as a μ heavy chain protein together with a surrogate light chain, called the pre-BCR. Then, rearrangements of the light chain V and J genes takes place, first in the kappa locus, and if no in-frame arrangement is produced, the lambda locus[31]. After several checkpoints for positive and negative selection for fitness and autoreactivity, respectively, immature B-cells start producing surface-bound IgM[32]. Together with allelic variation in the V, D and J genes, V(D)J recombination provides a high diversity to the B-cell repertoire, allowing the individual to respond to a broad range of different pathogens[33].

At the start of an infection, antigen-specific B-cells are activated in an extrafollicular response, resulting in their proliferation and differentiation into short-lived plasmablasts[34]. In the Germinal Centers (GC) of secondary lymphoid organs, the B-cells undergo somatic hypermutation (SHM), which introduces random mutations in the V(D)J region of the genome[35]. Simultaneously, class switching ensures that the B-cell produces antibodies with a different isotype than the initial IgM, depending on the type of infection and helper T-cell response[35]. The resulting B-cell receptor is tested for antigen-binding and the best binders selectively receive survival signals, causing a rigorous selection and optimization of the B cell repertoire[34]. These selected B-cells differentiate into memory B-cells that serve as a long-term mechanism of defense, and long-lived plasma-cells that start to produce antibodies against the pathogen.

Antibody structure

Antibodies are symmetrical structures with two identical heavy chains and two identical light chains that are connected to each other by non-covalent and covalent bonds[36]. Both the light chain and heavy chain contain a constant and variable region. The variable region provides the antigen-specific binding capabilities, whereas the constant region of the heavy chain dictates the antibody isotype and its effector functions. The symmetric nature of the antibody allows the bivalent binding, leading to a high avidity[37]. Each variable region is composed of three complementarity determining regions (CDRs) and four neighboring framework regions (FRs). CDRs are most essential in antigen binding.

B-cells during SARS-CoV-2 infection

Antibodies are one of the main defense mechanisms against SARS-CoV-2, they are important markers for COVID-19 outcome and essential for preventing re-infection with the same or a different variant[3]. When examining the ratio of Spike-specific to nucleocapsid-specific antibodies, patients with higher ratios experienced a milder disease progression[3]. As can be expected, patients with a worse disease outcome have a higher absolute amount of neutralizing antibodies due to their higher virus levels and prolonged antigen stimulation[38]. Additionally, antibodies and memory B-cells are essential for protection against reinfection[39].

Protection against other variants is dependent on cross-reactivity of the antibodies[40]. An example is the Omicron variant, for which the pre-existing antibodies provide poor cross-neutralization[26]. Upon booster shots with an mRNA vaccine, more cross-neutralizing responses develop due to further affinity maturation of the antibody pool[41]. A decay in antibody titers is observed after infection and vaccination, as can be expected from contraction of the B-cell response after the trigger is gone[42,43]. These findings and further research in this field are essential to be taken into consideration when deciding the vaccination regimen for a population or developing a COVID-19 vaccine against new viral variants.

Antibodies targeting SARS-CoV-2

Several antibodies targeting SARS-CoV-2 have been described for humans. Most antibodies generated target the S-protein, however also M-specific antibodies have been found in patients[44,45]. Antibodies

differ dramatically in their neutralization potential, from no neutralization to potent (cross)neutralization[46,47]. Gene usage is of great importance for neutralization capacity. For example human V-genes often utilized in neutralizing antibodies are IGHV1-58, IGHV3-30, IGHV3-53[48,49]. Specific germline-encoded motives on are critical for binding to the spike protein, such as NY and SGGS in IGHV3-53[48]. A large amount of antibodies and their sequences are listed in the CoV-AbDab database, including their origin, neutralization capacities and V(D)J sequences[50].

Sequencing techniques

Recent advances in sequencing have led to a tremendous increase in knowledge about the human genome, with as a great example the development of cheaper and quicker long-read and whole genome sequencing techniques[51]. The ImMunoGeneTics (IMGT) database contains genes and alleles of BCRs and TCRs of a large proportion of vertebrate species and is frequently used as a reference database[52]. However, sequencing and aligning the BCR and TCR loci is challenging because of the large amount of insertions and deletions[53]. Therefore, despite the development of new sequencing technologies, a large proportion of the V-gene polymorphisms in humans are not cited in the IMGT database[52]. Assignment to an incomplete database can lead to an over- or underestimation of SHM[54], showing that using only a non-individualized reference database presents an incomplete picture[55]. This is especially true for other species, such as rhesus macaques, for which the IMGT database consists only of the alleles of two animals. Other databases such as Karolinska Institutet Macaque Database (KIMDB), which was generated from 45 macaques, are more complete[53]. To identify alleles that are not present in existing databases, the Karlsson Hedestam lab developed a tool for *de novo* individualized V-gene database generation from repertoire sequencing called IgDiscover[56]. With this tool, the germline sequence can be inferred from bulk-RNA sequencing of IgM-specific B cells.

Rhesus Macaques

Selecting the animal model carefully is an important aspect of immunological research, as a different model can have a big impact on the outcome[57]. In this research, the rhesus macaque (*Macaca mulatta*) was used. NHP models have several advantages over models such as rodents. The most important advantage is their close genetic proximity with humans (93% sequence identity)[58]. Whereas rodents were separated 70 million years from humans in their ancestry, the macaque-human ancestor diverged a relatively short 25 million years ago[59]. Another indicator of this similarity of rhesus macaques to humans is the cross-reactivity of antibodies targeting human and NHP immune populations[60]. Important principles to consider in NHP research are the 3 R's for more humane animal research; Replacing animals with in vitro methods were possible, refining research to make the animal suffer as little as possible, and reducing the amount of animals needed[61]. The research group aims to implement these 3Rs in all animal research, including this project.

Aims

- Molecular characterization of SARS-CoV-2 spike-binding antibodies produced by rhesus macaques in response to inoculation with prefusion stabilized SARS-CoV-2 Spike protein. By determining the monoclonal antibody:
    - Binding to SARS-CoV-2 Spike protein and RBD
    - (Cross)-neutralizing capacity
    - Broad epitope mapping

Results

Current research on SARS-CoV-2 immunity is mostly based on patient samples obtained after infection or vaccination. In this setting there is a high level of variability and uncertainty about the precise timing of the initial exposure, the individual genetic differences and lifestyles. Here, we describe a pipeline to discover and characterize SARS-CoV-2 targeting antibodies in a controlled setting. Application of this pipeline on rhesus macaques provides a possibility to trace the antibody producing B-cells at different time points and in different immune compartments.

Experimental design

To characterize and trace antibody responses after inoculation with prefusion stabilized spike and the Matrix-M adjuvant, obtained through an MTA with Novavax, three rhesus macaques were immunized three or four times with 100 μg soluble prefusion stabilized WuHu-1 spike with adjuvant (Figure S1). Blood, lymph node and bone marrow samples were taken at several time points before, between and after the immunizations (Figure S1). Bulk IgM RNA libraries were sequenced using the Illumina MiSeq, to determine the VDJ germline sequences for each macaque (Figure 2A). This sequencing output was run through the IgDiscover software, which generated a V-gene germline database for each individual animal.

To isolate spike-specific antibodies, memory B-cells were sorted from post-inoculation samples using FACS, based on binding of the antibodies to a spike trimer with a fluorescent probe (Figure 2B). Subsequently, single memory B-cells were sequenced using the 10x Genomics Chromium microfluidic system, providing us with paired heavy and light chain sequences for each B-cell. 57 of these paired heavy and light chain sequences were selected to be produced and characterized. During the selection of paired sequences, several criteria were taken into account; different V and J genes, closest orthologues to relevant human V genes described in literature (3-53, 3-66, 5-15), relevant V genes described for macaques (3-73) and including a wide range of SHM-levels.

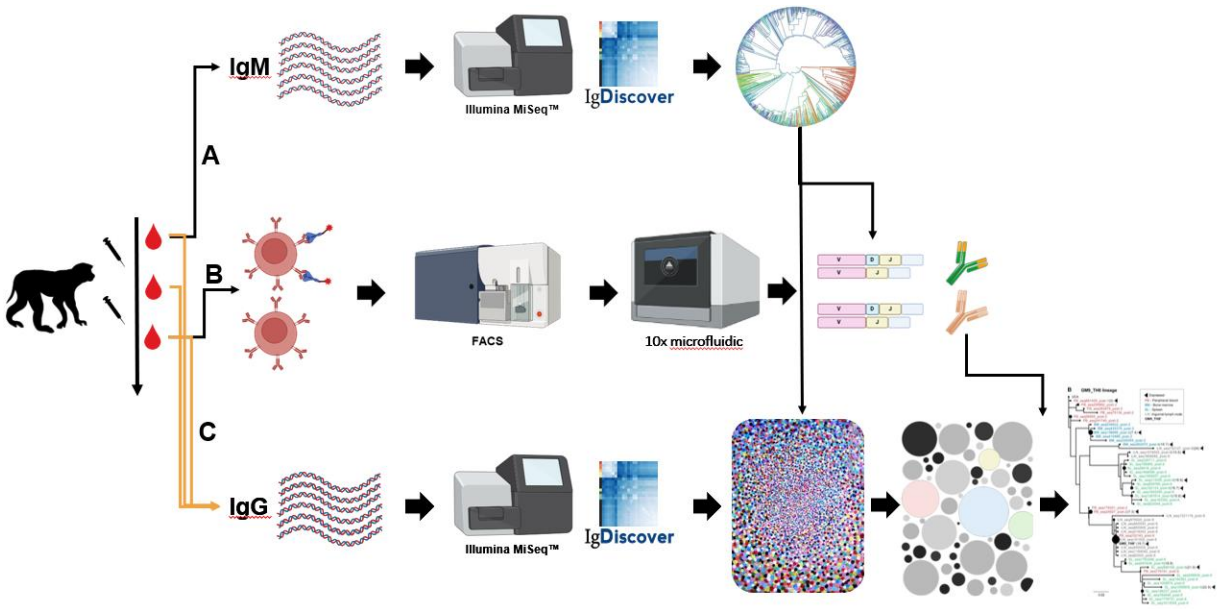

Figure 2: experimental design. Inoculated Rhesus Macaques are sampled before, between and after inoculations. A. IgM libraries before inoculation are bulk-sequenced and germline V-databases are generated with IgDiscover. B. Spike-specific B-cells are sorted with a fluorescent spike-probe, sequenced with the 10x Genomics Chromium microfluidic system, and antibodies were produced with the paired heavy and light chain sequences. C. IgG libraries were made from all time samples and bulk sequenced. Then, all sequences were assigned to a germline and clones were defined.

