From the Department of Microbiology Tumor and Cell Biology
Karolinska Institute, Stockholm, Sweden

IMMUNOGLOBULIN GENE USAGE AND AFFINITY MATURATION IN ANTIVIRAL ANTIBODIES

Pradeepa Pushparaj

Karolinska Institutet

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Cover illustration: The front page shows two potent antibodies bound to SARS-CoV-2 RBD. These antibodies were isolated during my doctoral research and are described in papers II and III.
Cryo-EM panels by Dr. Hrishikesh Das.

Immunoglobulin Gene Usage and Affinity Maturation in antiviral antibodies

Thesis for Doctoral Degree (Ph.D.)

By

Pradeepa Pushparaj

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Principal Supervisor:
Prof. Gunilla Karlsson Hedestam
Karolinska Institutet
Department of Microbiology Tumor and Cell Biology

Co-supervisor:
Prof. Karin Loré
Karolinska Institute
Department of Medicine, Solna

Opponent:
Prof. Penny Moore
University of Witwatersrand
National Institute for Communicable Diseases

Examination Board:
Associate Prof. Mats Bemark
University of Gothenburg
Department of Microbiology and Immunology

Prof. Olle Kämpe
Karolinska Institute
Department of Medicine, Solna

Prof. Francesca Chiodi
Karolinska Institute
Department of Microbiology Tumor and Cell Biology

முயற்சி திருவினை ஆக்கும் முயற்றின்மை

இன்மை புகுத்தி விடும்.

- திருவள்ளுவரின் திருக்குறள் 616

Effort brings forth results,

Its absence brings to nothingness.

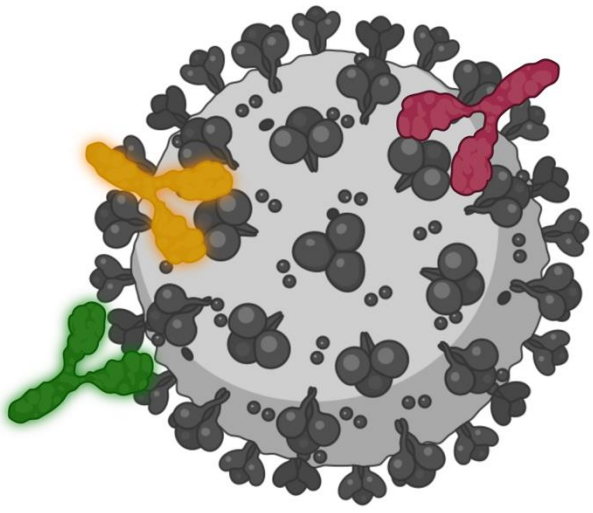
- Thirukural 616 by Thiruvaluvar

To my family

POPULAR SCIENCE SUMMARY OF THE THESIS

Viruses - The emergence of new infections with pandemic potential has occurred time and again throughout history and is a major threat to human health and prosperity. Many of these outbreaks have been caused by viruses, such as Influenza A in 1918, 1957 and 1968, and in late 2019, by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). This illustrates the capacity of viruses to spread rapidly in the population. Inside the host, they attach to molecules on the cell surface, before entering and reprograming the cellular machinery to create more viruses able to infect many more cells, leading to more or less severe disease and activation of the host immune system. While there are antibiotics to successfully treat bacterial infections, curing viral infections is more challenging as only a limited set of antiviral drugs exists. Therefore, the best way to limit the damage caused by viruses is through vaccination.

Vaccines - Vaccines can take credit for the near eradication of several deadly viruses, such as smallpox and polio, and are one of the most successful medical interventions ever produced, thanks to the advancements in life science research. Vaccination is the process of introducing a biological product (e.g., a viral protein molecule) into the body so that the immune system can safely induce a pathogen-specific response. This exploits the ability of our immune system to respond to, and remember, a large number of foreign structures. Thus, after vaccination, the immune system can more rapidly control potential re-infections with the same pathogen thanks to immunological memory.

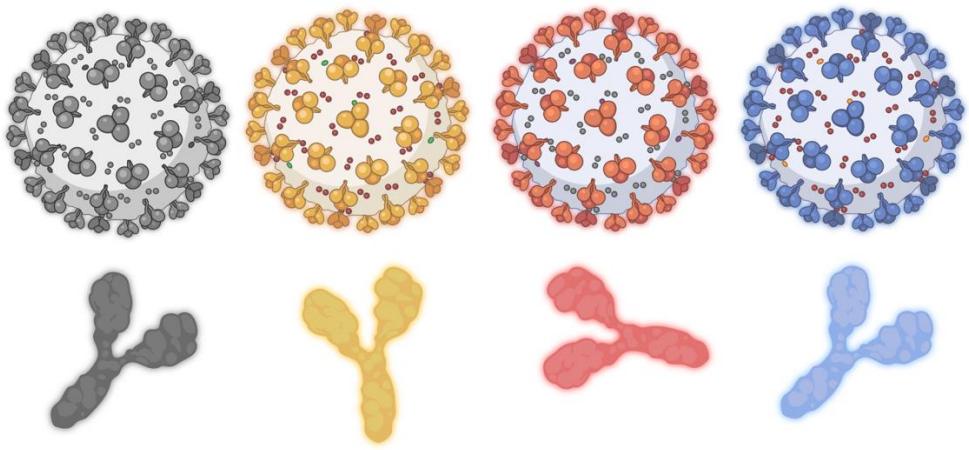


Antibodies - Protection against re-infection is largely manifested by secreted proteins called antibodies, produced by immune cells called B cells. Antibodies are highly specific to a given pathogen, and if they bind certain structures of the virus, they can block the ability of viruses to enter cells. Most of the licensed vaccines in use today provide protection through antibodies. Hence, measuring the quality of induced antibodies is one of the main ways to assess the efficiency of vaccine candidates. Some classes of vaccines also stimulate T cell responses, which cooperate with antibodies to protect against disease.

Challenges - Despite remarkable developments in vaccine technology, vaccines against many viruses do not yet exist, such as human immunodeficiency virus type 1 (HIV-1), a virus that is highly evolved to escape antibody recognition. As viruses mutate inside the

host, the greater the number of infections in the population, the greater the risk of mutation and selection of antibody escape variants. There are, however, certain features that viruses must keep constant to be able to function, which can be exploited during vaccine design. Modern molecular biology tools have allowed us to isolate individual antibodies produced by a single B cell from subjects who were previously infected or vaccinated – so-called **monoclonal antibodies** (mAbs). A highly efficient infection-blocking mAb, also termed a neutralizing antibody, can be administered as a drug to prevent, or control infection for a short period of time. For longer-lasting protection, antibodies need be naturally produced by our immune system after infection/vaccination. Detailed studies of neutralizing mAbs induced by vaccination or infection can provide valuable information for guiding vaccine design. The four papers included in this thesis are focussed on understanding the quality of antibodies that are induced by the surface structures of HIV-1 and SARS-CoV-2. In addition, my research has focused on the subtle differences in antibody genes between individuals and how this variation can influence a person's ability to respond to infection.

In **paper I**, we studied antibody responses to engineered HIV-1 surface envelope glycoproteins (Env) in rhesus macaques. Macaques were vaccinated with Env several times and blood samples were collected two weeks after each vaccination. Using these samples, I extracted many efficient Env-specific mAbs that had the capacity to block infection of a subset of HIV strains. Using next generation DNA sequencing technology, we were able to trace the trajectory of these mAbs, monitor their evolution, and better understand their distribution across different immunological compartments (e.g., blood, spleen, bone marrow, lymph nodes, intestine) over time. Our results showed that many relatives of these mAbs were present in blood, spleen, and lymph nodes, but not in the gut, and that many mAbs become more efficient after vaccine a boost. I detected very selective mAb variants in the bone marrow, as this is a specialized organ where B cells producing antibodies (plasma cells) can survive for very long periods of time. Studies of the binding specificity of these antibodies may help re-engineering the HIV-1 Env protein to elicit more broadly neutralizing antibodies.



In **paper II**, we studied antibody responses to the SARS-CoV-2 spike protein in humans who were previously infected with the virus. We obtained a set of highly effective spike-specific mAbs that were able to bind and block several SARS-CoV-2 strains. Just like the virus mutates, antibodies also possess the ability to mutate so the best binders can be selected – a process known as antibody affinity maturation. To study the role of affinity maturation in one antibody lineage of interest, I generated a 'naïve' version of this antibody by removing all the mutations generated by affinity maturation. I then added one mutation at a time or in combination on this backbone and tested their neutralizing activities. We identified key mutations that improved the ability of the antibody neutralize SARS-CoV-2 variants – from Alpha to Omicron, even though these variants did not exist at the time this antibody was elicited.

Antibody germline gene variation – Antibodies are made up of a combination of genes called variable (V), diverse (D) and joining (J) genes. Human DNA contains many V, D and J genes that come together to form a functional antibody. Through different V, D and J combinations, there can be up to 100 billion different antibodies in our body with different specificities. To add to this diversity, two people can have slightly different versions of the same V gene, which may alter antibody binding capacities. Do these subtle differences make the antibodies behave differently? In **papers III and IV,** I investigated the effect of this variation on SARS-CoV-2 spike-specific antibodies.

Some antibodies need to mutate relatively more to acquire virus neutralizing activity, while other antibodies are effective already in their initial naïve form. It can be useful to have this latter type of antibodies as they can be produced quicker and can help controlling the infection. For such antibodies, their naïve state is predominantly determined by the V gene they use.

In **paper III**, we demonstrate that some types of neutralizing antibodies can only be made by a specific V gene variant, which is only present in some people. Through cryo-electron microscopy, we could define the precise binding mode of such an antibody to the SARS-CoV-2 spike, explaining the molecular basis for the preferential V gene variant usage. Understanding the frequency of different antibody gene variants in the population is therefore an important line of work for the future. Finally, in **paper IV**, we studied a SARS-CoV-2 neutralizing antibody that uses a V gene that is entirely absent in many individuals. Contrary to the previous study, I could show that this V gene could be compensated by another very similar V gene, demonstrating a degree of redundancy between some V genes. Altogether, these results show that antibody V genes are highly variable among individuals and specific gene variants one has can influence the quality of antibodies you make to SARS-CoV-2 and likely to other viruses. Understanding this antibody gene variation is crucial as it may help explain why certain individuals respond better or worse to infection

or vaccination. Eventually, this could help identify persons who are at greater risk during a pandemic.

The results from the studies described in this thesis provide important insights into the characteristics of antiviral antibodies - how they develop, expand, disseminate, mature, and how their genetic features influence their functions. These results may help design vaccines that induce higher quality antibodies, and therefore more effective protection. Additionally, identification of potent antiviral antibodies such as those described in this thesis can aid the development of novel antiviral drugs.

ABSTRACT

The ability of antibodies to block infections makes them highly relevant for successful vaccine development. Through the papers described in this thesis, I attempt to characterize the functional and genetic aspects of antiviral antibodies induced by infection and vaccination.

In **Paper I**, we characterized the distribution and maturation of HIV-1 envelope glycoproteins (Env)-specific antibody lineages post-vaccination in different immune compartments of rhesus macaques. Vaccine-induced Env-specific antibody lineages were disseminated across the periphery, lymph node, spleen, and bone marrow (BM) but not in gut tissue. We observed a consistent increase in the somatic hypermutation (SHM) levels of Env-specific antibody sequences after each boost and the SHM levels strongly correlated with the affinity of members from a potent neutralizing antibody lineage.

In **Paper II,** we set out to understand the role of SHM in a broad, potent, public class of antibodies isolated from a healthcare worker who was previously infected with SARS-CoV-2. I selected a potent neutralizing antibody and reverted the heavy chain (HC) to the germline sequence. I then sequentially introduced individual or combinations of SHM so that we could test the functional impact of this. We found a substantial gain of antibody potency and breadth when certain SHM mutations were reintroduced, and we identified two key mutations that largely contributed to the breadth of this lineage. Furthermore, we showed that the mature antibody retained neutralizing activity against potential future viral variants by deep mutational scanning (DMS) experiments. A high-resolution structure of this antibody obtained by cryo-electron microscopy (cryo-EM) confirmed important interactions made by the identified SHMs with the SARS-CoV-2 spike (S).

In **Papers III and IV**, we investigated the effect of immunoglobulin heavy chain variable (IGHV) gene polymorphisms on the function of human SARS-CoV-2 antibodies isolated post-infection. We genotyped a cohort of previously infected healthcare workers and evaluated the neutralization activity of germline-reverted and allele-swapped S-specific IGHV1-69*20-using antibodies from two independent donors carrying this allele. Neutralization was retained when reverting the IGHV region to the germline IGHV1-69∗20 allele but lost when reverting to other IGHV1-69 alleles demonstrating a strong allele-dependence in these antibodies. A high resolution cryo-EM structure of one of the antibodies revealed significant contacts made by two IGHV1-69*20-germline encoded amino acid residues with the S, illustrating the impact of IGHV polymorphisms on antibody functions. We next focused on the IGHV3-30 group of genes, which are frequently used by S-specific antibodies. By IGHV genotype and haplotype analysis we observed that IGHV3-30-3 gene was deleted in many individuals, and the IGHV3-30 alleles were heterogeneously distributed in our cohort. When the IGHV region of an

IGHV3-30-3*01 neutralizing antibody was swapped with IGHV3-30 alleles, the neutralization remained unaffected demonstrating functional redundancy within this gene group, at least for this antibody lineage.

The results from my doctoral research provide insight into functional and genetic properties of antibodies induced by viral antigens, which have important clinical relevance both for guided-vaccine design and monoclonal antibody therapeutics, and for our general understanding of antibody responses in the population.