In parallel, bulk IgG libraries from samples at all time points were produced to trace the development path of selected antibody sequences between and after inoculations (Figure 2C). The IgDiscover module clonotype was used to define the clones in these sequences and to assign clones to each of the sequences. However, because tracing and germline database generation was largely performed by Marco Mandolesi, my supervisor, only the antibody production and characterization is presented here.

Gibson cloning and monoclonal antibody expression

The paired heavy and light chain sequences selected for antibody production were inserted in an expression plasmid containing human IgG constant regions. All 57 heavy and 57 light chain sequences were successfully cloned using Gibson cloning, showing a high cloning efficiency. Subsequently, we transformed ultracompetent bacteria with the cloned plasmids and picked single colonies for expansion. As a quality control, the inserts were expanded by colony PCR and the product was run on an agarose gel to confirm the presence of the insert and the absence of primer dimers (figure 3A). Next, colony PCR products were sent for Sanger sequencing to confirm the sequence of the insert. If the colony PCR product could not be confirmed, full plasmid was sent for Sanger sequencing. Once the sequences were confirmed, positive colonies were scaled up to produce more plasmid. The plasmid was isolated and HEK293F cells were transfected to start producing antibodies. Once the antibodies were purified from the supernatant, as another quality control, we ran a subset of the antibodies through an SDS-PAGE gel to confirm the presence of the heavy and light chain as well as correct folding (figure 3B). 56 out of 57 constructs were successfully expressed and purified for characterization, showing the efficiency of this pipeline.

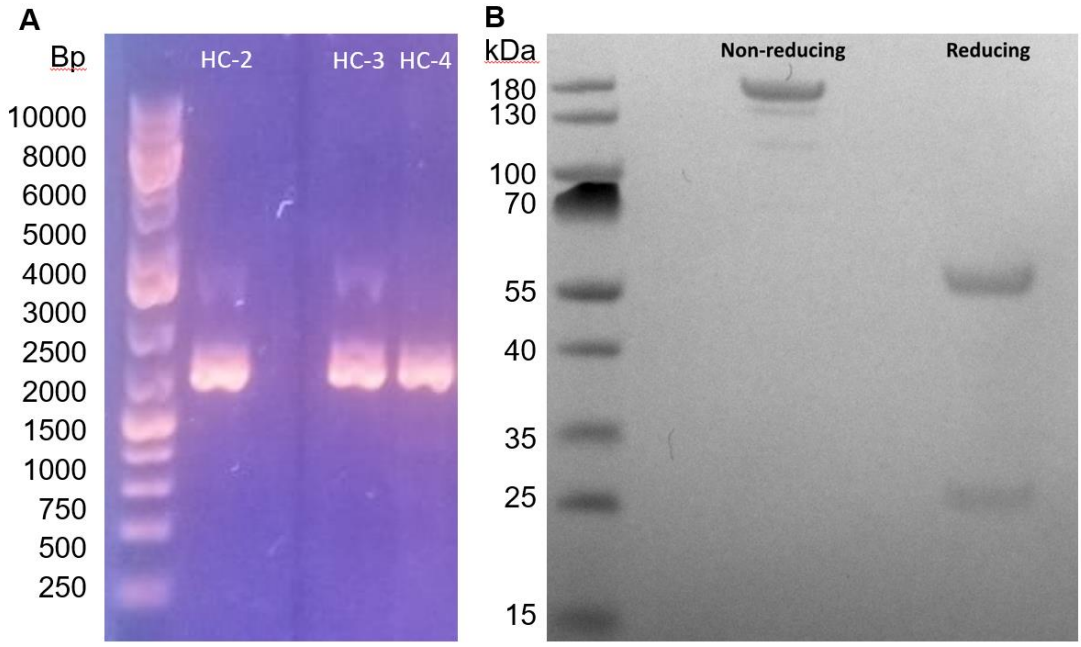

Figure 3: quality controls. A: agarose gel confirming the presence of the plasmid and the absence of primer dimers. B: SDS-PAGE of the isolated antibodies in non-reducing and reducing condition.

Spike-specific B-cells selected effectively

Neutralizing antibodies target the spike trimer, explaining why most vaccines use spike as a target. In this study, the macaques were inoculated with prefusion stabilized SARS-CoV-2 spike protein in adjuvant and memory B-cells were sorted based on their binding to the spike-trimer. To assess antibody spike-binding, we performed an indirect ELISA. Potential variation between plates was normalized through a max-min normalization with a previously characterized mouse serum. A dilution curve, indicating spike-binding, was detected with 44 of 56 cloned antibodies (Table S1), indicating that the probe was very effective at selecting memory B-cells with spike-specificity (figure 4A,B, S2). There was however a wide distribution of the concentration at which the antibodies had their half-maximal binding (EC50), suggesting a difference in binding efficiency (Figure 4C).

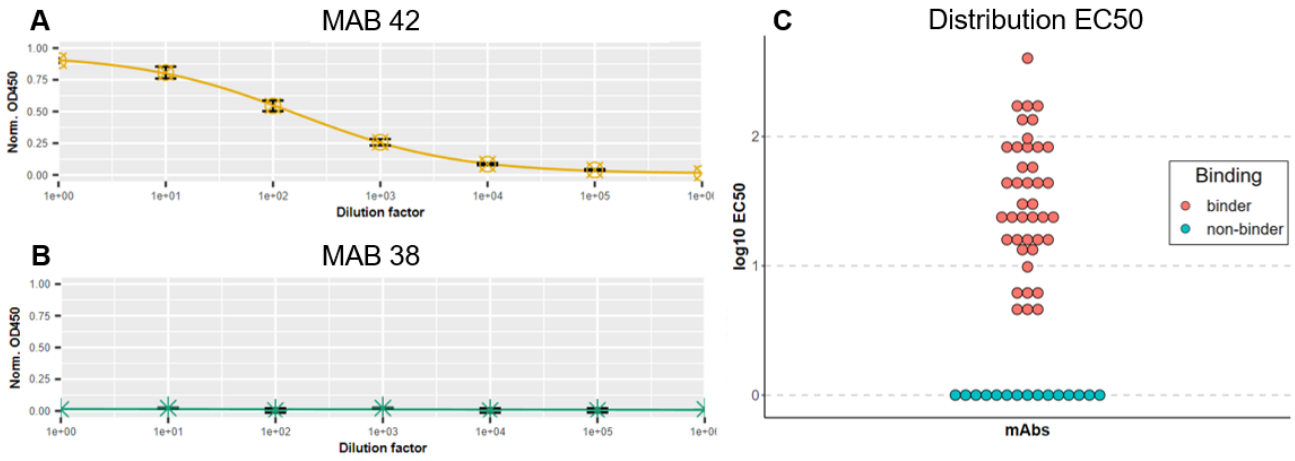

Figure 4: indirect ELISA spike-binding. A: dilution curve of a spike-binding antibody (n = 3). B: dilution curve of a non-binding antibody (n=3). Dilution factor for the antibody on X-axis against normalized OD450 for spike binding on the Y-axis. C: distribution of EC50 spike-binding antibodies (pink) and non-binders (blue).

RBD-binding profiles

Previous studies have shown that most neutralizing antibodies bind to the RBD of the spike protein[62,63], indicating that RBD-binding is a good predictor for neutralization. Nevertheless, there have also been reports of neutralizing antibodies binding outside of the RBD[64,47]. To determine whether our antibodies bind to the RBD, we performed an indirect ELISA testing RBD-binding on the 44 previously found spike-binders of which 2 antibody dilution curves are shown (Figure 5A, B, S3). Variability in between plates was again normalized through a max-min normalization with previously characterized mouse serum, which was present on all plates. From 44 spike binders, 16 bound to the RBD (Table S1). These RBD-binders make primary candidates for neutralization.

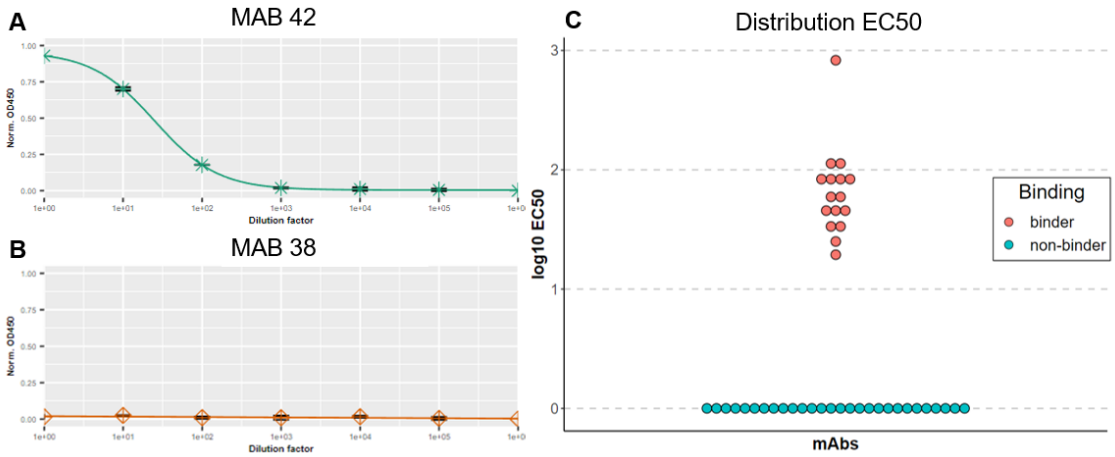

Figure 5: indirect ELISA RBD-binding. A: dilution curve of a RBD-binding antibody (n=3). B: dilution curve of a non-binder (n=3). Dilution factor for the antibody on the X-axis against normalized OD450 for spike binding on the Y-axis. C: distribution of EC50 RBD-binding antibodies (pink) and non-binders (blue).

Neutralizing capability

Binding to RBD alone does not ensure that these antibodies prevent the virus from entering cells. Several RBD-binding antibodies have been described that do not neutralize SARS-CoV-2[65]. Therefore, it is essential to test their neutralizing activity. Neutralization assays can be performed using the full virus; however, this requires a biosafety level 3 laboratory, which slows down the experiments and is less practical. Therefore, here, we used pseudotyped virus containing SARS-CoV-2 spike to enter cells and a luciferase reporter for the neutralization assay. All 44 spike-binding antibodies were tested for their neutralizing potential. 13 antibodies had neutralizing activity (Figure 6A), all of which were RBD binders (Table S1). The concentration at which the antibodies neutralized 50% of the virus differed between antibodies with a factor 10, showing that although all 13 antibodies can neutralize the virus, the concentration to do so effectively was variable (Figure 6B).

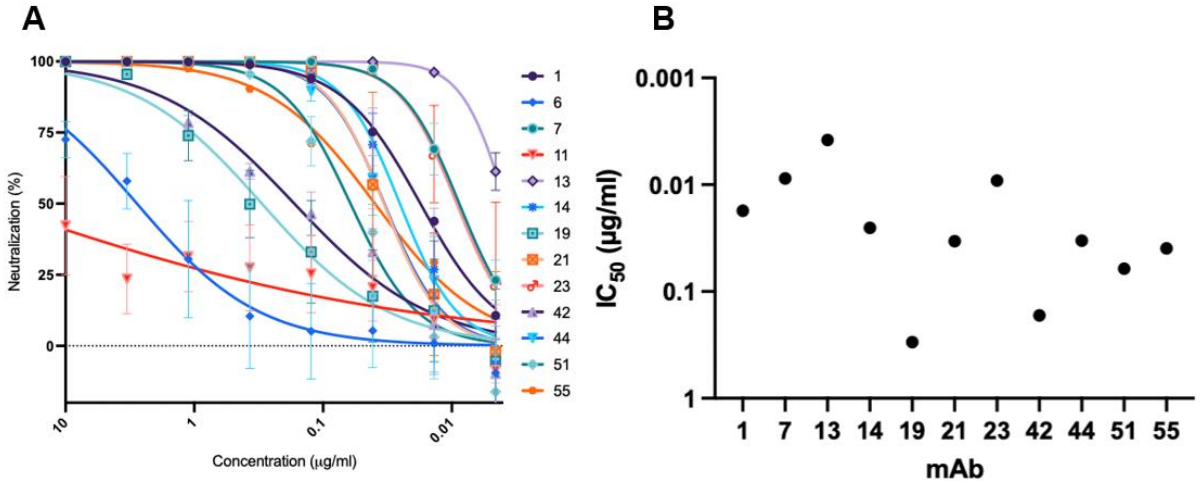

Figure 6: pseudotyped virus neutralization assay. A: dilution curves with antibody concentration (X-axis) and neutralization percentage (Y-axis). B: IC50 (µg/mL)(Y-axis) for each cloned antibody (X-axis) (n= 3) . B: monoclonal antibody on the X-axis, concentration of antibody where there is a 50% reduction of pseudoviral infection (IC50) on the Y-axis. Neutralization experiments were performed by Daniel Sheward and Julian Fischbach.

Preliminary epitope mapping

Epitope mapping of neutralizing antibodies is essential. Clinically, it is important to know the binding site of each of the monoclonal antibodies, to make sure they have distinct binding sites and therefore complement each other. Additionally, when new variants arise, the mutations can be mapped on the spike-protein, and the information about antibody epitopes will help predict which antibodies might be broadly neutralizing. Related to this, it is important to know the binding footprint of the antibodies and whether the antibodies generated by a vaccination or infection target conserved spots on the Spike protein.

Human antibodies against SARS-CoV-2 have been classified in four different S-binding classes, each of these classes have a distinct way of neutralizing SARS-CoV-2 by binding different epitopes[66]. Class 1 represents antibodies that bind to the receptor binding motif (RBM), thereby directly competing for binding with the human ACE2 receptor. This class can only bind to the RBD when it is in an "up" conformation. Class 1 usually contains antibodies that use IGHV3-53 and have a short HCDR3. A related but slightly different binding happens by class 2 antibodies. Similar to class I antibodies, their binding site overlaps with the RBM, revealing direct ACE2 competition as a neutralization mechanism; however these antibodies have an additional neutralization capacity by also binding to the RBD in the "down" conformation, which locks the RBD in a conformation incapable of binding ACE2[66].

In addition to this direct competition, antibodies can bind to different sites on the RBD, for example to sterically hinder structural changes in the RBD and spike protein, not allowing the fusion peptide to change in conformation, and therefore blocking the fusion of envelope with the host membrane. Class 3 antibodies fall into his category; they bind outside of the RBM and can bind in both the "up" and "down" RBD-conformation. Class 4 antibodies also bind outside of the RBM, however they differ from class 3 antibodies because they bind a buried epitope only while RBD is in the "up" conformation[66].

To determine in what class our antibodies most likely fall, we performed a competition ELISA with two previously characterized nanobodies (Nb) (Figure 7B). Nb consist of only a single variable domain on the heavy chain, thus they are smaller than conventional antibodies. Nb, like antibodies, can bind to different motifs on the RBD. When they bind, they will prevent antibodies from binding to the same exact motif of the RBD; however, because of their small size, they are less likely to hinder antibodies binding to other epitopes (Figure 7A). This property makes them excellent candidates for antibody epitope mapping. For this competition ELISA, we used Nb C7 and G6, kindly provided by Leo Hanke. Nb C7 has strong binding to amino acids 487 and 496, thereby overlapping the RBM, and falling under class 1- or 2-like binding (Figure 7C). Whereas Nb G6 binds around amino acid 380, consistent with a class 4-like binder[67]. Our results demonstrate that most antibodies compete for spike binding with nanobody C7 (Figure 7D). Antibodies 19 and 42 do not compete with C7 for binding. Complementary to this result, antibodies 19 and 42 do compete with G6 for binding, whereas the other antibodies do not (Figure 7E).

Although the nanobody competition assays clarify a part of the neutralizing capabilities of our antibodies, it is still a preliminary epitope mapping, as the antibodies provide only a broad view on the epitope and the C7 antibody might not only compete with class I and II but also with other classes of antibodies. Additionally, the Nb epitopes were mapped using deuterium exchange with mass-spectrometry, which cannot distinguish between interaction with the Nb or a conformational change resulting from the Nb binding, introducing more uncertainty[67]. Structural assays are planned to further characterize the antibody-RBD interactions of the neutralizing antibodies.