List of scientific publications

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  * These authors contributed equally

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CONTENTS

LIST OF ABBREVIATIONS

ACE-2	Angiotensin-converting enzyme 2
ADCC	Antibody-dependent cell-mediated cytotoxicity
AID	Activation-induced deaminase
AIDS	Acquired immunodeficiency syndrome
APC	Antigen-presenting cell
APRIL	Proliferation inducing ligand
ASC	Antibody-secreting cell
BCG	Bacille Calmette-Geurin
BCR	B cell receptor
BM	Bone marrow
bNAb	Broadly neutralizing antibody
C	Antibody constant region
CCR5	Chemokine receptor 5
CD4bs	CD4-binding site
CDRs	Complementarity determining regions
CXCL12	Chemokine ligand 12
DMS	Deep mutational scanning
ELISA	Enzyme-linked immuno sorbent assay
ELISpot	Enzyme-linked immuno spot
Env	HIV-1 envelope glycoproteins
ER	Endoplasmic reticulumn
FACS	Fluorescence-activated cell sorting
FcR	Fc receptor
FDC	Follicular dendritic cell
GC	Germinal center
HC	Heavy chain
HDX-MS	Hydrogen–deuterium exchange mass spectrometry
HEK	Human embryonic kidney

HIV-1	Human Immunodeficiency Virus type 1
HRP	Horse radish phosphate
Ig	Immunoglobulin
IgH	Immunoglobulin heavy
IGHV	Immunoglobulin heavy chain variable
IgK	Immunoglobulin kappa
IgL	Immunoglobulin lambda
IMGT	ImMunoGeneTics
LN	Lymph node
mAbs	Monoclonal antibodies
MERS	Middle east respiratory syndrome
MHC	Multiple histocompatibility complex
MPER	Membrane proximal external region
MZB	marginal zone B cells
NFL	Native flexibly linked
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
pHSCs	Pluripotent haematopoietic stem cells
PRR	Pattern recognition receptor
RAG	Recombination activating enzyme
RBD	Receptor binding domain
S	SARS-CoV-2 spike
SARS-CoV-2	Sever acute respiratory syndrome coronavirus-2
SHM	Somatic hypermutation
SPR	Surface plasma resonance
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
Tfh	T follicular helper
TMB	3,3',5,5'-Tetramethylbenzidine

TMPRSS2	Transmembrane serine protease
Treg	Regulatory T cell
V1/V2	Env variable loops 1 and 2
V3	Env variable loop 3
VDJ	Variable Diverse Joining genes
VH	Heavy chain variable region
VK	Kappa chain variable region
VL	Lambda chain variable region
VoCs	Variants of concern
WHO	World health organization

1 INTRODUCTION

Vaccines are a proven, cost-effective way to prevent infections and they continue to be a global health success story through the near eradication of diseases such as smallpox and polio. According to World Health Organization (WHO), millions of lives are being saved each year through vaccination (1,2). Due to the lack of antiviral treatments, rapid production of vaccines remains the most viable solution to existing and emerging viral outbreaks.

Vaccines are versatile in the way they exploit the immune system's extraordinary ability to establish strong, specific immune responses and memory against infections. In general, vaccines assume two roles - long-lasting protection in individuals and, if the vaccine coverage is high enough, stimulation of herd immunity in the population. Ever since Edward Jenner, vaccine development has taken an empirical approach, which has been fruitful for some pathogens. Increasingly, an immunologist-guided approach is used to design state-of-the-art vaccines to tackle antigenically complex pathogens such as HIV, Influenza, SARS-CoV-2. All viable vaccines in use, except for BCG, confer protection through the induction of antibodies, the only biological component of immune system that can block and prevent infection. The efficiency of a vaccine candidate is traditionally assessed by the quality of antibody responses it induces, in most cases at a polyclonal level (3). However, only few vaccines provide sterilising immunity. The other arm of the immune system, represented by T cells, is essential to clear infected cells, and control the infection. Hence, it is desirable to have vaccines that can induce both B and T cell responses. Antigen-specific antibodies have been shown to be maintained in the circulation for decades post-vaccination demonstrating longevity of antibody-producing cells (4). Waning of antibody titres over time may be compensated by the existence of long-lived memory cells that can provide a rapid recall response upon exposure to the same antigen again (5). The topic of durability and quality of vaccine-induced responses is not fully understood and remains a research area that is regularly revisited when testing candidate vaccines.

When designing and evaluating vaccine candidates to challenging pathogens, a parallel evaluation of induced antibody responses at a single cell level can be highly valuable and perhaps essential. Such an in-depth characterization of antigen-specific antibody responses requires cutting-edge technologies and a strong groundwork in immunogenetics. During my doctoral research, I studied the quality of the infection/vaccination-induced antibody responses against HIV-1 and SARS-CoV-2 viral proteins. We exploited a highly versatile antibody germline gene inference tool, IgDiscover, which provided a solid foundation to perform highly accurate antibody characterization (6). In this thesis, I address antibody responses to infections and vaccinations from two angles – functional dynamics of antigen-specific antibodies and the functional impact of antibody germline polymorphisms.

2 LITERATURE REVIEW

2.1 AN OVERVIEW OF THE IMMUNE SYSTEM

The immune system is primarily responsible for the protection of the host from harmful foreign agents including infectious pathogens such as viruses. Through an intricate array of mechanisms, our immune system has the extraordinary ability to discriminate self from non-self. There are two major arms of the immune system - innate and adaptive. The innate immune system is the first line of defence against invading agents and can act within hours or days of infection. Though the cells of innate immune system lack the ability to develop immune memory, they are highly robust and are specific enough to recognize self from non-self through the expression of common receptors namely pattern recognition receptors (PRRs). Phagocytosis and the process of antigen presentation by the innate immune cells are crucial for pathogen clearance and the activation of adaptive immunity (7,8). Some of the classic physicochemical innate immune barriers include the skin, mucus layer, soluble proteins, small molecules, epithelial linings of respiratory, gastrointestinal, and urinary tracts.

Adaptive immunity is acquired post-birth, during the lifetime and unlike the innate immune responses, it manifests exquisite ability to specifically recognize and remember pathogens. B cells and T cells are two major cell types of the adaptive immune system that can specifically recognize a wide range of antigens through their highly diverse surface receptors – B and T cell receptors (BCRs and TCRs). The antigen-specific B and T cells can differentiate into memory cells capable of providing protection from subsequent exposures.

2.1.1 T cells

T cells originate from the common lymphoid progenitors in the BM and develop in the thymus. Depending on their TCRs, T cells commit to αβ or a γδ lineage. αβ T cells acquire either a $CD8^+$ or $CD4^+$ phenotype and circulate, while γδ T cells mostly become tissue-resident. Through their unique TCRs, T cells recognize processed antigenic peptides that are presented on receptors known as major histocompatibility complex (MHC) class I or II. MHC class I is expressed on all cells, except red blood cells, while MHC class II is selectively expressed by the antigen-presenting cells (APCs).

$CD8^+$ T cells are cytotoxic and can specifically recognize pathogen-ridden cells or cancer cells and kill them through MHC-I-mediated antigen presentation. $CD4^+$ T cells are MHC-II restricted and provide helper functions that support both $CD8^+$ T cells and B cells through the production of cytokines and other factors. T cells are therefore critical for the control and clearance of infections (9). CD8+ T cells can different into long-lived effector memory T cells and their reactivation triggers robust cytotoxic responses (10,11). Hence

both $CD4^+$ and CD8+ T cell responses are crucial parts of vaccine-induced immunity (12–14). Regulatory T cells (Tregs) are a subset of CD4+ T cells that regulate T cell activation. T follicular helper cells (Tfh) localize in the B cell rich zones of secondary lymphoid organs and facilitate B cell affinity maturation (15). In this thesis, I make the occasional reference to T cells, but I have not included an in-depth discussion of their important role in responding to and controlling infections since this was not the topic of my investigations. Instead, I focus on B cell and antibody immunology, which were my main areas of interest.

2.2 DYNAMICS OF ANTIGEN-SPECIFIC B CELL RESPONSES

2.2.1 B cells

B cells and their antibodies make up the humoral immune responses and remain indispensable for our ability to limit re-infection by previously encountered pathogen. B cells recognize and neutralize pathogens through the production of antibodies – the secreted form of the BCRs. Due to the ability of neutralizing antibodies to bind and block infection, they are the most common correlates of protection and therefore are highly relevant in the context of vaccine development (3,16).

The development of B cells begins in the fetal liver and progresses within the BM in a stepwise manner. The pluripotent hematopoietic stem cells (pHSCs) of the BM first become committed to the B cell lineage through the expression of lineage-specific markers and develop a functional BCR through a series of immunoglobulin (Ig) heavy and light chain gene recombination processes (17)(18). The immature B cells, each with a unique recombined BCR, undergo intense negative selection by self-antigens, a highly regulated process known as central tolerance. During this process, BCRs that bind and recognize self-antigens are identified and these self-reactive B cell clones undergo clonal deletion or become anergic. The immature B cells that manage to cross this checkpoint are the ones that carry BCRs specific for a large array of foreign antigens. They eventually migrate to the secondary lymphoid organs such as spleen, lymph node and some to the gut to become mature B cells. During this maturation process, remaining self-reactive B cell clones are discarded and mature naïve B cells start to circulate within the periphery to encounter a myriad of non-self-antigens (19).

2.2.2 B cell activation

The activation of mature naïve B cells is triggered by their interaction with a cognate antigen. The activation of B cells can be T-dependent or T-independent based on the type of antigen they encounter. The former is usually prompted by protein antigens while the latter is triggered by polysaccharides or highly repetitive structures (20,21). Antiviral antibody responses tend be T-dependent. The three major subsets, as defined in mouse studies, of mature naïve B cells are follicular B cells, marginal zone B cells (MZB), B-1 cells.

The MZBs and B-1 cells are located at the marginal sinus of the spleen and at the mucosal sites respectively. Due to the nature of their location, they mainly encounter T-independent antigens and provide immune surveillance at the tissues susceptible to environmental pathogens without T cell help (22). In humans and other primates, the definition of these B cell subsets is less clear even though counterparts to these cells are likely present in these species also.

Within the follicular regions of the secondary lymphoid organs such as the lymph nodes are the T and B cell zones. Naive B cells are present at the centre of these follicles and are bordered by the T cells. Viral antigens are generally presented on the follicular dendritic cells (FDCs) and provide activation signals to T and B cells within the lymph nodes (23). Recent advanced microscopic studies show that the B and T cells start to move and interact in the inter-follicular region already one day after immunization. Antigen-activated B cells upregulate CCR7, migrate to the T cell zones and form long-standing interactions with the CD4+ T helper cells to become fully activated. B cells also take up the antigen, process and present the peptides to T cells thereby acting as antigen-presenting cells (24)(15).

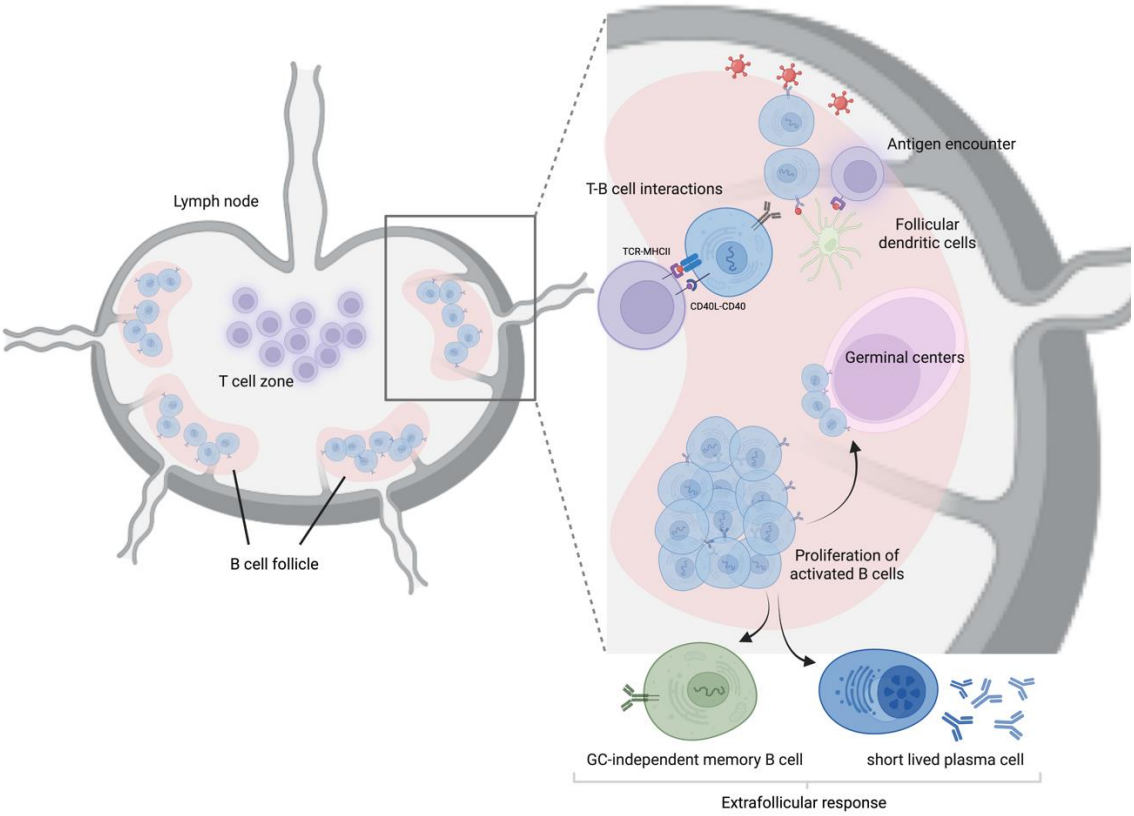

Figure 1. B cell activation. *B and T cell zones exist in the secondary lymphoid organs. B cells get activated by cognate antigens and receive signals from T cells at the B-T cell border. Activated B cells take one of three paths –they differentiate to short-lived plasma cells or become GC-independent memory B cells, or they enter GCs where they undergo affinity maturation and differentiation.*

Depending on the co-stimulatory signals, antigen availability and the BCR affinity levels, the activated B cells take one either of these three paths. Some differentiate to short-lived

antibody-producing plasmablasts or enter GCs to undergo affinity maturation. Some activated B cells are also shown to become memory B cells that are GC-independent (25,26). A subset of activated B cells enter GCs to undergo a cascade of events to establish a solid, long-standing humoral immune response (**Figure 1**).

2.2.3 Germinal centres

GCs are dynamic, transient structures that are formed typically within the secondary lymphoid organs, where they prevail from days to months until antigen clearance. Their functional significance is evident by the immunodeficiency syndromes caused in people that have defective GCs (27). They provide a unique microenvironment that supports the interaction of various cell types. Initial GCs can be formed within 4-8 days of antigen exposure with a few hundred initial B cell clones as a seeding (28). More B cell lineages can enter established GCs during later stages (29,30). GC structures are spatially divided into the dark and the light zones based on their cell surface phenotypes (31). The dark zone of the GCs is the site of B cell proliferation, where the Ig variable regions of the activated GC B cells are edited and further diversified. This process of BCR alteration is termed as somatic hypermutation (SHM) and is centrally mediated by the expression of the enzyme activation-induced deaminase (AID) (32,33).