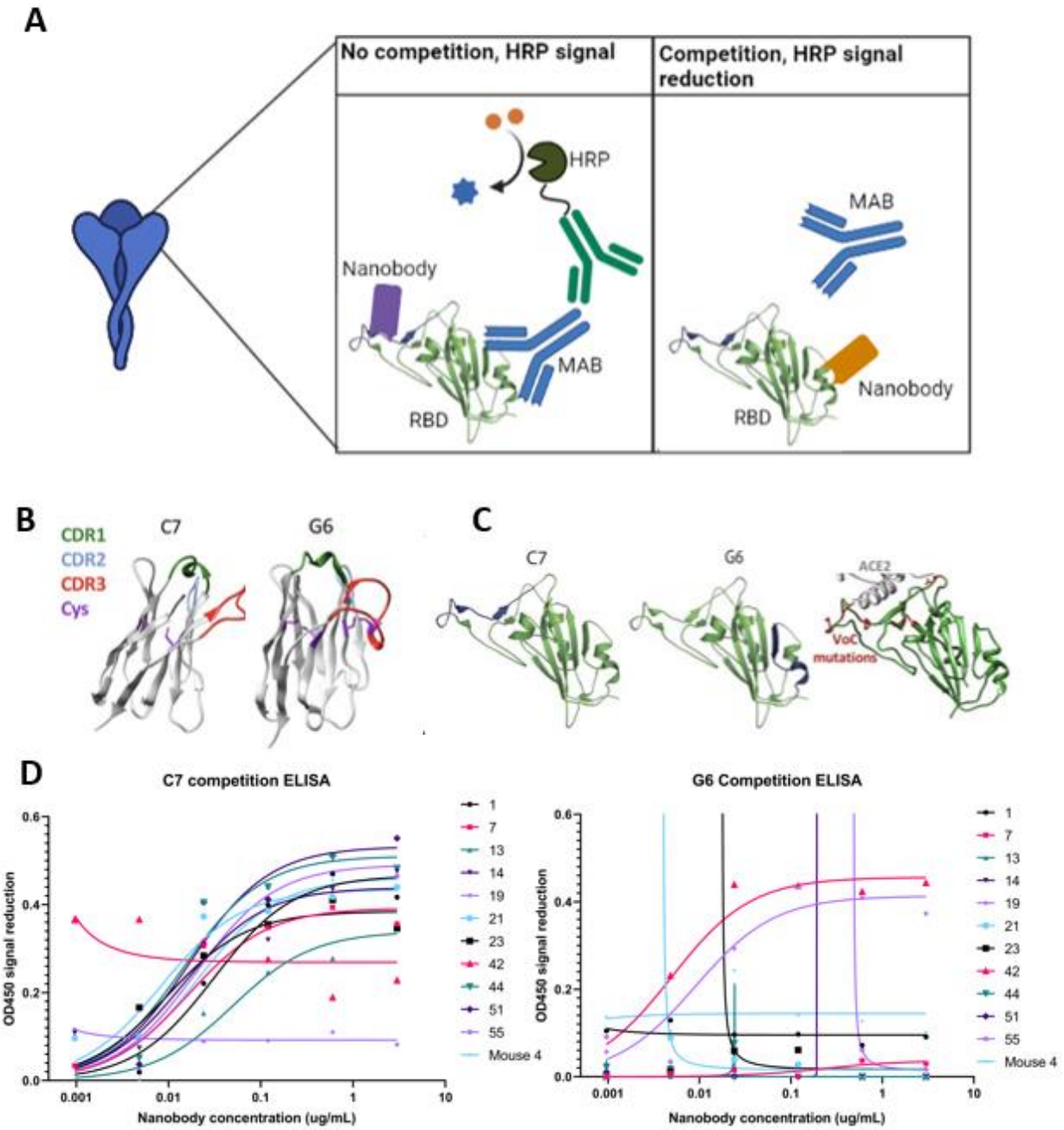

Figure 7: Competition ELISA for preliminary epitope mapping. A. Competition ELISA setup, a different Nb and Ab epitope leads to little competition, allowing the secondary antibody to bind with as a result signal (left panel). Same epitope for Nb and Ab leads to competition, where the primary antibody cannot bind, is washed away, and therefore no secondary antibody can bind (right panel). B. Nanobody structures including CDR1 (green), CRD2 (blue) and CDR3(red), as well as a Cys residue(purple). C. RBD structure with nanobody and ACE2 receptor epitopes. D. Competition ELISA results. B and C adapted from Leo Hanke et al., Multivariate mining of an alpaca immune repertoire identifies potent cross-neutralizing SARS-CoV-2 nanobodies. 2022, Science Advances.

Neutralization capacity for different variants

The COVID-19 pandemic is a developing situation, with new variants appearing constantly. These variants are increasingly able to evade the immune response developed by vaccines and previous infections[68]. As of July 2022, Omicron is the most frequent circulating variant. Monoclonal antibodies showed a 5-fold reduction in neutralizing capabilities, when comparing Omicron variants BA.2 to BA.4/5, and some antibodies lost their neutralization capacity completely[28]. Therefore, it is essential to determine what characteristics define cross-neutralizing antibodies. Until this point, we performed all the ELISA and neutralization assays with the Wu-Hu-1 strain spike and RBD, which does not show whether these antibodies will also bind to other (sub)variants. To address this, an indirect ELISA using omicron (BA. 1) spike protein was performed on all previous neutralizing antibodies. The ELISA results suggest that not all antibodies still bound to spike (Figure 8A). In addition, the antibodies that bound needed to be present at a higher concentration to reach the same binding strength. It should however be noted that the Omicron spike protein was reported to be less stable than the spike protein from the founder strain, therefore the lower presence of correctly folded spike could provide an alternative explanation for these results. To test the neutralization capacity of the antibodies against different Omicron sub-variants, neutralization against the Omicron variants BA.1, BA.2, BA.4/5 and, as a control, the original Wu-Hu-1 founder strain were evaluated (Figure 8B). Although a large subset of the antibodies still neutralized the BA.1 and BA.2 subvariants, the neutralization capacity was reduced for the BA. 4/5 variant. Only one of the tested antibodies remained capable to neutralize all variants at an equally high level; antibody 23 (Figure 8B).

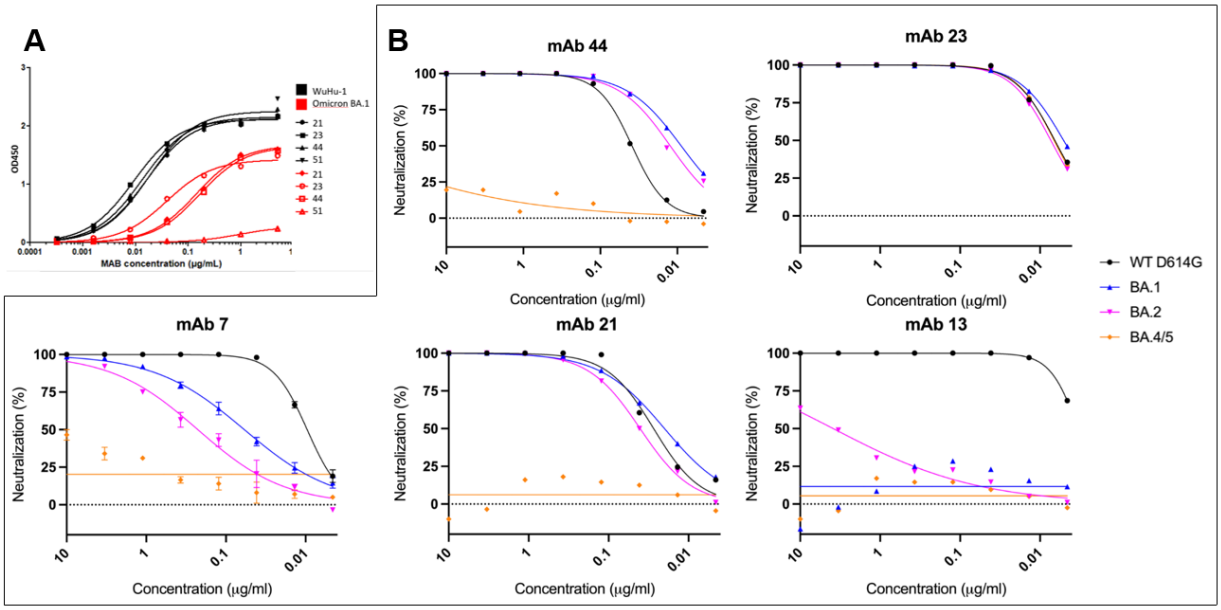

Figure 8: omicron S-binding and neutralization by monoclonal antibodies. A: ELISA showing antibody binding to SARS-CoV-2 Omicron BA.1 spike (red) and Wu-Hu-1 spike (black), with the antibody concentration on the X-axis and the OD450 on the Y-axis. B: neutralization assay for several antibodies against pseudotyped virus of the Wu-Hu-1 strain (black), BA.1 (blue), BA.2 (purple) and BA. 4/5 (yellow). Neutralization assays performed by Daniel Sheward and Julian Fischbach.

Properties of antibody 23

The remarkable cross-neutralizing capacity of antibody 23 makes it an ideal candidate to study the characteristics of cross-neutralizing antibodies. To determine how much the sequence differs from the germline sequence, the antibody heavy chain sequence is aligned with the germline sequence (Figure 9A). These amino acid changes have occurred through SHM. We found that there was no apparent relationship between SHM percentage and neutralization, or SHM percentage and spike binding (Figure 9B). Antibody 23 has an SHM percentage difference in amino acids from the germline sequence of 12%, which does not differ significantly from the other antibodies. H-CDR3 length of MAB 23 is 10 amino acids, which is shorter than most of the other antibodies. Although antibody 23 competes with C7[66], it is too early to conclude with certainty that it binds in a class 1 mode until more detailed structural experiments are performed.

Further studies into the phylogenetic trees generated by lineage tracing will provide valuable information about how cross-neutralizing antibodies like MAB 23 develop, after what inoculation the antibody appeared in the dataset and the dissemination of the MAB 23 producing B-cells in different compartments. However, lineage tracing falls outside of the scope of this thesis.

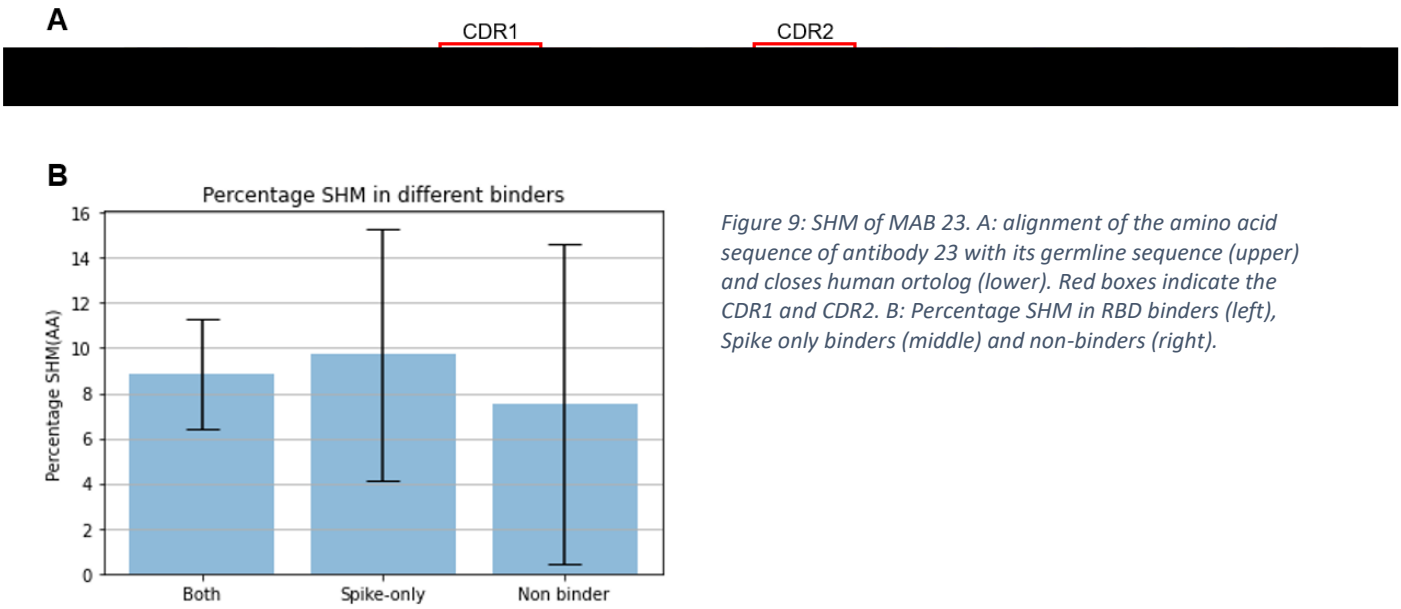

Figure 9: SHM of MAB 23. A: alignment of the amino acid sequence of antibody 23 with its germline sequence (upper) and closes human ortolog (lower). Red boxes indicate the CDR1 and CDR2. B: Percentage SHM in RBD binders (left), Spike only binders (middle) and non-binders (right).