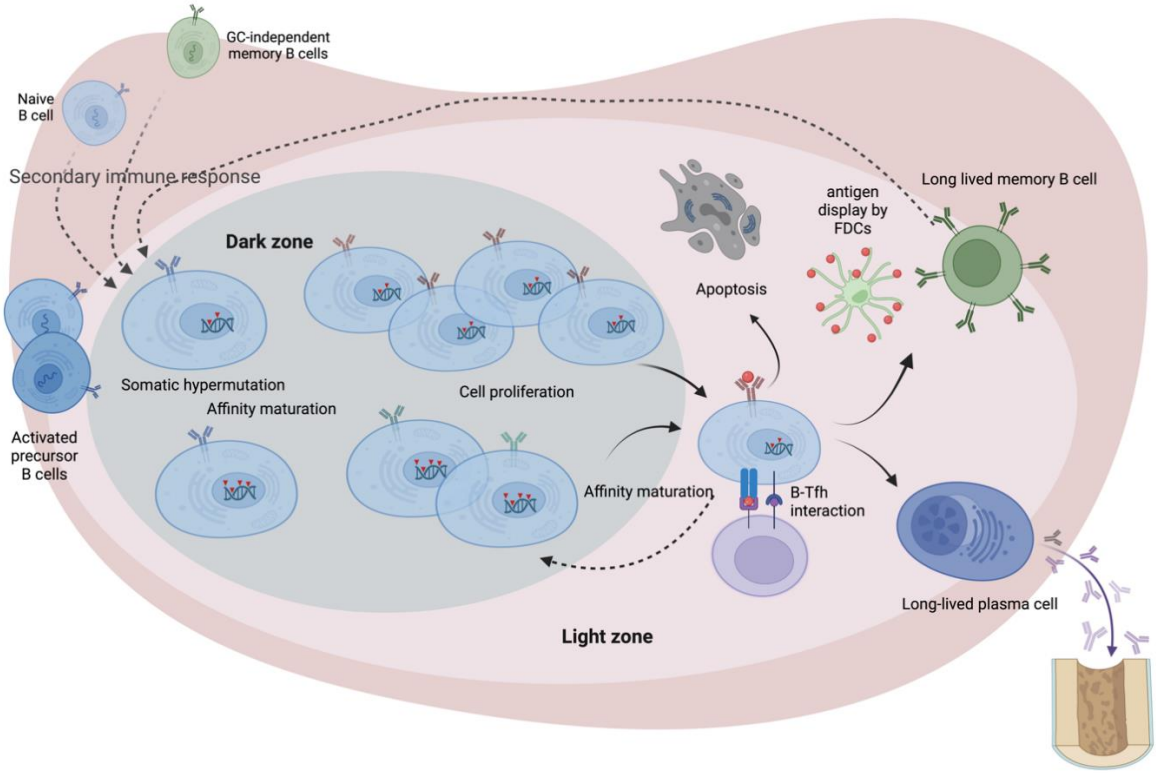

Figure 2. *Germinal center reactions. Activated B cells enter the dark zone of the GC to undergo SHM and cell proliferation. B cells with modified BCRs enter the light zone to get selected based on their affinities to antigen displayed by FDCs. B cells also receive soluble stimuli from Tfh cells in the light zone. B cells with disadvantageous SHMs undergo apoptosis. Affinity matured B cells differentiate into memory B cells or plasma cells.*

B cells clones with modified BCRs are cycled through the light zone where they competitively bind to the antigens displayed by the surface receptors of FDCs, resulting in selection of the best binders (34,35). The Tfh cells in the light zone provide soluble and membrane bound stimulus to the B cells (33). The presence of a small population of CD8+ T cells is also observed in the GC light zones ensures the killing of virus-infected cells and prevent B cell lymphomas (36,37). The B cell clones with an increased affinity to the antigen post-SHM get positively selected while the ones with disadvantageous mutations undergo apoptosis (38). The selected B cell clones either re-enter the dark zone to undergo further affinity maturation or they exit GCs by differentiating to plasma cell and memory B cells. Single cell sequencing analyses identifies that 2-3% of GC B cells have distinct plasma cells and memory B cells phenotypes (39). The plasma cells and memory B cells derived from the antigen-driven GC events are generally long-lived and form the two major pillars of immunological memory. GC reactions are summarised in **Figure 2**.

2.2.4 Plasma cells

The fate of B cells that exit GCs is at least partially based on their antigen-affinity. B cells with relatively higher affinity are selected to differentiate to antibody secreting plasma cells (40,41). Terminally differentiated plasma cells from GCs are generally long-lived and can sustain antibody production for long periods of time (42). A subset of plasma cells migrate to BM by their expression of surface receptors and secreted chemokines, where they receive special survival signals. The survival period of antigen-specific plasma cells is highly debated. Independent observations indicate that their survival can range from months to years to decades or even a life time (43–45). The presence of long-lived plasma cells possibly contribute to the vaccine-specific serum antibody titres maintained for up to 60 years post-vaccination (4,45). It has been suggested that the niches within the BM are limiting, possibly explaining why not all plasma cells survive long-term. The BM plasma cell survival niche is characterized by the expression of chemokine ligand 12 (CXCL12), VCAM1, stromal cells and proliferation inducing ligand (APRIL) that support the plasma cell survival (46). The massive antibody production that characterizes plasma cells causes endoplasmic reticulum (ER) stress; thus, the unfolded protein response is important to prevent cell death of plasma cells (47). The detailed mechanisms underpinning the recruitment, migration, and maintenance of plasma cells in the BM remains to be unravelled.

2.2.5 Memory B cells

Single pathogen encounter provides a long-lasting protection against subsequent exposures in most cases. Memory B cells majorly contribute to this phenomenon by providing excellent immune surveillance and mounting enhanced immune responses upon pathogen re-exposure. Activated B cells can give rise to memory B cells in a GC-dependent and GC-

independent manner as discussed above. During a secondary immune response, the antigen-specific memory B cells rapidly differentiate into antibody-producing plasma cells or re-enter GCs to gain affinity. Antigen accessibility and the strength of BCR signalling determine the path that activated B cell precursors will take. High affinity B cells under antigen-rich circumstances rapidly differentiate into antibody-secreting plasmablasts. Such an antigen controlled early B cell differentiation is perhaps useful during an active viral replication. A recent study claimed that the majority of activated low-affinity B cells become non-GC early memory B cells and that low antigen availability greatly favours this process. Early memory B cells are detected as early as 2.5 days after infection, typically have no SHM and are mostly unswitched (48).

GC-dependent memory B cells are formed within 5-6 days of infection and can be formed continually within GCs for up to 6 months or more. It is becoming increasingly clear that low-affinity B cells are preferentially selected to enter GCs to affinity mature (49,50). The observation that complex antigens induce more GCs (51) and that this allows consistent diversification of memory B cells, resulting in a considerable gain in affinity and breadth is essential to protect against constantly mutating viruses such as SARS-CoV-2. The generation of antigen-specific memory B cells of remarkable breadth against viral variants after just a single exposure to the ancestral virus demonstrates the versatility of the GC processes (52–55). The majority of the GC memory B cells are long-lived and circulate within the periphery and secondary lymphoid organs. The long-lived memory B cell pools are highly stable and significantly contribute to the plasmablast generation during secondary responses (56,57). Recent studies have discovered a tissue-resident memory B cell population within infection prone areas such as the lung and mucosa that is phenotypically different from the circulating memory B cells (58).

Vaccination often requires one or more boosts to achieve high quality, durable responses. The re-entry of memory B cells into GCs and the engagement of naïve B cells upon vaccine-boost is largely debated (59,60). It is generally believed that memory B cells do re-enter GCs upon reactivation depending on factors such as antigen affinity, vaccine boost locations (61,62). A recent study claimed that the re-entry of memory B cells post-boost is rare and that more than 90% of the B cells that enter GCs during the secondary response are naïve (63). These data illustrate the knowledge gaps that exists in our understanding of memory B cells. Overall, it is likely that a good balance of naïve activation and memory B cell reactivation will result in a highly diverse, updated BCRs.

2.3 ANTIBODY GENETICS

2.3.1 Immunoglobulin structure

Thanks to the advancements in crystallographic and electron microscopy-based studies of protein structures, thousands of antibody structures have been solved and deposited,

constantly informing us of the fine details of different antibody conformations (64–66). Antibodies are Ig heterodimeric protein molecules composed of two copies each of a heavy chain (HC) (55 kDa) and a light chain (24 kDa). Each of the light chain pairs with a HCs and the two HCs pair with each other through disulphide bonds to fold into a 'Y' shaped protein.

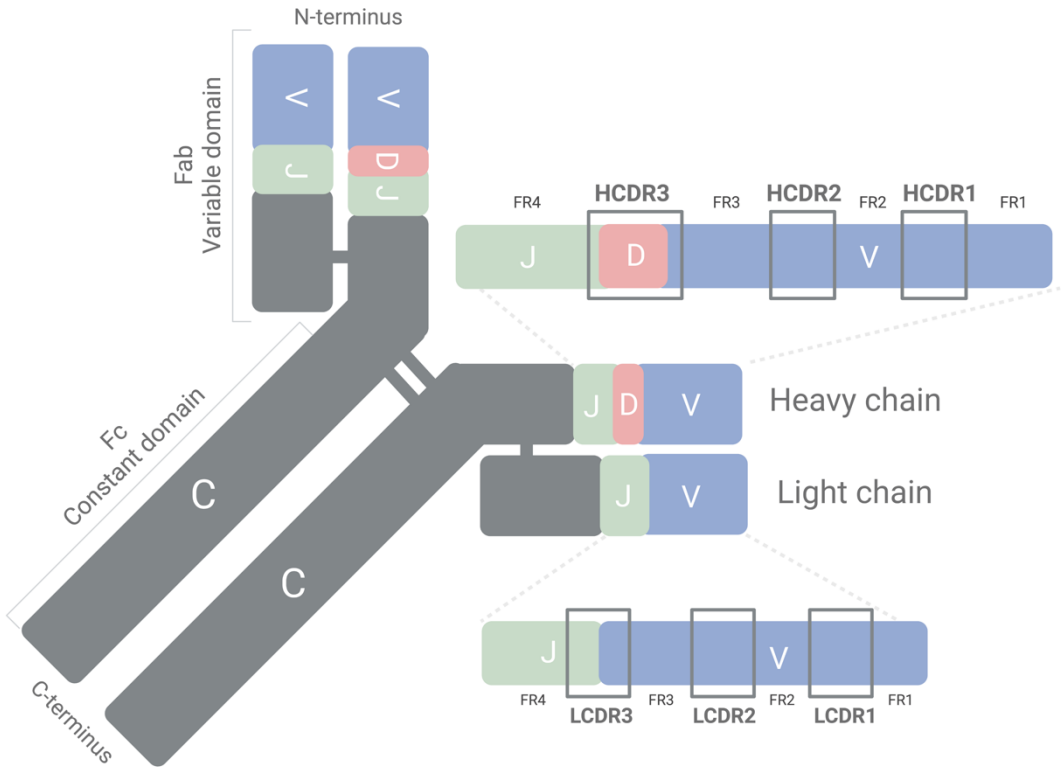

Figure 3. Immunoglobulin structure. *Igs are heterodimers linked by disulphide bonds consisting of antigen-binding Fab domains and an effector Fc domain. The variable region of the antibody HC is encoded by rearranged V, D, J genes, while the variable region of the antibody light chain consists of the V and J genes. Both the heavy and light chain regions encompass CDRs and FRs.*

thereby creating the structural domains of an antibody - two antigen binding variable domains called Fabs and one constant Fc domain (**Figure 3**). Like most Ig family proteins, antibodies are composed of beta pleated sheets connected by disulphide bonds. Hence, the two Fab domains are connected to each other and to the constant domain by a flexible hinge region made of disulphide bonds (67). The variable regions of an antibody are present in the Fab domain and contain short protruding loops that form the main antigen-binding regions of antibodies called the complementarity determining regions (CDRs). The heavy and light chain variable regions have three CDRs each (68). The variable domain also contains framework (FR) regions interspersed between the CDRs and may or may not interact with antigens.

2.3.2 The human immunoglobulin heavy chain loci

The Fab domain is the most studied when it comes to the antigen-binding properties of antibodies. Generally, HC plays a greater role in antigen binding due to its slightly more sophisticated gene organization compared to light chains (LC) (69) (70–74). In my doctoral research, I have studied the genetics, distribution, and maturation of antiviral antibodies with a focus on HCs.

The Ig loci are regions on the human chromosome that contain the genetic components to make up a functional BCR. The human Ig HC (IgH) locus contains genes that encode for HCs and are present on the chromosome 14 (14q.32.33). The human LC Ig kappa (IgK) and lambda (IgL) loci contain genes that encode the kappa and lambda LCs and are present on the chromosome 2 (2p11.2) and 22 (22q11.2) respectively (75–77). The IgH locus comprises of four different gene segments - variable (V), diverse (D), joining (J) and constant (C), while the antibody IgK, IgL consists of three gene segments, V, J and C. The rearranged V(D)J regions of an antibody heavy and light chains are often collectively referred to as the variable region.

The first full-length physical mapping of the IgH locus generated by Matsuda *et al.*, describes a 975 kilobase (kb) long stretch of DNA at the end of chromosome 14 consisting of highly repetitive, interspersed, closely resembling, functional and non-functional V, D and J gene segments. Subsequently, IgK and IgL loci were also mapped revealing convoluted organization of V and J genes (78). Since then, the number of studies that have attempted to map the IgH locus is limited but this has recently become a more active field of research (79–83). The IgH locus is characterized by extensive structural variations, segmental deletions and duplications that have occurred through evolutionary times, possibly making it one of the most polymorphic regions of the human genome. Studies of diverse population groups to comprehensively characterize the loci and fully realize the extent of inter-individual variation of germline V genes is much needed. The ImMunoGeneTics (IMGT) database is a widely used reference database for immunogenetic information to define the genetic properties of antibody sequences. Currently, IMGT reports the presence of 52 IGHV genes, 23 IGHD genes and 6 IGHJ genes that are functional in humans (84). Although constantly being updated, the IMGT database is not well validated and is known to contain erroneous sequences (85). In addition, it is only derived from limited number of individuals thus poorly representing global allelic diversity (84,85). Continuous developments in sequencing approaches and computational methods to infer novel alleles (6,86) have increased the number of individuals from whom antibody gene variants have been identified, thereby revealing an astounding level of allelic and copy number variation, which will be addressed in the later chapters (6,80,81,87,88).

2.3.3 VDJ recombination

Antibody repertoire diversity results from the random rearrangement of V, D and J genes in different combinations. This process is initiated during the developmental stages of a B cell in the BM by the expression of recombination activating enzymes (RAG 1 and 2). It is a highly regulated, site specific, stepwise process, where the IgH recombination precedes the IgK/IgL recombination. During the IgH rearrangement, the D and J genes assemble first, followed by recombination of the V gene with the recombined DJ segment. The stepwise recombination is regulated by the recombination signal sequences (RSS). The RSS are composed of palindromic heptamers and AT rich nonamers separated by either 12 or 23 base pair (bp) spacer sequences corresponding to one and two turns of a DNA helix. The D genes are flanked on both sides by 23 bp RSS while the V and J genes contain a 12 bp RSS on their 3' and 5' ends respectively. This controls the recombination process by allowing it to occur only when two sequences are flanked by a 12 and 23 RSS - the 12/23 rule (89). The V and the J segments contain a 23 RSS flanking them thus preventing their pairing before the DJ assembly at the IgH locus.

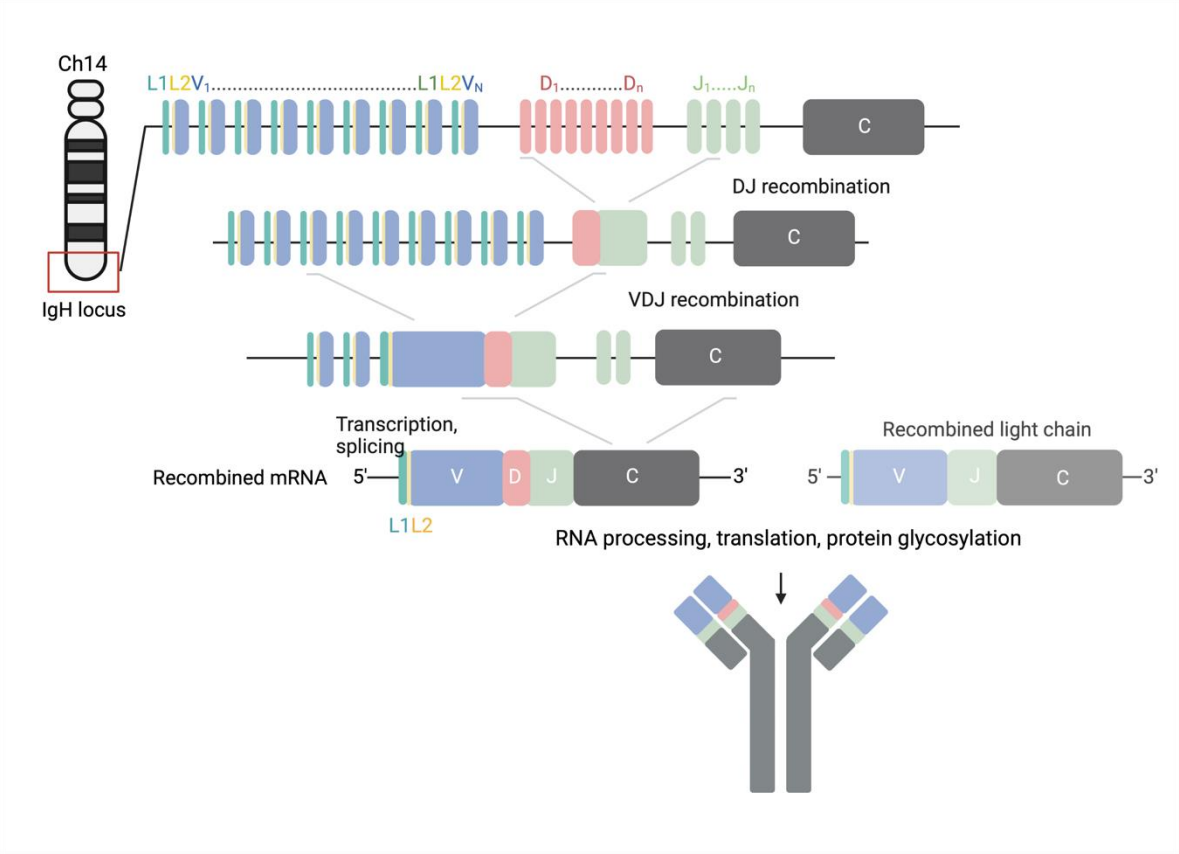

Figure 4. VDJ recombination. *The V, D and J genes of the IgH locus are present at the end of the Ch14. VDJ recombination takes place in a stepwise manner. The D and the J genes recombine first. The V gene joins the recombined DJ segments generating a post-transcriptional spliced mRNA. Similar process happens at the IgK/IgL locus generating recombined LC mRNA. The HC and LC are then co-expressed as antibody protein molecules.*

The recombined V(D)J segment is then spliced to recombine with the downstream constant gene, which is typically an IgM-domain in developing B cells (**Figure 4**)(67).

The above process is highly regulated and controlled by enzymes, which effectively catalyse the reactions causing DNA break and repair. During V(D)J recombination, the ends of the adjoining gene segments can vary in length due the endonuclease digestion. Furthermore, the introduction of random nucleotides by terminal deoxynucleotidyl transferase (tdT) at the VD and DJ junctions of HCs and the VJ junctions of the LCs results in CDR3 regions that are of different lengths, highly diverse and unique to a given rearrangement (**Figure 5**). One deep sequencing study identified 3 to 9 million unique HCDR3s in circulation in a single individual (90). Antibodies with long HCDR3s can have an advantage in terms targeting shielded epitopes as illustrated by several neutralizing antibodies against HIV (91–93). CDR3s substantially add to the diversity of antibody repertoires, and they are of special interest in immunological evaluation of antibodies since they help determine the clonal relationships between sequences.

B cells, like all somatic nucleated cells, are diploid and contain both a maternal and a paternal chromosome. V(D)J rearrangement occurs locally on a given chromosome and hence successful rearrangement on both chromosomes would result in two productive BCRs rendering the given B cell dual specific. The theory of allelic exclusion was proposed as a means to explain the one BCR-per-B cell rule and several models demonstrate this (94–96). Studies using transgenic mice models show that the expression of IgM on an immature B cell inhibits the further V(D)J recombination (97,98).

Following these intricate somatic recombination events, productively rearranged HC and LC are co-expressed as heterodimers that are either presented on the cell surface as BCRs or as secreted in the form of soluble antibodies.

2.3.4 Somatic Hypermutation

Upon antigen-encounter, a mature naïve B cell becomes activated and a subset of them enter the GCs in the secondary lymphoid organs. As mentioned in the previous section, among the series of events in the GCs, is the antibody affinity maturation process during when the rearranged BCR of the activated B cell undergoes random mutations throughout the HC and LC variable regions to diversify the antigen-specific repertoire. The process of SHM is catalysed by the enzyme called activation-induced deaminase (AID). While most SHMs are nucleotide substitutions, sometimes deletions and insertions are observed in antibodies isolated from chronic HIV patients (99). SHM occurs throughout the V(D)J coding regions but are mostly avoided at site comprising regulatory genetic elements and the constant region. Gene transcription levels, selective targeting of AID, high-fidelity repair of AID-generated uracils are some of the ways through which B cell genome is protected from acquiring SHMs (100–102).

2.3.5 Antibody diversification

Combinatorial diversity – The major part of the naïve B cell repertoire diversity is generated by V(D)J recombination and subsequent HC-LC pairing - collectively called as combinatorial diversity, which can generate up to 1.9 x 10^7 different BCRs (103). It can still happen that two naïve BCRs have the same heavy VDJ and light VJ genes. However, the additional junctional diversity generated during the V(D)J rearrangement makes BCRs with the same gene usage distinct from each other (104,105).

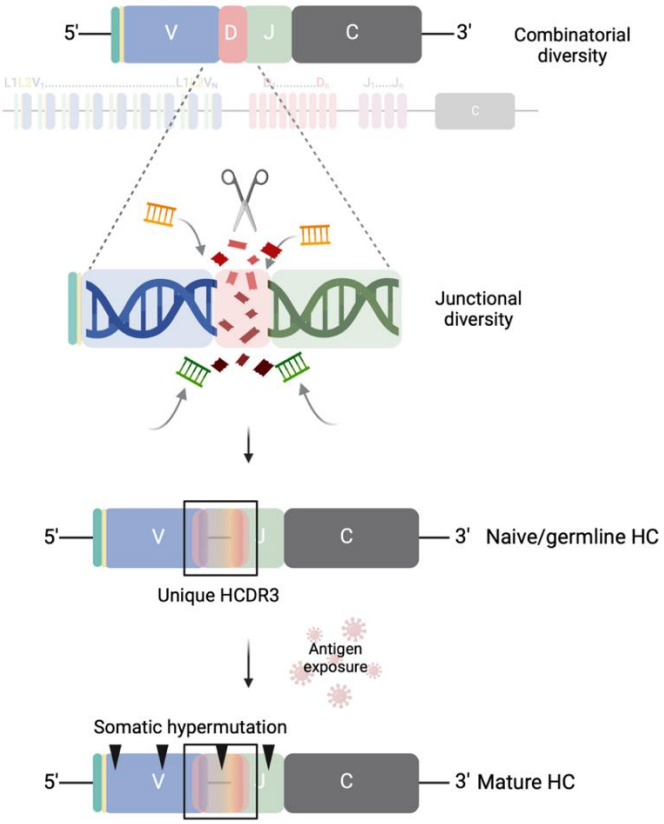

Figure 5. Antibody diversification. *The uniqueness of a BCR is the result of combinatorial and junctional diversity. Combinatorial diversity denotes the VDJ recombination machinery. Junctional diversity is the nucleotide insertions, deletions and substitutions that happen at the V-D, D-J junctions during the recombination process.*

Junctional diversity - During the joining of D-J and the V-DJ gene segments, there is a substantial amount of nibbling by exonucleases at the V-D-J junctions. P addition process, mediated by Artemis DNA-PK complex, is the generation of stretches of complimentary nucleotides, which is then followed by the TdT mediated random addition of nucleotides as extensions to the ends of the single strand. Followed by these two nucleotide addition processes, the unpairing bases are removed and the existing gaps are filled up by ligation and DNA synthesis to make up a coding joint. The generation of junctional diversity therefore is highly random and makes up the hypervariable CDR3 regions of the HC and LCs. In addition to these, rare events such as receptor editing and V gene replacement can contribute to further diversity of the naïve B cell repertoire **(Figure 5**) (106).

The above processes in the end theoretically make up for up to 10^{15} unique BCRs, which are further modified post-antigen exposure by SHM. As discussed earlier, antibody germline gene variation will considerably add to the diversity of the naïve B cell repertoire between subjects.

2.3.6 Antibody isotypes and their effector functions

There are five different antibody isotypes dictated by the type of the constant domain of the antibody HCs - IgM, IgD, IgG, IgA, IgE (67). The different isotypes are formed through class switch recombination (CSR) depending on the environment they are in. IgM and IgD are the isotypes often found to be secreted by naïve and recently activated B cells. Pentameric IgM increases avidity to antigen due to the availability of more binding sites (107,108). IgA exists in a monomeric and a dimeric form, where the dimeric form, secretory IgA (sIgA) is found at the mucosal surfaces (109). Around 75% of the antibodies present in the circulation are IgG and most of these are affinity-matured antibodies (110).

The Fc domain of antibodies perform a range of effector functions critical for a wholesome immune response. Antibody-bound antigens can be marked for phagocytosis initiated by the interaction between the bound antibody Fc region and Fc receptors (FcR) present on macrophages, NK cells and neutrophils. Fc receptor engagement results in the phagocytosis of the antigen-antibody complex followed by subsequent intracellular oxidative damage, enzymatic digestion and pathogen killing. This process is called antibody opsonization and one of the main antibody effector functions.

Antibodies Fc regions are also excellent at complement activation. IgMs and most IgGs can bind to complement glycoprotein molecules and activate them, triggering a series of effector reactions that lead to pathogen killing and removal (107).

Furthermore, antibodies can mediate cytotoxicity through Fc receptors. This process of antibody-dependent cell-mediated cytotoxicity (ADCC) occurs through the recognition of pathogen-bound antibody Fc region by Fc receptor-expressing cytotoxic cells such as natural killer (NK) cells (111).

Presence of neutralizing antibodies at mucosal surfaces is critical for protection from infections and this requires the translocation of antibodies through mucosa. Acquisition of IgG mediated passive immunization by the fetus from the mother also requires the placental translocation of antibody molecules. IgA and IgG Fc regions mediate efficient translocation of antibodies in these contexts (112).

2.4 VACCINES

For many decades, vaccination against viruses has involved the introduction of harmless inactivated or attenuated forms of whole viruses to mount safe immune responses that can

protect against future infections. Nowadays, vaccine design increasingly relies on molecular biology-based approaches where a single target antigen, usually the surface glycoprotein of viruses, can be selected and modified as necessary to optimize the stimulation of neutralizing antibodies. During the COVID-19 pandemic, several new vaccine platforms have been approved for clinical use, many of which were tested pre-clinically in the context of other viral infections prior to the pandemic.

2.4.1 Viral antigens as vaccine candidates

Viral surface proteins, often referred to as 'spikes' are fusion proteins that attach to the host cell surface and initiate entry. The viral spikes therefore contain a receptor binding region that accommodates epitopes binding to the target receptors of the host cell. Around these receptor binding regions of the spike surfaces are the epitopes that targeted by neutralizing antibodies. Moreover, constantly mutating viruses must keep a substantial part of the receptor binding region conserved in order to retain their ability to attach to and enter the host cell (113,114). To induce antibodies of high potency and breadth, it is critical to present these conserved neutralizing epitopes to the immune system. Most spike-based vaccines rely on this principle.