Discussion

Taken together, the data produced here show that inoculation with spike adjuvanted with the Matrix-M adjuvant leads to a robust antibody producing immune response. These antibodies can be efficiently isolated and produced. After production, the characterization of these antibodies demonstrated that there are potent spike binders, as well as RBD binding antibodies present in inoculated macaques. When looking at the neutralizing activity of these antibodies against the founder strain Wu-Hu-1, the results show that all neutralizing antibodies in this group are RBD binders, confirming previous reports that RBD-binding correlates with neutralization capacity[69]. In this study, no non-RBD binding antibodies were found to be neutralizing. However this does not exclude the possibility of non-RBD binding neutralizing antibodies, as they have been previously described[47]. The results also showed that most of the antibodies that were produced were class I or class II binders, whereas two antibodies (42 and 19) compete with nanobody G6, and therefore most likely are class IV binders[67].

An essential part of current SARS-CoV-2 research is finding out how new appearing SARS-CoV-2 variants evade the existing antibody response generated by either vaccination or previous infection. Finding ways to induce antibodies that neutralize across different variants would bring us one step closer to ending the SARS-CoV-2 pandemic. In our data, we found that 4 out of 5 antibodies tested were cross-reactive to SARS-CoV-2 Omicron BA.1 spike. However, only 1 of the cross-reactive spike antibodies also neutralized all 3 different omicron sub-variants. While most antibodies neutralized Omicron BA. 1/2, only antibody 23 was able to neutralize Omicron variant BA. 4/5. Importantly, the antibody maintained its ability to neutralize not only the other Omicron variants, but also the founder strain Wu-Hu-1. This shows that it is possible for macaques to generate antibodies that have a broad cross-neutralization capacity.

In the study of neutralization capacity, there was no replicating virus challenge. The antibodies were characterized by ELISA and pseudotyped virus, neither of which is a replicating viral particle. However, it has been shown that pseudotyped viruses are an excellent tool to test for neutralizing capabilities[70]. The pseudoviruses deliver a luciferase-reporter to the cell, allowing measurements of luciferase activity as a measurement of infection[70]. Moreover, it has been shown that some neutralization assays can be even more precise than live assays[71]. Additionally, the pseudotyped virus is a much safer and more time-efficient method than the complete virus challenge.

Antibody 23 was shown to compete with C7, suggesting that it is a class 1-like or class 2-like binder. Several class 1-like and class-2 like binders have been reported to cross-neutralize different variants[66]. Interestingly, the C7 nanobody with which antibody 23 competes for an epitope does not have cross-neutralizing activity, albeit that the most current variants were not tested[67]. This shows that the characteristic of being a class I-like or class II-like binder does not always lead to cross-neutralization. The G6 antibody was reported to bind to a highly conserved epitope outside the RBD, consistent with class IV-like binding. As a result, G6 is broadly cross-neutralizing, including the Beta strain and related SARS-CoV-1 virus[67]. Therefore, in future studies it will be important to test the cross-neutralization capacities of antibodies 19 and 42, which competed with G6 for binding.

Finding out the epitope that antibodies bind to is especially important in a clinical setting, because a complementary treatment with a monoclonal antibody binding to a class 1-like or class 2-like and another class binding antibody will target the virus at multiple sites. Although these antibodies are not meant for clinical use because of their non-human origin, they will show more of an insight into how these classes of binders develop. Additionally, it was reported that viral escape from one class of binders did not provide escape from another class of antibodies, underscoring the importance of having a vaccine that does not only induce one class of antibodies[66].

Another important factor to consider when interpreting the competition ELISA results, is that a spike protein consists of 3 different RBD's, that can all individually be in an 'open' (up) or 'closed' (down) position[72]. Additionally, "semi-open" states of the RBD, that are also capable of ACE2 binding, have been described[18]. Which class of antibodies can bind depends on whether the RBD is in the open or closed state, and therefore the ratio of these states within the spike on the plate may influence the ELISA results. It would be difficult to determine the ratio open/closed state the RBD's are present, especially because structural analysis could be skewed by the stabilization process of the protein. Additionally, the open/closed state ratio might be subject to conditions such as acidity, mutations in spike, spike density on the envelope and antibody/nanobody binding. Indeed, it was shown that several mutations could cause a transition from the down- to the up- RBD states[73]. Additionally, SARS-CoV-1 was shown to have a more stable Down-down structure[74]. This could influence antibody neutralization characteristics, even though the actual binding capacity of the antibodies will not go down, the opportunity for binding does[74]. Additionally, during in vivo infection, there are pre- and post-fusion conformations[75,76]. Here, pre-fusion stabilized spike was used, because most described antibodies target pre-fusion spike, however it has to be kept into consideration that most vaccines also contain pre-fusion stabilized spike, therefore only eliciting pre-fusion target antibodies[77].

Alignment of the antibody amino acid sequences to their germline sequence showed the SHM percentage. There was no clear association between SHM percentage and neutralization, confirming previous reports stating that neutralization was more dependent on which germline genes were recombined than the on the SHM percentage[29,78,79].

The last two decades, great leaps in B-cell and V(D)J germline analyses have taken place. An important development in rhesus macaque B-cell research has been the creation of a more complete germline database based on 45 animals[53]. This is a significant improvement when compared with the information taken from the frequently used International ImMunoGeneTics Information System, (IMGT) database, which only contains a limited number of alleles. As shown in Vazquez Bernat *et al*[53]. the discovery of novel germline IG alleles has not reached saturation and for every new animal studied, additional alleles are expected to be identified. In addition to providing information relevant for SARS-CoV-2 vaccine development, this dataset contributes to the completion of the V(D)J genome analysis of this important model organism.

Limitations

An important limitation to the competition ELISA is that it cannot be ruled out that the nanobodies sterically hinder antibodies that bind to another epitope. Although the complementarity in the results increases the credibility, there is a need to validate these data with structural data. Additionally, although the competition ELISA results give an indication about the broad epitope that the antibodies bind, C7 competition cannot distinguish between antibodies with class-I like or class-II like binding. For this, a higher resolution is needed, which will be obtained by future structural studies.

A limitation intrinsic to the pipeline used here, is that for bulk sequencing analysis, no heavy and paired chains can be obtained, because bulk sequencing does not provide data on the cell that the sequence belonged to. For the monoclonal antibody production, this limitation is circumvented by single cell sequencing, however lineage tracing cannot be performed from single cell sequencing data, as there is a need for a large dataset. Therefore, lineage tracing is only performed with the heavy chain.

Future

In future research, it would be interesting to further characterize antibody binding sites by determining the structure of antibody 23. From this structure, it will become clear what characteristic of the binding provides the antibody with its cross-neutralization capacity. Conversely, the structure of the other not

cross-neutralizing antibodies could shed a light on how SARS-CoV-2 escapes neutralization. Furthermore, to see the contribution of SHM to cross-neutralization activity and general binding, antibody 23 could be converted to its germline and intermediate stages. The comparison between the germline-reverted, intermediate and final antibodies will show when the cross-neutralization ability appeared, indicating important residues within the antibody.

This research shows that NHP models are suitable for SARS-CoV-2 vaccine studies. The dataset created in this project contains a lot more information than what was presented for the scope of this thesis, for example RNA libraries were isolated from mesentery, axiliary, mediastinal, paraortic lymph nodes and the spleen, containing information about the physical distribution of B-cells during an immune response. These libraries are especially suited to learn more about immunity in these different compartments and the behavior of B-cells after inoculation with an antigen. There are still several gaps in our understanding of adaptive immunity in general; why do some memory B-cells return to the lymph node germinal centers for another round of SHM, whereas other memory B-cells differentiate into plasma cells during reinfection? Is the polyclonal nature of the repertoire maintained over time and after vaccine boosting or does it become more narrow and oligoclonal? Does an intranasal booster contribute to a better tissue-resident B-cell response against SARS-CoV-2 in the lungs? These are important questions for both the general field and the approach to tackle the SARS-CoV-2 pandemic.