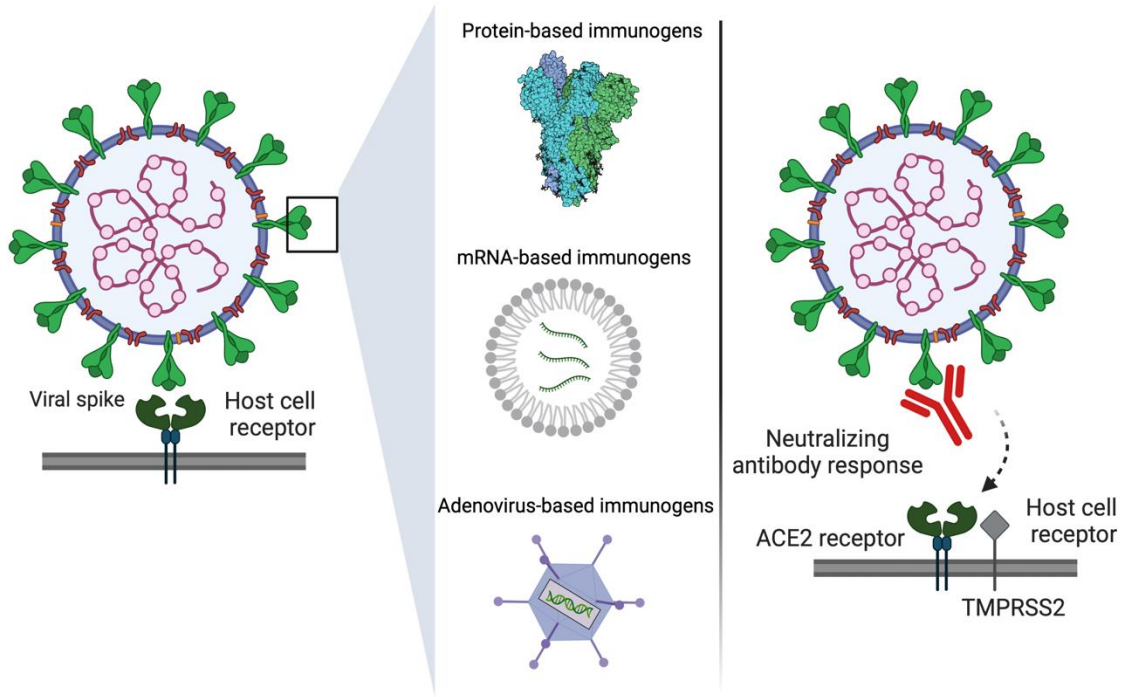

Figure 6. Viral antigens as vaccine candidates. *Viral fusion proteins are arranged on the surface of the virus. These surface proteins can be delivered as vaccines in several forms, including as recombinant protein, in lipid-coated mRNA, or with adenovirus-based delivery. The spike-based vaccines induce neutralizing antibodies that can bind to the viral surface spikes and block infection.*

The two major conformations of viral spike proteins are pre-fusion and post-fusion. The pre-fusion conformation is the native state of spike on the infectious virus that exposes relevant neutralizing epitopes and therefore, the desired form to include in a vaccine. To

illustrate this, studies of antibodies against the respiratory syncytial virus (RSV) F protein have demonstrated that most of the neutralizing antibodies target the pre-fusion conformation (115). The inherent metastability of the native pre-fusion spike means that unless stabilized, spike glycoproteins tend to acquire the post-fusion conformation. Through the introduction of trimerization domains and mutations that prevent this conformational change, successful stabilization has now been achieved for several immunogens (116–123). For SARS-CoV-2, most but not all of the licensed vaccines are based on stabilized pre-fusion spikes that are administered in the form of recombinant adenovirus vectors, mRNA or protein (**Figure 6**) (124–129). Structural biology and protein engineering were fundamental research fields for this advancement.

2.4.2 Human Immunodeficiency virus type I (HIV-1)

HIV-1 is the causative agent of AIDS and causes approximately 600,000 deaths per year (130). HIV-1 belongs to the family of *Retroviridae* and contains two positive sense RNA strands encoding the *gag, pol* and *env* proteins as well as a several accessory elements. The genetic material of HIV-1 is enclosed in a cone shaped capsid contained within an assembled icosahedral viral symmetry. The *gag* gene encodes for the viral matrix, capsid, and nucleocapsid proteins while the *pol* gene encodes viral enzymes: protease, reverse transcriptase (RT) and integrase. The *env* gene encodes the envelope glycoprotein (Env) (131,132). The primary target cells are CD4+ T cells but antigen presenting cells such as marcophages and DCs can also be infected. Env is the only virally encoded protein exposed on the surface of the virus and is responsible for binding to the primary CD4 receptor and a co-receptor, usually chemokine receptor type-5 (CCR5), mediating viral entry (113,133). Upon infecting host cells, the 9.2kb RNA is reverse transcribed by the error-prone viral RT enzyme, which lacks proof-reading capacity. This introduces random mutations in the viral genome, resulting in the generation of a swarm of diverse viral variants within each host. HIV-1 pathogenesis is discussed in length in this review (134).

2.4.3 Structure of envelope glycoproteins (Env)

For decades now, HIV-1 has been a central research topic in vaccine development. While successful anti-retroviral drugs are available today (135), we still lack a prophylactic vaccine. Antibody-mediated vaccines are entirely based on the HIV-1 surface protein Env as it accommodates several neutralizing epitopes and is the sole target of the neutralizing antibodies (113). However, owing to their swift evolution, high genetic variability, and structural complexity, eliciting broadly neutralizing responses against Env is an unprecedented challenge and an effective vaccine is still elusive. In paper I and in several collaboration projects during my doctoral research, I worked on understanding the B cell responses to engineered Env-based protein vaccines.

Env is a heterodimer consisting of two main subunits – gp120 and gp41. They are trimer molecules with three gp120 subunits covalently linked to the three gp41 molecules. Env is highly variable with up to 30% difference between strains. Particularly, the gp120 subunit is heavily glycosylated and camouflaged by hypervariable regions (**Figure 7**).

Most antibodies induced against the virus are strain-specific due to the amino acid deletions/insertions/substitutions and glycan shifts on Env. Highly conserved Env epitopes exist and are targets of antibodies that neutralize a broad array of HIV-1 variants. While such cross-reactive antibodies, called broadly neutralizing antibodies (bNAbs), develop in some HIV-1-infected patients years after infection, often after extensive affinity maturation, they are induced too late to be effective (136,137). For bNAbs to productively block infection, they must be induced prior to infection, which is the goal of a prophylactic vaccine. So far, this has only been achieved in engineered mouse models (138) and in rare rabbit (139) or macaque (140) studies. Nevertheless, isolation and characterization of bNAbs from chronically infected persons have provided valuable insights into the conserved, relevant neutralizing epitopes to target by vaccination and some of the best bNAbs are under development as therapeutics as reviewed in these references (141,142).

Like many viral surface glycoproteins, the pre-fusion state of Env is highly metastable and processing into the gp120 and gp41 subunits requires cleavage by furin during biosynthesis (143). Several approaches have been taken to engineer soluble Env immunogens to mimic the native prefusion form, such as the SOSIP trimers and the native flexibly linked (NFL) Env trimers (121–123). In my PhD studies, I used the NFL Env trimers designed in the Wyatt laboratory at the Scripps Research Institute as the immunogen. These trimers are cleavage independent and stabilized by the introduction of several mutations to maintain the protein in its native conformation (122,123). So far, some vaccine studies with either the SOSIP or NFL trimers were shown to induce potent neutralizing antibodies; however, these antibodies were largely strain-specific (144–146). It is possible that longer immunization regimens are needed, and/or sequential administration with different Env immunogens to drive responses against conserved determinants on Env more effectively.

2.4.4 Env-specific antibody responses

When evaluating neutralizing antibody responses against HIV-1 Env, it is critical to distinguish between Tier-1 and Tier-2 viruses. The former have easy-to-neutralize open conformations and often arise after culturing the virus *in vitro*, so called lab-adapted Envs (147). The latter are more representative of real-world circulating viruses, which tend to be neutralization resistant.

bNAbs - Most isolated bNAbs that neutralize a large palette of HIV-1 strains target one of the following epitopes - CD4 binding site (CD4bs), variable loops 1 and 2 (V1/V2), variable loop 3 (V3) glycan, membrane proximal external region (MPER), gp120-gp41

interface or the fusion peptide. Structural resolution of antigen-antibody complexes reveals their binding properties.

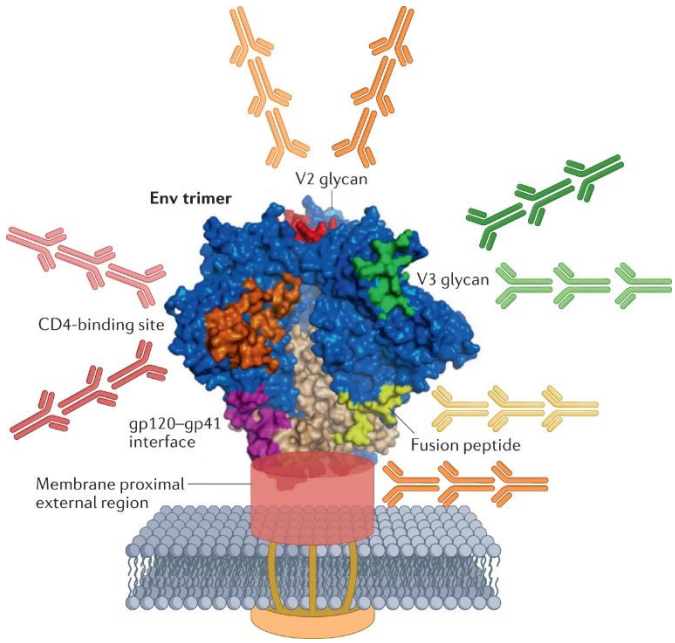

Figure 7. HIV-1 Env bNAb epitopes *(adapted from (148)). An overview of the HIV-1 Env epitopes targeted by bNAbs.*

VRC01 is a widely known CD4bs-directed mAb of high potency and breadth. Several similar antibodies, collectively referred to as VRC01 class antibodies, have been isolated from different donors since then (149,150). This class of bNAbs has been shown to bind through structural mimicry between their HCs V-region and the CD4bs of the Env, through beta strand interactions on the HC encoded HCDR2 (151). This structural mimicry of VRC01-class bNAbs is dependent on the immunoglobulin heavy chain variable (IGHV) germline gene-usage. IGHV1-2, but only certain alleles of this gene have the necessary binding motif (152). Similar antibodies can also be made with IGHV1-46 (153–155). There have been significant efforts to design immunogens that can engage naïve B cells carrying IGHV1-2, which have the potential to eventually affinity mature to become bNAbs (156–158). This is highly challenging especially because of the loss of binding of the germline-reverted VRC01-class antibodies to most vaccine candidates (150,159,160). A clinical trial using an immunogen designed to specifically engage the germline-reverted form of VRC01 recently showed that naïve precursor B cells with genetic properties that are characteristic for this class of Ab were successfully induced (161). Thus, this will be an interesting approach to follow as future clinical trials are performed.

V1V2-directed bNAbs are characterized by extraordinarily long HCDR3s that are often more than 20 amino acids and can penetrate the Env glycan shield (92). V3 glycan-directed mAbs bind to the glycan patches around the V3 region also typically have long HCDR3 and high levels of SHM (162). Less than 0.2% naïve human BCR HCs have this HCDR3 lengths thereby dramatically reducing the possibility of engaging these naïve BCRs through

targeted immunogens (163). MPER-directed mAbs are some of the broadest HIV-1 neutralizing mAbs isolated to-date. However, MPER bNAbs are also known to be some of the most polyreactive HIV-1 Env mAbs and have been shown to bind to host lipids and proteins (164,165). Fusion peptide is a recently discovered site of vulnerability on Env that can be targeted by bNAbs. Fusion peptide-based immunogens and several fusion peptide-directed bNAbs have been of particular interest in the field recently (166–168).

Whether it will be possible to elicit bNAbs against HIV-1 through vaccination remains an open question, but with the improvements in Env trimer immunogen design the field remains highly active.

2.4.5 Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)

SARS-CoV-2 was first detected in the late 2019 and is believed to have emerged from an animal market in Wuhan, China (169). Due to its high transmissibility, the outbreak quickly escalated into becoming a global pandemic. SARS-CoV-2 is the causative agent of COVID-19, a respiratory tract inflammation that can lead to a life-threatening pneumonia if it reaches the lower respiratory tract. The devastating effects of this global pandemic was intersected with an extraordinary, swift response from the scientific community and has revolutionized the speed at which research is conducted and shared. Within 10 days of the outbreak, the viral genome was released (GenBank Accession number MN988668) (170). The target receptor of SARS-CoV-2 was identified to be angiotensin-converting enzyme-2 (ACE2) on host cells and viral entry is mediated through the receptor binding domain (RBD) of the SARS-CoV-2 spike (S) (171). The structural resolution of S and its molecular interactions with ACE2 was a significant progress and fundamental for the development vaccine candidates (172). Vaccines were rolled out already in 2021 and soon thereafter, including those from Pfizer-BioNTech (mRNA), Moderna (mRNA), AstraZeneca (DNA), Novavax (protein), CoronaVac (whole inactivated virus), Sinopharm (whole inactivated virus) (124–129).

SARS-CoV-2 is an enveloped, single-stranded positive-sense RNA virus belonging to the family of betacoronaviruses. The genome of SARS-CoV-2 encodes for viral structural and non-structural proteins. The structural proteins include the nucleocapsid, membrane, envelope, and spike (S) proteins. The entry and fusion of the virus is manifested by the interaction of S with its host receptor ACE2. The S protein is class 1 fusion protein composed of two subunits - S1 and S2. The S1 subunit consists of the receptor binding domain (RBD) that binds to the angiotensin-converting enzyme-2 (ACE-2) and S2 contains heptad repeat regions and fusion protein that mediate the membrane fusion. Sequential proteolytic cleavage of S protein is essential for the virus entry. The initial S1-S2 cleavage is facilitated by furin and the second cleavage at the S2' site is dependent on the host proteases. The second cleavage is mediated through either of the two proteases

depending on the entry route of the virus- transmembrane serine protease (TMPRSS2) (at the plasma membrane) or cathepsin L (in the endolysosome) resulting in two possible virus entry modes with the former being more common than the latter. This results in the shedding of S1 and dramatic confirmation changes in S2, thereby exposing the fusion peptide. The viral RNA is released into the host cell followed by its replication and translation. The translated structural proteins translocate into the endoplasmic reticulum (ER) to assemble the virions the ER-Golgi interface. The virions exit the cell through exocytosis. The pathogenesis of SARS-CoV-2 is discussed in detail in these reviews (173,174).

2.4.6 Structure of SARS-CoV-2 Spike

SARS-CoV-2 S is a 180-200 kDa protein with substantial glycosylation that is meant for evading antibody binding (175). The S protein contains 1273 amino acids of which 391 to 541 are RBD residues. The prefusion i.e., the native conformation of S is highly metastable and undergoes major, irreversible restructuring triggered by the binding of S1 to ACE2. The native S showcases hinge-like movements that transiently expose the receptor binding elements on the S1. These movements are referred to as 'RBD up' and 'RBD down' with the latter being the receptor distant conformation. Introduction of two consecutive proline stabilization mutations facilitated the expression of S protein in its native, prefusion state (172). This was eventually further improved by the introduction of six proline substitutions in a version of S called HexaPro that increased the stability, expression and yield of S proteins approximately ten-fold (176).

2.4.7 Existing and emerging variants of concern (VoCs)

Continuous global transmission and replication of SARS-CoV-2 have led to numerous mutations in the virus genome, not the least in the S protein, the primary component of all licensed SARS-CoV-2 vaccines in use. This extensive virus evolution has led to the existence of several viral variants. Variants of concern (VoC), as defined by WHO, are the variants with mutations that increase the transmissibility, virulence or tamper public health measures (177). The emergence of VoC has significantly reduced the efficacy of all licensed vaccines, creating a need for vaccine updates. Some of the mutations in spike enables the virus to transmit more efficiently. Up until a certain point, the WHO named the VoCs that emerged in different geographical locations with Greek letters - Alpha (United Kingdom), Beta (South Africa), Gamma (Brazil), Delta (India) and Omicron (South Africa). After the emergence of Omicron, the virus has diversified considerably and current subvariants are mostly named using the Pango nomenclature (178). The effects of key mutations in the above VoC, with a specific focus on Omicron will now be discussed.

D614G was one of the first S mutations detected within just a couple of months of virus emergence (179). This mutation was associated with increased transmission by favouring

RBD-open conformation and quickly outcompeted its ancestral strains to become the globally dominant strain (180–182). The effect of the mutation on antibody neutralization as measured by several studies generate contradicting data but is very minimal (183,184). **N501Y** is one of the early mutations present in Alpha, Beta and Gamma and resulted in an increase in ACE2 affinity (185–187). This mutation does not affect antibody responses (188,189). **E484K** is an early S mutations that occurred in the Beta and Gamma variants (190,191). It is in one of the most immunodominant regions and targeted by majority of anti-S antibodies. This mutation resulted in a 10-fold reduced susceptibility to neutralizing antibodies present in convalescent plasma (192). The significant effect of this mutation on the polyclonal responses suggests that many antibody lineages target epitopes involving E484, which likely have exerted pressure on the virus to both mutate this residue and to retain this mutation in future VoCs. **K417N/T** is present in Beta and Gamma variants and similarly to E484K, this mutation is part of epitopes that are frequently targeted by the host antibody response. Several clinical mAbs have been shown to be susceptible to these mutations (189,192,193). **N439K, Y453F, S477N** are additional mutations that increased ACE2 binding and has some effect on antibody response. Furthermore, **N-terminal domain (NTD) mutations**, mostly deletions, increase viral replication and interfere with anti-NTD antibodies (194).

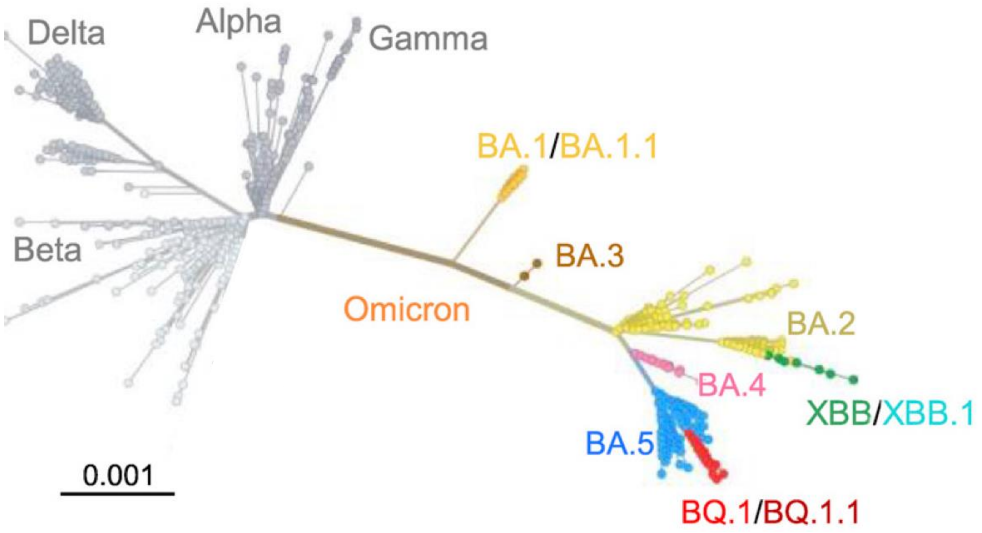

Figure 8. SARS-CoV-2 variants. *The figure shows the evolutionary tree of SARS-CoV-2 Omicron subvariants along with the other major variants. (adapted from (195)).*

The emergence of Omicron (B.1.1.529) with approximately 30 S mutations in the end of 2021 completely changed the landscape of SARS-CoV-2 VoCs and greatly threatened vaccine effectiveness. Omicron quickly became dominant globally and showcased high levels of immune evasion. Several Omicron subvariants have emerged since further impacting S-specific antibody responses. Overall, the subvariants BA.2, BA.2.75, BA2.75.2, BA.4/BA.5 show significant immune evasion compared to BA.1 (196–198). At the time

of writing, the latest subvariants of Omicron that are under monitoring include BA2.75.2, BQ.1, XBB, XBB1.5, BF.7, XBF and showcase extraordinary immune escape (177).

2.4.8 Deep mutational scanning (DMS)

DMS is technology that enables the simultaneous measurement of the functional impact of millions of mutant protein molecules in a single experiment. In brief, it involves the generation of DNA libraries encoding mutated proteins followed by the expression and display of the proteins on the yeast surface, which can then be screened for binding properties using cell sorting techniques (199). As a general tool, the large scale data obtained from DMS can be highly useful to screen for drug molecules, identify advantageous mutations for protein engineering or screen for rare mutations that may have deleterious effects (200,201). Owing to the versatility of this technique, it was quickly adopted by several labs to comprehensively analyse binding properties of SARS-CoV-2 S mutants during the pandemic. By generating RBD mutant display libraries on the background of the ancestral SARS-CoV-2 S, the group of Jesse Bloom identified mutations that were tolerated and enhanced RBD binding to ACE-2 (185). This was swiftly followed up with a comprehensive mapping of the antibody escape mutations on the RBD enabling the screening of antibodies based on breadth and potency against future VoCs (192,202). Through a productive collaboration with the Bloom lab, as described in **paper II**, the DMS technique was used to predict potential RBD escape mutations from a public class of antibodies isolated and described in this thesis.

2.4.9 S-specific antibody responses

The indispensable role of the SARS-CoV-2 S protein in mediating the first step of the infection makes it a highly biologically relevant vaccine candidate. As a consequence of its biological function, S accommodates several neutralizing antibody epitopes. Isolation and characterization of potent and broad neutralizing antibodies against S showed that the majority of the neutralizing antibodies target RBD and often overlap with the ACE2-binding site (192,203,204). The presence of RBD-specific neutralizing antibodies after immunization correlated with protection in animal models (205,206). RBD-specific antibodies isolated after SARS-CoV-2 infection can be very potent and possess extraordinary breadth against the future variants (207). Many of the early RBD-specific antibodies have very low levels of SHM indicating the ability of naïve BCRs to bind RBD with high affinity (208–210). In several studies, RBD-specific antibodies have been shown to preferentially use certain IGHV genes including IGHV1-69, IGHV5-53, IGHV3-66, IGHV3-30, IGHV3-30-3, IGHV5-51 (211–216). A recent study showing the presence of RBD-specific B cells in seronegative individuals confirms the RBD-affinity of certain naïve BCRs (217). Cryo-EM analysis of RBD-mAbs have categorized them into different classes based on their gene-usage, HCDR3 lengths and binding modes (218,219).

Besides the RBD specificity, potent neutralizing mAbs against NTD of SARS-CoV-2 have been isolated that primarily bind to a single large glycan free epitope called the NTD supersite (220–222). Neutralizing mAbs have also been isolated against SARS-CoV-2 S1 C-terminal domain (CTD) and S2, with most of them having relatively low potency. Recently, S2 stem helix-directed, IGHV1-46 using bNAbs were isolated and shown to protect against SARS- CoV-1, SARS-CoV-2, and MERS-CoV (223).

2.5 ANTIVIRAL MONOCLONAL ANTIBODIES

2.5.1 Monoclonal antibody isolation and characterization

Serological assays designed to measure polyclonal antibody responses are suitable for understanding the overall efficacy of vaccine candidates. To fully understand the antigenicity of vaccines and the quality of the induced antibody responses, mAb isolation is fundamental, especially if candidate vaccines fail to work. Antiviral antibodies have many applications. They help dissect vaccine-induced responses and identify vulnerable epitopes to design better immunogens. Owing to their high specificity, mAbs have been extremely successful as targeted therapies for a range of clinical conditions including infectious diseases, autoimmune diseases, and malignancies. Antibodies have also played crucial role in the field of diagnostics.

Immunologists have taken advantage of the surface BCR on the memory B cells, which can be bound by fluorescently labelled protein antigens to isolate single or bulk cells of desired specificities. Paired heavy and LCs from single sorted cells can be cloned into expression vectors to produce mAbs. An alternative to this technique is the use of a droplet-based single cell sequencing by platforms such as that provided by 10x Genomics. During my doctoral work, I isolated large panels of mAbs against both the HIV-1 and SARS-CoV-2 surface proteins that form the central part of the papers included in this thesis.

Characterization of mAbs is often done on three levels. The first step is to define the genetic properties of a mAb by V, D, and J gene assignments and determining the level of SHM. The second step involves assessing the binding and neutralization activity by ELISA and a suitable assay for measuring virus neutralizing activity. The third step is to examine the structure of the mAb, ideally by solving high resolution structures of the mAb/antigen complex, thereby dissecting its binding modes. In all papers, I use cutting-edge technologies to characterize the most interesting mAbs on all these three levels.

2.5.2 Antibody binding modes

Monoclonal antibodies are diverse and have different binding modes. For most antibodies, the HCDR3 forms the important contacts with the antigen, at least prior to affinity maturation. However, studies have described very potent and broad mAbs that, entirely or

partially, mediate binding through their HCDR1 and HCDR2, and less or not at all through the HCDR3. These studies will be covered in detail in the upcoming chapter. The HCDR1 and HCDR2 sequences are determined by the IGHV gene. It is becoming increasingly clear IGHV genes accommodate huge allelic variation and some of the polymorphisms within gene families occur around the HCDR1 and HCDR2 and can therefore influence antigen-binding properties of antibodies. Hence, using highly reliable, accurate genetic information to characterize mAbs is fundamental.

2.6 HUMAN IGHV GENE VARIATION

There are approximately 52 functional human IGHV genes that fall into 7 different gene families, IGHV1-IGHV7, depending on their phylogenetic relationships as established by IMGT (84). During evolution, these genes acquired mutations that were kept, resulting in substantial amount of allelic variation in IGHV genes in the population. Structural variation in the form of deletions and segmental duplications arose too, and together the allelic and structural diversity of IgH locus translates to high inter-individual Ig germline variation (**Figure 9)**. Due to the challenges of sequencing the IgH locus, the real scale of antibody germline gene diversity still remains to be determined (82). It is especially important to identify alleles present in persons of non-European ancestries as such alleles are very spare in the current public databases. While there are now many efforts underway to improve this (224,225), systematic characterization of large population groups is needed to create a comprehensive database of alleles. Such a resource is essential to accurately characterize antibody responses to infections and vaccinations.

Certain regions of the IgH locus appears more susceptible to variation than others. IGHV1-69 is one of the most polymorphic IGHV genes containing the highest number of allelic variants as well as copy number variation (81,226,227). As a result of this, it is common for individuals to carry more than two alleles of this gene. There are currently 20 IGHV1-69 alleles present in the IMGT database, of which 16 are functional. These alleles vary at 6 amino acid positions, of which 4 are present around the CDRs (84). Yet another highly variable region on the IgH locus encompasses IGHV3-30, IGHV3-30-3 and IGHV3-30-5, IGHV3-33 genes. IGHV3-30 has a high level of allelic variation and are also often duplicated (228–232). The IGHV3-30-3 gene is found to be homozygously deleted in several individuals. The functional relevance of this deletion is discussed in the paper IV (87).

IGHV1-69 and the IGHV3-30 group of genes are highly used in the human B cell repertoire and among antiviral antibodies (203,211,233). To study the how IGHV polymorphisms affect antigen-binding properties of antibodies and to better understand the affinity maturation process on a DNA level, accurate antibody germline gene assignment is indispensable. Personalized Ig genotyping is the best solution to this since all

individuals have a different set of alleles. Thus, assignment to a personal Ig germline allele database provides a strong foundation for antibody characterization. IgDiscover is a highly versatile germline gene inference tool that can efficiently generate personalized Ig germline genes through the processing of individual IgM libraries derived from cDNA. This has led to the identification of several novel alleles both in macaques and humans (6,234). This significant advancement can help uncover the scale of IgH variation and lead to the construction of better databases for germline alleles. In all papers described in this thesis, I used IgDiscover to genotype the subjects and then dissect their antibody responses.

There is a slow and steady realization of the functional impact of IGHV polymorphisms, which is a major topic of my doctoral research. IGHV gene usage bias by antibodies in certain infections is becoming increasingly evident and future studies will reveal if certain alleles of these genes are preferentially used. Furthermore, Ig variants have been associated to several disease conditions. Both these topics will be reviewed in upcoming sections. The functional consequences of this variation and deletion these IGHV genes have been examined in papers III and IV.