Methods

Animals

Two rhesus macaques (*Macaca Mulatta*) were housed at the Astrid Fagraeus Laboratory at the Karolinska Institutet. Procedures complied with the guidelines of the Swedish Board of Agriculture. The macaques were inoculated intramuscularly with 100 µg prefusion stabilized spike protein adjuvanted with 75 µg matrix M adjuvant (Novavax). The first three inoculations happened at weeks 0, 4 and 9 in all macaques. Macaque H03 received another inoculation in week 31, macaque I10 received another inoculation at week 30. Blood samples were collected at week 0, 2, 4, 9, 11, 19, 23, 27 for all macaques. Additionally, macaque I10 was sampled at week 32 and 33. Lymphe node and bone marrow were sampled at weeks 6 and 11. Sampling and inoculation was performed by the facility veterinarian, Marco Mandolesi and Xaquin Castro Dopico.

FACS

Spike specific memory B-cells were selected with FACSAria cell sorter (BD Bioscience) based on their binding to prefusion stabilized spike trimer conjugated to streptavidin-allophycocyanin (SA-APC)(Invitrogen) and CD3-, CD14-, CD20+, CD27+, IgG+ staining. This protocol has been described by Sundling *et al*[80]. The cells were bulk sorted in PBS (10% FBS) and used as input for 10X single cell sequencing. Cell sorting was performed by the Karolinska Institute cell sorting facility, Marco Mandolesi, Benjamin Murrell and Jonathan Coquet.

Single cell sequencing

Single cell sequencing was performed by SciLifeLab in Stockholm with the 10X Genomics Chromium microfluidic system. Raw data were polished by Benjamin Murrell.

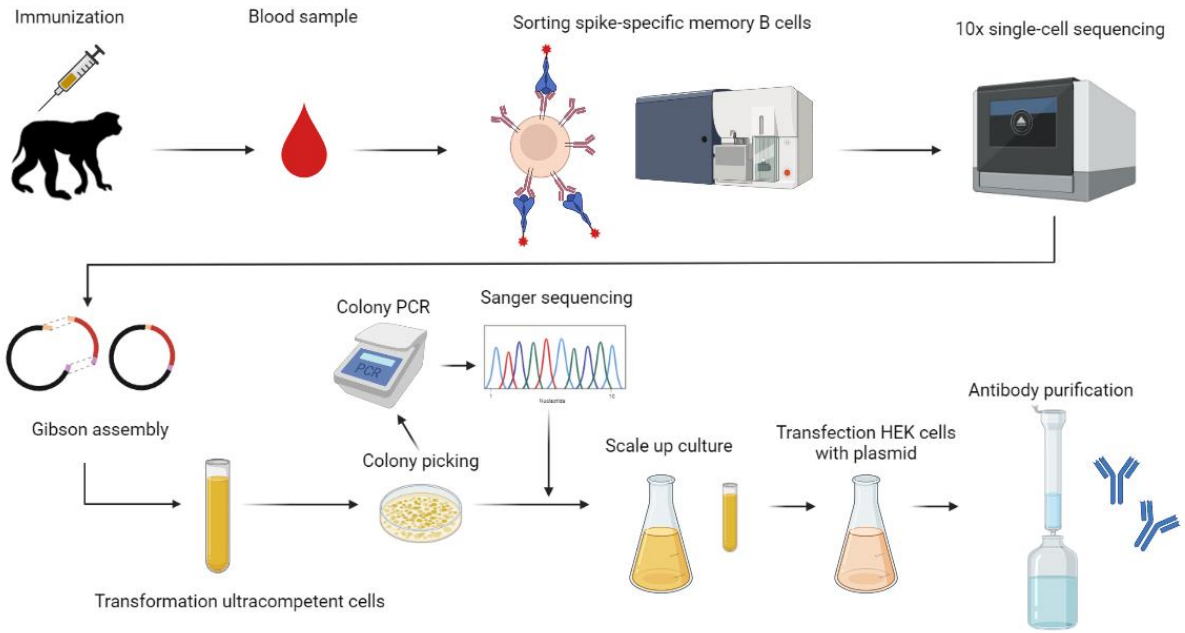

Figure 10: workflow from B-cell isolation to monoclonal antibody production.

Antibody Cloning

Antibody cloning was performed in several steps (figure 7). Paired heavy and light sequences obtained from the 10x single-cell sequencing were selected based on several selection criteria described in "experimental design". Heavy (addgene, 80795), kappa (addgene, 80796) and lambda (addgene,

99575) chain vectors containing a V(D)J leader sequence and ampicillin resistant gene were expanded in XL10-Gold Ultracompetent cells (Agilent). The inserts were designed to have homologous overlapping ends with the vectors. Heavy and light chain V(D)J sequences were introduced in heavy, kappa and lambda chain vectors with the Gibson assembly Master Mix (New England BioLabs) and incubation for 1 hour at 50 °C. Gibson assembly mixture was diluted 3X in ddH_2O. 5 µg of vector was added to 10 µL XL10-Gold Ultracompetent Cells (Agilent), quickly spinned down and incubated on ice for 30 minutes. The mixture was incubated at 42°C for 30 seconds and quickly transferred back on ice for 5 minutes. 200 µL of SOC media (Karolinska Universitetslaboratoriet) was added to the cells and they were incubated at 37°C for 1 hour in a shaking incubator set at 200 rpm. 50 µL of the cells were plated on an LB-AMP plate (Karolinska Universitetslaboratoriet) and incubated ON at 37°C.

Per V(D)J sequence, 2 colonies were picked and transferred to 200 µL LB-AMP media (Karolinska Universitetslaboratoriet) in a U-bottom 96 well plate and incubated at 37°C ON in a shaking incubator set at 200 rpm.

Colony PCR

PCR mixture was prepared containing 2 µL bacteria containing the plasmid, 1 µL forward primer (SP6), 1 uL reverse primer specific for heavy, lambda or kappa chain (Table 2), 10 µL of 2X Kapa HiFi ReadyMix (Roche), diluted in 6 µL ddH_2O. The tube with the mixture was placed in a thermocycler and ran through several cycles of heating (table 1). Success of amplification was verified by running 1 µL of the product in a 1% Agarose gel. 400 µL EtOH was prepared for each sample. Plasmid was attached to beads by mixing 19 µL AMPure XP beads (Beckman Coulter) for 20 µL of PCR reaction and incubating for 5 minutes. The tube or 96-well plate was placed in a magnetic rack for 5 minutes, and the supernatant was removed. The beads were washed twice with 200 µL 80% EtOH and airdried for 5 to 7 minutes. The tube or 96-well plate was removed from the magnet and the beads were homogenized with 30 µL elution buffer. The tube or 96-well plate was placed back in the magnet for 3 minutes. The supernatant containing the plasmid was transferred to a new tube and the final concentration of plasmid was measured with Qubit dsDNA BR kit (Invitrogen, Thermofisher Scientific). 5 µL of plasmid dilution was sent to Genewiz for confirmation of the sequence through sanger sequencing.

Table 1: colony PCR thermocycler conditions

Temperature (°C)	Time (min:sec)	Cycles
96	8:00	
95	0:30	30
66	0.30	
72	1:00	
72	7:00	
4		

Table 2: primer sequences.

	Sequence
Forward primer (SP6)	CGATTTAGGTGACACTATAGAATAACATCCAC
Reverse primer heavy chain	GGTGTGCACGCCGCTGGTCAGG
Reverse primer kappa chain	GAGGGCGTTATCCACCTTCCACTG
Reverse primer lambda chain	GGAGGGTGTGGTGGTCTCCAC

Scale up bacterial culture

After sequence confirmation, the bacterial cultures were expanded, first in 2 mL LB-AMP media for 5-7 hours at 37°C, 200 rpm, then in 30 mL LB-AMP media at 37°C, 200 rpm overnight. Glycerol stocks were created for each of the plasmids with 100 µL bacterial culture and 100 µL of 50% glycerol as a back-up and stored at -80°C. The plasmid was then isolated using Midi-Prep Plus kit (Qiagen) and plasmid concentration was measured using the Qubit dsDNA BR kit (Invitrogen, Thermofisher Scientific).

Cell culture

HEK293F cells (Gibco) were used for monoclonal antibody production. HEK293F cells were grown in suspension in FreeStyle 293 Expression Medium (Gibco) supplemented with Penicillin/Streptomycin. The cells were placed in a shaking incubator at 37°C, 200 rpm, 5% CO2.

Antibody production

The HEK293F stock was diluted to a million cells per mL in 30 mL. Transfection was achieved by first incubating 500 µl Opti-MEM (Gibco) with 50 µl FreeStyle MAX (Gibco) for 5 minutes at RT. In a separate tube, 18 µg of plasmid encoding the heavy chain, 18 µg encoding the light chain plasmid was diluted in Opti-MEM until the total volume was 50 µL. After the incubation, the plasmid mixture was mixed together with the FreeStyle-MAX mixture and incubated for 30 minutes at RT. The plasmid-MAX mixture was slowly added to the HEK293F cells while swirling the flask and the cells were incubated for 5-7 days at 37 °C with 8% CO2 at 125 rpm.

Antibody purification

The transfected cells were centrifuged at 3000 rpm for 15 minutes at RT and filtered through a 0.45 µm minisart syringe filter (Sartorious). A Pierce centrifuge column was washed with 5 mL PBS. 200 µL protein G Sepharose 4 fast flow (Cytivia) was added onto the filter of a Pierce centrifuge column and incubated for 5 minutes at RT. 10 mL PBS was added to the Pierce column to remove excess ethanol from the filter. Then, the filtered supernatant was added to the centrifuge column. The liquid was run through by gravity and collected, this step was repeated 6 times. The centrifuge column was washed twice with 25 ml PBS. 300 µL neutralization buffer (1M Tris-HCl, pH 9) was added to a new 50 ml falcon tube and the pierce centrifuge column is placed on top of the falcon tube. 2 mL elution buffer (0.1M Glycine-HCl, pH 2.7) was added to the column and the eluted IgG was transferred in a Protein concentrator tube (Thermofisher Scientific) (15-30 kDa). The tube was filled up to 4 mL with PBS and centrifuged at 4000 rpm for 6 min. The concentration of IgG was measured with Qubit Protein kit (Invitrogen, Thermofisher).