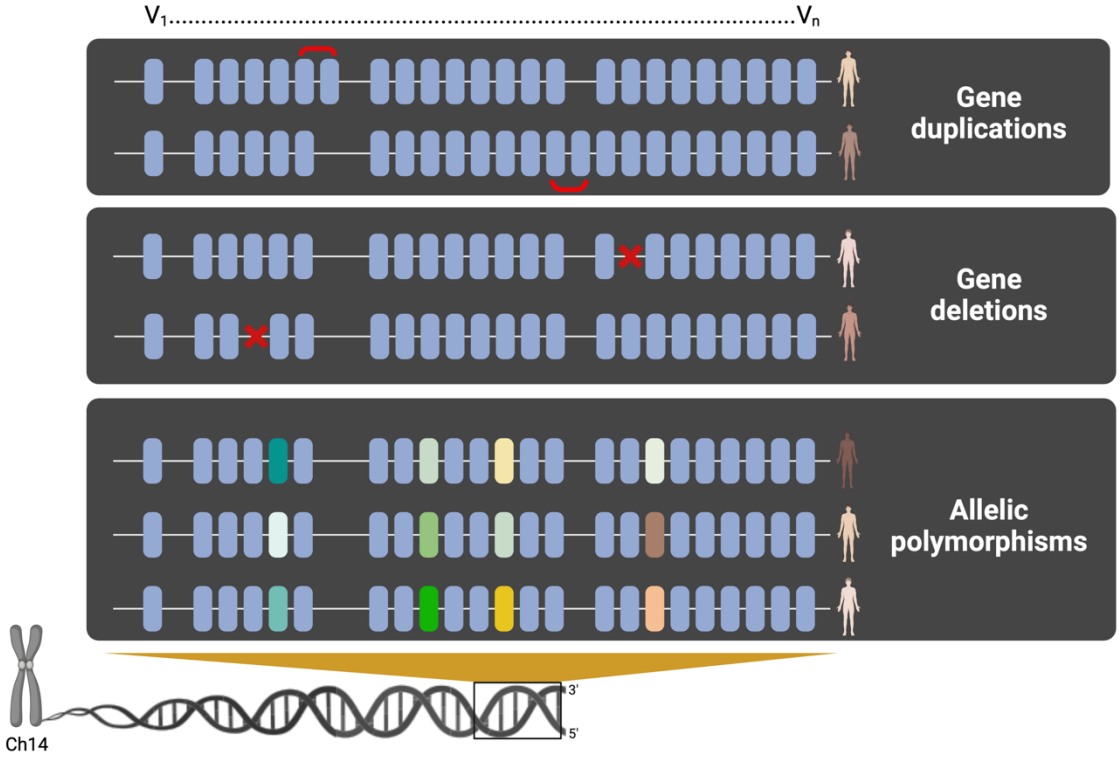

Figure 9. IGHV germline variation. *The IGH locus is present at the telomeric end of Ch14. The inter-individual variation of IGHV genes is attributed to gene duplications, gene deletions and allelic polymorphisms within genes.*

2.6.1 Role of IGHV germline-encoded motifs in antiviral antibody responses

Some infection/vaccination-induced antibodies have HCDR1 and/or HCDR2 antigen-binding modes. The fact that HCDR1 and HCDR2 are determined by the IGHV gene used means that germline polymorphisms within IGHV genes can influence antigen-binding (**Figure 10**) (152,214,235,236). The importance of IGHV germline configurations and their usage-bias by antiviral antibodies have been implicated in several infectious diseases.

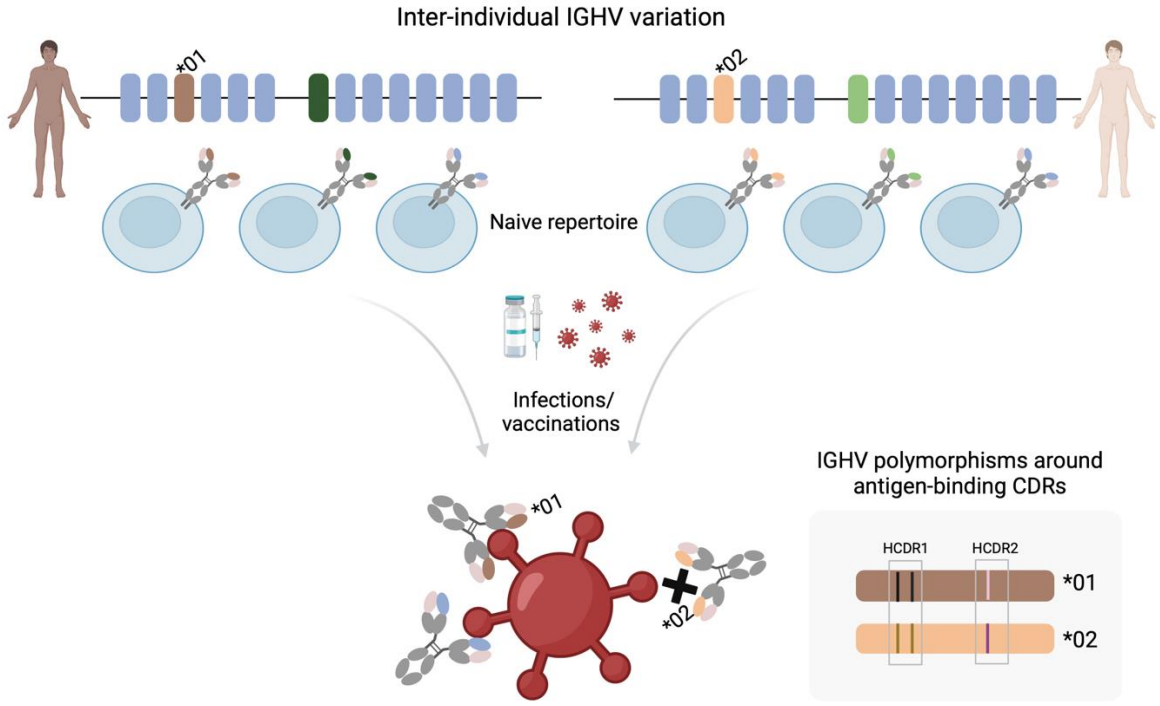

Figure 10. Functional significance of IGHV polymorphisms*. The IGHV germline allele content shapes the naïve B cell repertoire and therefore the antibody responses to infections or vaccinations.*

The IGHV1-69 gene is often used by anti-Influenza antibodies. Broadly neutralizing antibodies against the highly conserved Influenza hemagglutinin stem region prefer IGHV1-69 alleles that have a phenylalanine (F) instead of a leucine (L) at the polymorphic position 54 of the CDR2. Analysis of IGHV1-69 polymorphisms in the 1000 genome project shown a high prevalence of the F-allele in individuals of African ancestry (227,237). IGHV3-30 is another germline gene that is used by protective anti-Influenza antibodies (238,239).

Convergent antibody rearrangements were observed in IGHV3-13-using neutralizing antibodies against glycoprotein-1 core of Ebola (240). Germline-encoded residue Y58 of alleles, in combination with the LC gene IGKV1-5, was found to be recurrent in Zika virus memory B cell responses (241). The surface protein of *Plasmodium falciparum* expresses a highly repetitive, immunodominant repeat region NANP. A majority of anti-malaria

antibodies targeting this epitope use the IGHV3-33 gene and the germline-encoded W52 of the HCDR2 is critical for this interaction (242).

The VRC01 mAb described above, targets the conserved CD4bs of HIV-1. VRC01 and similar bNAbs, referred to as VRC01-class antibodies, are germline-dependent and use certain CDR features of the genes IGHV1-2. Specific residues IGHV1-2*02 and IGHV1-2*04 alleles that are shown to be important to make VRC01-class antibodies are W50, N58 in the CDR2 and R71 in the FR (153,154). Individuals lacking germline alleles with the above motifs are expected to be unable to make VRC01-class antibodies. This was also shown to be the case in a clinical trial that evaluated the IGHV1-2-germline targeting immunogen eOD, where one individual who was heterozygous for IGHV1-2*05 and IGHV1-2*06, two alleles that do not have the W50, N58 and R71 motifs, did not respond to the immunization, while all other trial participants did (161,243).

S-specific antibodies isolated early after infection typically have very low SHM and can be very potent, demonstrating that germline BCRs can display high affinity towards S. Several IGHV germline genes and specific motifs have been implicated in S-specific responses. SARS-CoV-2 neutralizing antibodies have been shown to preferentially use IGHV1-69, IGHV3-30, IGHV3-66, IGHV3-53, IGHV5-51 (211–216). The IGHV3-53/IGHV3-66 germline motifs 'NY' and 'SGGS' of the HCDR1 and HCDR2 are known to contribute to binding to the RBD by of a public class of antibodies (235,244). The highly similar IGHV3-30/IGHV3-30-3/IGHV3-33 germline-encoded residues N57 and K58 are shown to contact the RBD residues at positions 473 and 475 that are highly conserved across SARS-CoV strains (236). Recent study shows an enrichment of IGHV1-46 and IGHV3-23-usage among bNAbs that neutralize all three pathogenic betacoronaviruses, MERS-CoV, SARS-CoV, SARS-CoV-2 by targeting conserved regions on the stem helix of S2. These S2-directed bNAbs are observed in multiple individuals and are thought to be a public class of mAbs (223). Another study describes a highly potent IGHV1-69-using, MERS-CoV targeting mAb that is almost germline (one amino acid change) and its binding is found to be 85% dependent on the HC derived residues (245). Paper III of the thesis shows how certain IGHV1-69-using S-specific mAbs neutralize the virus only when specific allelic variants are used (215).

2.6.2 Disease associations of IGHV polymorphisms

IGHV polymorphisms have been associated with several autoimmune disease conditions. Polymorphisms or deletions around the IGHV3-30, IGHV4-31 region have been linked to rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) in Korean and Caucasian population groups (246,247). Deletion around this region was also found to be frequent in people with chronic idiopathic thrombocytopenic purpura (248). As observed in genome wide association studies (GWAS) in a Chinese population, SNPs in the IGHV1-

69, IGHV2-70 regions were associated to Kawasaki disease (249). GWAS studies from an indigenous South Pacific population showed that nonsynonymous variant of IGHV4-61 gene could be linked to rheumatic heart disease risk (250).

While these studies suggest biological consequences of IGHV germline-encoded variation, the complete interpretation of these observations awaits better definition of the IG locus and the frequency of functional alleles in different population groups. Genetic and functional examination of larger cohorts in these contexts will shed more light on the clinical relevance of Ig germline variation.

3 RESEARCH AIMS

The overall objective of this thesis was to outline the importance of affinity maturation and germline gene-usage of antiviral antibodies using state-of-the-art methodologies. The specific aims of the papers described in this thesis are described below.

Paper I - To understand the distribution and affinity maturation of HIV-1 Env-induced antibody lineages in rhesus macaques.

Paper II - To outline the molecular interactions and the detailed role of SHM of a public class of broadly neutralizing anti-RBD antibodies induced infection by ancestral SARS-CoV-2.

Paper III - To investigate the impact of germline-encoded polymorphisms in IGHV1-69 on the neutralization activity of human anti-SARS-CoV-2 antibodies.

Paper IV - To examine the functional impact of IGHV3-30-3 deletion and IGHV3-30 variation on an anti-SARS-CoV-2 neutralizing antibody.

4 RESEARCH METHODOLOGIES

4.1 Isolation of immune cell populations from biological samples

For **paper I**, rhesus macaque samples from various immune compartments were obtained. The blood samples were centrifuged at 1500 rpm for 10 minutes to retrieve the plasma. Density-gradient centrifugation was used to isolated peripheral blood mononuclear cells (PBMCs). Cell populations from other immune compartments such as spleen, lymph node and gut were obtained by the mechanical disruption of tissues followed by cell filtrations using a 70μm filter and several washing steps. For **papers II, III, IV**, PBMCs and plasma samples from human donors were collected as described above. Plasma samples were frozen and stored at -80C for subsequent analysis. The cell samples were frozen with 10% dimethyl sulphoxide (DMSO) and stored in liquid nitrogen. All immunizations and samplings were performed at KI.

4.2 Antibody binding and affinity measurements

Preliminary analysis of mAb/plasma binding was performed using ELISA in all the papers. ELISA is a standard, robust immunoassay that broadly involves direct, or, in the case of His-tagged antigens, anti-histidine-based coating of antigen (1-2 μg/ml) at 4°C overnight. Serially diluted plasma samples were incubated onto the coated plate for 1-1.5 hours at room temperature. The antigen-bound antibodies are then captured through polyclonal anti-NHP/human reagents conjugated with the enzyme horseradish peroxidase (HRP). The HRP substrate, 3,3',5,5'-Tetramethylbenzidine (TMB) was added to obtain a signal after the enzymatic reaction. The absorbance values were measured at 450nm on an ELISA reader.

In **paper III**, we used surface plasmon resonance (SPR) to measure the binding kinetics of Fabs using the Biacore 2000 instrument. All experiments were performed in a running buffer of pH 7.4 containing 10 mM HEPES, 150 mM NaCl and 0.005% Tween-20 at 25°C. The biotinylated RBD probes were immobilized on streptavidin sensors. Serially diluted Fabs were injected at a flow rate of 30μl/min. The measurements were performed after regenerating the immobilized RBD using glycine-HCl buffer. BIAevaluation Software was used to analyse the data. The binding kinetics experiment was performed in collaboration with McInerney group at KI.

4.3 Virus neutralization assay

Pseudovirus neutralization assays are a widely used methodology to quantify the ability of antibodies to neutralize virus. Pseudoviruses are replication-incompetent viral particles capable of a single round of viral entry, displaying viral spike proteins of choice, and a genome expressing luciferase reporter. In addition to replacing the need to handle live viruses, they specifically measure virus entry into the host cell allowing robust and

quantitative assessment of neutralizing activity. In our assay, the pseudovirus particles were generated by co-transfecting one plasmid expressing the SARS-CoV-2 spike protein and one plasmid expressing a lentiviral packaging construct based on the HIV gag and pol genes and a firefly luciferase reporter gene. The generated single-round infectious pesudoviruses are incubated with serial dilutions of the plasma preparation to be tested and cells that stably express the host receptors. The output is measured by luciferase luminescence and ID50 values are determined as the plasma dilution required to inhibit 50% virus neutralization activity. For monoclonal antibodies the set-up is similar, but the activity is measured as IC50 values, i.e., the concentration of antibody required to inhibit 50% of virus neutralizing activity.

In **paper I,** the neutralizing assays used different HIV-1 Env pseudotyped viruses with TMZ-bl target cells and serial dilutions of purified mAb or plasma in collaboration with the Mascola lab at the NIH.

In **papers II, III, IV,** the neutralizing assays used different SARS-CoV-2 spike pseudotyped viruses with ACE2 expressing human embryonic kidney (HEK) 293T target cells and serial dilutions of purified mAb or plasma in collaboration with the Murrell group at KI.

4.4 Assessment of polyclonal antibody responses by serology

Initial assessment of antigen-specific antibody responses to vaccinations or infections is performed at polyclonal level using serological assays. This is performed on the plasma/serum samples that contain many different antibodies. Serological data help obtain a broad picture of the quality and quantity of antibodies present and are fundamental for antibody characterization studies. In all the papers, the binding and neutralization activity of the polyclonal antibodies were assessed through ELISA and pseudovirus (PSV) neutralization assays respectively.