Proteins

Wuhu-1 and Omicron BA.1 prefusion stabilized spike protein as well as RBD protein was kindly provided by the Gerarld McInerney lab.

SDS-PAGE

In the reduced condition, samples were reduced with NUPAGE sample reducing agent 10X (Invitrogen) in a 1:20 proportion with the sample. NUPAGE LDS loading dye (4X)(Invitrogen) was added to all samples in a 1:4 proportion. The proteins in the denatured condition were incubated at 98°C for 2 minutes. The proteins were ran through a NUPAGE 4-12% Bis-Tris acrylamide gel (Invitrogen) in a NUPAGE MES SDS running buffer(20X) (Invitrogen). A PAGERuler prestained protein ladder (Thermo Fisher Scientific) is used for size standards. The gel is ran with 120 V for 70 minutes. The gel was stained with a SimplyBlue SafeStain (Thermo Fisher Scientific) for 20 minutes, and destained with ddH2O overnight.

ELISA & competition ELISA

The ELISA assays were performed in clear Flat-Bottom Immuno Nonsterile Maxisorp 96-well plates (#442404, Thermofisher Scientific). The plates were coated with 100 ng Spike/RBD or 25 ng Spike in case of the competition ELISA per well ON at 4°C. The wells were washed by adding and removing 200 µL 0.2% Tween in PBS 6 times. 200 µL 5% non-fat dry milk in PBS was added per well and incubated for 1 hour for blocking. The wells were washed again. In the case of the competition ELISA, dilutions were prepared from a starting concentration of 3 µg nanobody per mL, diluted 5x with 5% non-fat dry milk in PBS for each new row. 100 µL of the dilutions was added to the wells of the coated 96-well plate and the plate was incubated for 10 minutes at room temperature. Nanobodies were kindly provided and described by Hanke *et al*[67]. Without washing, 100 µL primary antibody was added with a concentration of 0.05 µg/mL in 5% non-fat dry milk in PBS. During the indirect ELISA, there were no nanobodies added, after blocking, the wells were washed and incubated for 2 hours with 100 µL primary antibody with a concentration of 0.5 µg/mL. Because the antibodies contained a human Fc region encoded in the vector, a secondary anti-human IgG HRP antibody (Southern Biotech, cat# 2015-05) could be used to visualize binding of the antibody to a spike-trimer coated plate After washing, the secondary antibody was added with a 1:10.000 dilution in 0.2% Tween in PBS and incubated for 1 hour at room temperature in the dark. For development, 100 µL of TMB-ELISA (Life technologies) was added to the wells and incubated for 2-5 minutes. Then, the reaction was stopped with 100 µL 1M Sulphuric acid and the plate was read in a spectrophotometer (Asys Expert 96) at 450 nm.

Neutralization assay

The neutralization assay was performed by Daniel J. Sheward and Julian Fischbach as described by Hanke *et al*[81]. HEK293T cells were co-transfected with a plasmids encoding firefly luciferase, a SARS-CoV-2 protein containing an 18 amino acid truncation of the cytoplasmic tail. The HEK293T cells were also co-transfected with lentiviral packaging plasmid (Addgene #8455). The co-transfection was performed using Lipofectamine 3000 (Invitrogen).

Library preparation and bulk sequencing

Samples for library preparation were handled in an RNAse-free environment. Library preparation for bulk sequencing was performed according to the 5' Multiplex (MTPX) protocol described in detail by Bernat *et al.*[82]. RNA was extracted using RNeasy Mini Kit (Qiagen). The Sensiscript RT Kit (Qiagen) was used for cDNA synthesis. The cDNA was purified using the PCR purification kit (Qiagen), after which the cDNA was amplified with PCR. The PCR product was ran in a 1% Agarose gel and the product was purified with the GeneJET gel extraction Kit (Thermo Scientific). KAPA HiFi HotStart ReadyMix PCR Kit (Kapa Biosystems) was used for index PCR. The concentration DNA was measured with the Qubit High Sensitivity kit (Invitrogen, Thermofisher), after which 2 µL was loaded on an agarose gel to check if any primer dimers formed during the PCR steps. The PCR products are purified using MinElute PCR purification Kit (Qiagen) on the condition that there were no primer dimers visible on the gel. If primer dimers were visible on the agarose gel, all product was loaded on a gel, the PCR product was cut out of the gel and purified. The DNA was then purified using Illumina's bead purification. Library preparation was largely performed by Marco Mandolesi. Bulk sequencing was performed by Sanjana Narang and Martin Corcoran according to the protocol described in Phad *et al*[83].

IgDiscover and lineage tracing

To generate individualized germline sequences for each macaque, IgM libraries were prepared from blood samples before the inoculations. RNA transcripts were isolated from the blood and reverse-transcribed with the protocol described in "library preparation". Then, the DNA-converted transcripts were sequenced using illumina next generation sequencing (NGS) to obtain the individualized V(D)J germline sequences for each macaque. IgDiscover is a bioinformatic tool that is used to generate the

individualized germline sequences. Lineage tracing and individualized V(D)J sequence generation was performed by Marco Mandolesi using the Clonotypes and Clonoquery modules of the IgDiscover software. The software is openly available (http://docs.igdiscover.se).

Ethical considerations

When using animals for research it is always important to carefully weigh the ethical considerations. The rhesus macaques from this study were housed in the Astrid Fagraeus Laboratory at the Karolinska Institute, which is AAALAC accredited. The work performed for this study was approved by Stockholms Norra Djuförsöksetiska Nämnd, the local ethical committee on Animal Experiments, [Ethical permit number: 18427-2019, ethical permit extension: 10895-2020].

Acknowledgements

During my time in Sweden and performing this internship, I received a great amount of support and guidance. I want to express my sincere gratitude to **Gunilla Karlsson Hedestam**, for allowing me to perform the internship in her lab and for the great amount of feedback and support. In addition, I would like to thank **Marco Mandolesi** for his endless patience in answering my questions and teaching me the complete antibody cloning and characterization pipeline.

I would also like to thank **Daniel Sheward** and **Julian Fischbach** for performing the neutralization assays and **Leo Hanke** for providing the nanobodies. I would also like to thank **Marco Mandolesi, Benjamin Murrell, Jonathan Coquet** and **Xaquin Castro Dopico** for all the work that was necessary to start this project, including macaque inoculations, sorting and single cell/bulk sequencing. Furthermore I would like to thank the rest of the lab, **Sanjana, Uta, Pradee, Andrea, Mariia, Sung Yong, Mark, Mateusz, Martin, Xaquin, Monika** and **Martina** for the interesting discussions and conversations and the great atmosphere. I also would like to acknowledge the second reviewer of this thesis **Balthasar Heesters**.

Supplementary data

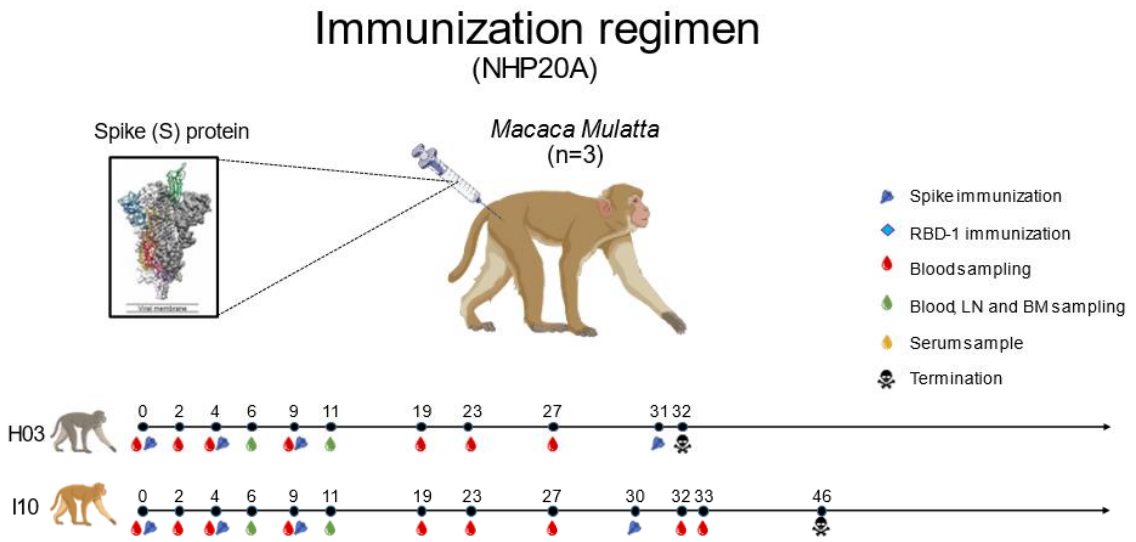

Figure S1: Immunization regimen of rhesus macaques

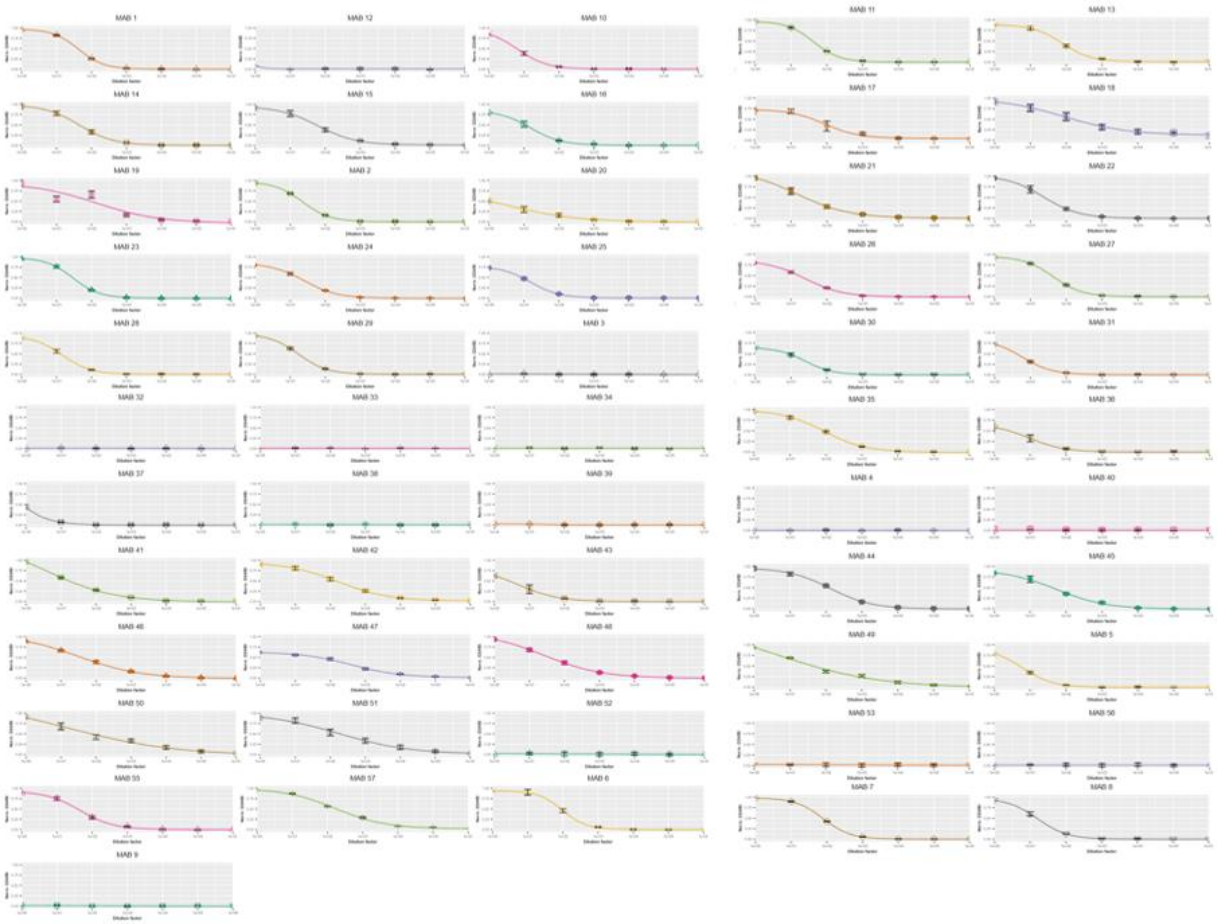

Figure S2: ELISA Spike protein dilution curves

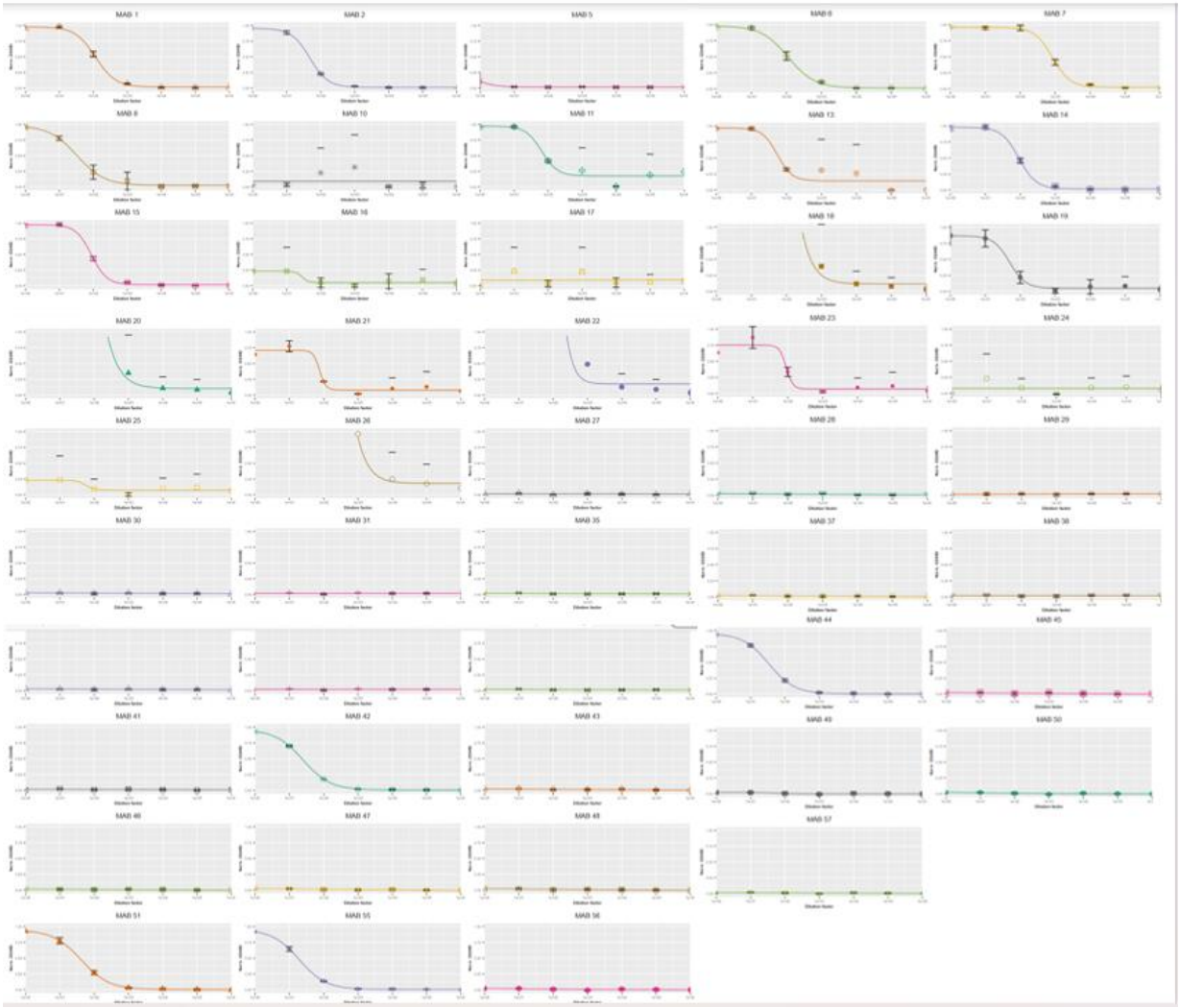

Figure S3: RBD ELISA dilution curves

Table S1: summary of spike binding, RBD binding, Wuhu-1 neutralization, Competition, Omicron BA.1 binding, Omicron BA. 1/2 neutralization and Omicron BA. 4/5 neutralization.

MAB	Spike binding	RBD binding	Wuhu-1 neutralization	Competition	Omicron BA.1 binding	Omicron BA. 1/2 neutralization	Omicron BA. 4/5 neutralization
1	Y	Y	Y	C7	-	-	-
2	Y	Y	N	-	-	-	-
3	N	N	N	-	-	-	-
4	N	N	N	-	-	-	-
5	Y	N	N	-	-	-	-
6	Y	Y	Y	-	-	-	-
7	Y	Y	Y	C7	~	Y	N
8	Y	Y	N	-	-	-	-
9	N	N	N	-	-	-	-
10	Y	N	N	-	-	-	-
11	Y	Y	Y	-	-	-	-
12	N	N	N	-	-	-	-

13	Y	Y	Y	C7	N	N	N
14	Y	Y	Y	C7	-	-	-
15	Y	Y	N	-	-	-	-
16	Y	N	N	-	-	-	-
17	Y	N	N	-	-	-	-
18	Y	N	N	-	-	-	-
19	Y	Y	Y	G6	-	-	-
20	Y	N	N	-	-	-	-
21	Y	Y	Y	C7	Y	Y	N
22	Y	N	N	-	-	-	-
23	Y	Y	Y	C7	Y	Y	Y
24	Y	N	N	-	-	-	-
25	Y	N	N	-	-	-	-
26	Y	N	N	-	-	-	-
27	Y	N	N	-	-	-	-
28	Y	N	N	-	-	-	-
29	Y	N	N	-	-	-	-
30	Y	N	N	-	-	-	-
31	Y	N	N	-	-	-	-
32	N	N	N	-	-	-	-
33	N	N	N	-	-	-	-
34	N	N	N	-	-	-	-
35	Y	N	N	-	-	-	-
36	N	N	N	-	-	-	-
37	Y	N	N	-	-	-	-
38	Y	N	N	-	-	-	-
39	N	N	N	-	-	-	-
40	N	N	N	-	-	-	-
41	Y	N	N	-	-	-	-
42	Y	Y	Y	G6	-	-	-
43	Y	N	N	-	-	-	-
44	Y	Y	Y	C7	Y	Y	N
45	Y	N	N	-	-	-	-
46	Y	N	N	-	-	-	-
47	Y	N	N	-	-	-	-
48	Y	N	N	-	-	-	-
49	Y	N	N	-	-	-	-
50	Y	N	N	-	-	-	-
51	Y	Y	Y	C7	-	-	-
52	N	N	N	-	-	-	-
53	N	N	N	-	-	-	-
54	N	N	N	-	-	-	-
55	Y	Y	Y	C7	-	-	-
56	Y	N	N	-	-	-	-
57	Y	N	N	-	-	-	-

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