4.5 Analysis of antigen-specific cellular responses

Antigen-specific plasma cell and B cell responses are assessed using two major techniques - enzyme linked immunospot (ELISpot) and flow cytometry.

ELISpot quantifies the number of antibody-secreting cells (ASCs) in a sample preparation. In **paper I** and several other collaborative projects during my doctoral studies, a so-called reverse B cell ELISpot setup was used. In brief, anti-IgG (Fc) antibodies were coated in ELISpot plates and then either stimulated (for memory B cell) or unstimulated (for plasma cell) cells were plated to capture ASCs. The plates are then washed, and a set of wells are probed with an antibody against IgG to capture the total number of IgG-producing cells and another set of wells are probed with a biotinylated antigen to determine the number of antigen-specific ASCs. Since memory B cells do not produce antibodies, they require

prior simulations for 72 hours. Human memory B cells were stimulated in the presence of CpG, CD40L and IL21 and macaque memory B cells were stimulated using CpG, pokeweed mitogen (PWD) and Staphylococcus aureus cowan strain I (SAC) to differentiate into plasma cells that can then we quantified.

Flow cytometry is a technique to identify different cell populations based on their expressed surface markers, or, after permeabilization, intracellular markers. Fluorescent tags are attached to the markers of interest and the cells expressing these markers can be detected by the laser in the instrument.

4.6 Antibody library production, genotyping and haplotyping

Antibody libraries were generated in all the studies described in this thesis for the germline gene inference (IgM) and repertoire (IgG) analyses. In brief, cDNA was synthesized from total PBMCs using the relevant constant gene-specific primer. Multiplex variable heavy, kappa lambda (VH/VK/VL) forward primer sets, and a universal reverse primer were used to generate full-length V(D)J amplicons, each with a unique molecular identifier (UMI). Illumina MiSeq 2x 300 bp kits were used to generate paired-end sequences. IgDiscover was used to derive the germline V and J allele content in study subjects of interest.

IgDiscover contains a module named '*plotalleles'* that allows the inference of individual haplotypes in certain cases. Haplotype analysis takes advantage of the principle that V(D)J recombination takes place locally on a given chromosome. Thus, heterozygous IGHVJ/IGHDs alleles can be used to link the IGHV alleles to a given chromosome and therefore generating a phased map of the IGHV allele distribution of individuals on the maternal and paternal chromosomes. The IG genotype analyses described in this thesis were performed by senior scientist, Dr. Martin Corcoran, from our group.

4.7 Isolation of antigen-specific memory B cells and their V(D)J sequences

Fluorescence activated cell sorting (FACS) was used in all papers to isolated antigen-specific single memory B cells. We used optimized FACS panels to identify and sort cells of interest. Fluorescent tags were introduced to the biotinylated HIV-1 or SARS-CoV-2 S or RBD probes by conjugation to Streptavidin-tagged allophycocyanin (APC)/phycoerythrin (PE) through stepwise incubations at 4°C. CD3-CD14-CD20+ CD27++IgG+ Env/S/RBD+ live cells were gated and sorted individually into 4 μl lysis buffer containing RNase inhibitor into 96-well plates. The sorted cells were immediately transferred to dry ice and then to -80°C.

Single cell cDNA synthesis was performed using random hexamers, dNTPs and Superscript IV enzyme. The V(D)J regions of the cDNA were amplified by nested 96-well single cell PCR reactions that amplify the outer and inner regions of the V(D)J. The forward primers of the nested PCRs contain a mixture of primers specific to different V

regions and the reverse primers specific to the constant region. Full-length paired/unpaired V(D)J sequences are obtained by the sanger sequencing of purified nested PCR product.

4.8 Expression and purification of mAbs

HC and LC V(D)J sequences were cloned into expression vectors using Gibson assembly reaction. Inserts with overhangs homologous to the ends of the linearized vector were designed and chemically synthesized (IDT technologies). Gibson assembly was set up according to instructions using 50 ng of freshly cut heavy/kappa/lambda vector (251) and 30 ng of insert. The Gibson reaction product containing the plasmid with insert was transformed into competent cells by heat shock at 42°C for 30 seconds. Positive HC and LC colonies were screened and used for subsequent transfections. 15 µg of natively paired heavy and LC plasmids were co-transfected using 30 ml HEK293F cells using Max reagent and cultured for one week. mAbs were purified after 7 days using protein G Sepharose columns and stored long-term in PBS.

4.9 Germline-reversion of mAbs

Reversion of mature V(D)J sequences to their germline configuration was performed in all papers in this thesis. To do this, the SHMs in the V and J regions were reverted to their germline residues. Because we generated individualized germline allele databases in all studies, we can be sure of that the gene and allele assignments are correct. The use of IgDiscover for individualized genotyping improves the accuracy of germline-reverted antibodies greatly. The CDR3 regions of the antibody sequences were kept intact as the non-templated modifications that occur at the V-D and D-J junctions during recombination for the HC (**Figure 11**) and the V-J junction for the LC prevent inference of the naïve BCR sequence. The reverted V(D)J regions were cloned into vectors and expressed as mAbs.

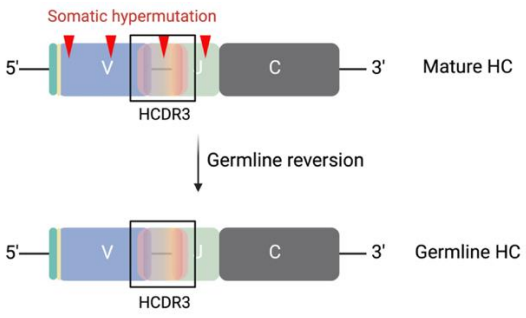

***Figure 11**. Germline reversion of HCs.*

4.10 Epitope mapping of mAbs

Molecular interactions between the mAb and the antigen are determined using hydrogen–deuterium exchange mass spectrometry (HDX-MS) and cryo-EM. Fabs obtained from the digestion of mAbs were used for these studies.

HDX-MS is a technique used to map the rough epitope targeted by antibodies. In **papers III and IV**, regions of interactions on the RBD were identified by this technique. The principle of the process is that proteins undergo rapid H-D exchange when exposed to deuterium oxide (D_2O). The procedure is performed using RBD with or without the Fabs bound. Both conditions are then exposed to D_2O. After exposure, the samples are quenched and subjected to digestion using 0.1% formic acid of different gradients. Peptides were analysed on a Exactive Plus Mass Spectrometer. A peptide library with the retention time and charge state was generated and analyzed using HDExaminer. HDX-MS experiments were done by Simon Ekström at the department of Biomedical Engineering, Lund University.

Cryo-EM analysis is one of the most sophisticated techniques for the high-resolution characterization of molecular interactions between protein binding partners, such as an antibody and its antigen. If the complex is stable and sufficient amount of data is collected, it can provide highly detailed information about how an antibody binds its antigen, such as which epitope region is targeted, what the binding angle is and, in some cases, it is also possible to define the fine details of which amino acids interact with each other at the protein-protein interact site. In **papers II and III**, we used cryo-EM to resolve the structures of two selected anti-RBS neutralizing antibodies and we obtained high resolution structural information (2.5 Å) of the Fab-spike complexes. In some cases, localized reconstruction and refinement are required to dissect the atomic details at the sites of interaction. Cryo-EM was performed in collaboration with the Hällberg group at KI.

4.11 Ethical considerations

In **paper I**, rhesus macaques were used to study vaccine-induced antibody responses. Rhesus macaques are suitable animal models to understand human immunology due to their high genetic homology to humans. However, the use of animal models and particularly higher vertebrates raises ethical concerns. Hence, their acquisition and usage are strictly monitored and regulated. The planning and implementation of our study was performed according to the 3R principle - Reduction, Replacement and Refinement. Maximum effort was taken to replace *in-vivo* analysis with *in-vitro* experiments. The lowest number of subjects needed to obtain significant scientific data was determined to avoid using excess animals. The research project was further refined to avoid unnecessary pain and discomfort of the animals for example, by reducing the frequency of immunizations, samplings. During the study, the animals were housed and cared for at the Astrid Fargaeus laboratory, Karolinska Institute, according to guidelines laid out by the Swedish board of Agriculture.

In **papers II, III and IV**, human samples were used to study antibody responses to SARS-CoV-2 infection. At the start of the pandemic, we obtained access to blood samples from

a set of healthcare workers at Karolinska University Hospital who were previously infected with SARS-CoV-2. Informed written consent was provided by each of the individuals in the study cohort and with the ability to withdraw from the study at any given point of time. The presence/absence of antibodies and memory B cells were reported back to the donors as agreed during the acquisition of informed consent. We consider these samples to be very valuable and highly protect the interests of the donors. Personal information of the participants is carefully protected by using codes as identifiers at samplings with the key not being available to the research group.

5 RESULTS AND DISCUSSION

5.1 Distribution of HIV Env vaccine-induced B cell responses in immune compartments

The biological and clinical relevance of HIV-1 Env is indisputable. The antigenic intricacy of the Env trimer creates unique challenges is designing immunogens that can induce protective responses as discussed in the previous sections. So far, vaccine studies using various forms of Env have not successfully elicited bNAbs and hence understanding the dynamics and evolution of Env-specific B cells in immunized subjects may provide valuable insights in vaccine development. Assessing polyclonal responses through serology is important but provides limited information. Additionally, mAb isolation is low throughput and often reveals only a part of the whole picture, which is a limiting factor. Higher resolution approaches, such as mAb isolation coupled with genetic and structural studies are needed to elucidate Env-specific antibody responses and to guide Env immunogen re-design. Over the years, our group has acquired in-depth knowledge about antibody germline gene variation in macaques and humans. This has highlighted that outbred species have significant inter-individual differences in their germline allele composition, which in turn influences how they respond to infections and vaccination. In **paper I**, we combined personalized antibody germline allele inference, repertoire sequencing analysis and isolation of antigen-specific HCs to investigate the diversity, distribution, and maturation of 180 Env-specific antibody lineages simultaneously. Four rhesus macaques were immunized six times at regular intervals using NFL Env trimers and sampled two weeks after each immunization. Serological analysis revealed detectable autologous plasma neutralization activity after the third immunization in all four animals. D20, one of the best responders, was pursued for the studies described in **paper I**. Isolation of single Env-specific memory B cells from PBMCs resulted in 672 productive HC sequences, which were then collapsed to 180 clonal lineages using a defined clonality criteria. Assignments of the antibody sequences to alleles known to be present in D20 added high precision to the V and J gene assignments.

Lineage tracing of the Env-specific antibody sequences in bulk IgG library from different immune compartments showed that the Env-specific antibodies were distributed throughout blood, BM, spleen, and LN (**Figure 12**). Most lineages were detected in spleen (102 lineages) and LN (111 lineages), which may indicate on-going GC reactions. The detection of both memory B cells and plasma cells by ELISpot in these compartments and time points is in line with this observation. The BM IgG libraries had 41 detectable lineages, most likely representing plasma cells as the BM had no detectable memory B cells by ELISpot analysis. The low number of traced Env-specific lineages in the BM may reflect a highly selective recruitment and limited space in BM survival niches. Clonotyping of the BM IgG library demonstrated a less polyclonal repertoire compared to IgG repertoires in

the other compartments (blood, spleen and LN). A total of 101 Env-specific lineages were detected in the PBMC IgG repertoire, which mainly reflects memory B cells as there were no Env-specific plasma cells in the blood at this time point as determined by ELISpot analysis. The gut samples stood out with only 8 minimally expanded Env-specific lineages detected, demonstrating its highly restricted overlap with other immune compartments as also observed in another study (252).

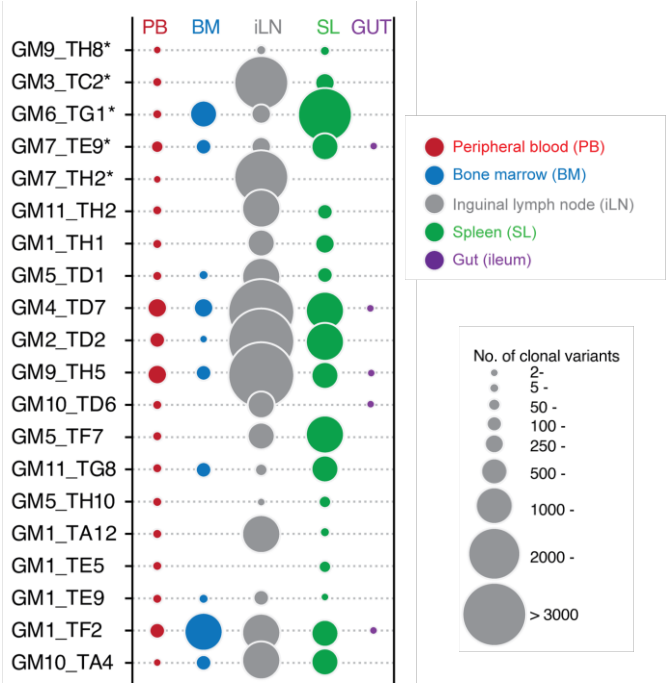

Figure 12. *Distribution selected Env-specific lineages within the IgG libraries of blood, BM, spleen, lymph node and gut.*

Together, these results illustrate the ability of NFL Env trimers to induced highly diverse antibody lineages that are distributed across compartments. Further, we found that boosting enabled increasing affinity maturation of Env-specific memory B cells. These results support the design of Env immunization regimens that rely on repeated boosting, preferably with heterologous Env immunogens, to drive antibodies that target conserved epitopes and hence may display greater breadth.

5.2 Can affinity maturation increase the breadth of antiviral antibody lineages?

Induction of GCs and therefore antibody affinity maturation is likely important for most vaccines to induce broadly protective responses. Affinity maturation processes increases the affinity to a given antigenic structure by selection of sequences that improves molecular contacts. Such vast affinity-induced sequence diversity within the somatic variants of an antibody lineage may be important to attain breadth against future pathogen variants. Hence both in vaccination and infection settings, affinity maturation and therefore attaining a diverse pool of clonal variants is highly desirable. The work in our group has focused on intraclonal antibody evolution since each lineage is unique and therefore has its

own requirements for SHMs to remain competitive in the polyclonal pool. The ability to genotype study subjects with IgDiscover provides the ability to accurately assign the germline sequence of a given antibody, which is the basis for understanding of antibody maturation process. The topic of antibody affinity maturation has been investigated in **paper I, III, IV** and in greater detail in **paper II**. A high level of SHM is a hallmark characteristic of Env-specific antibodies and is necessary to attain desired breadth and potency against HIV-1.

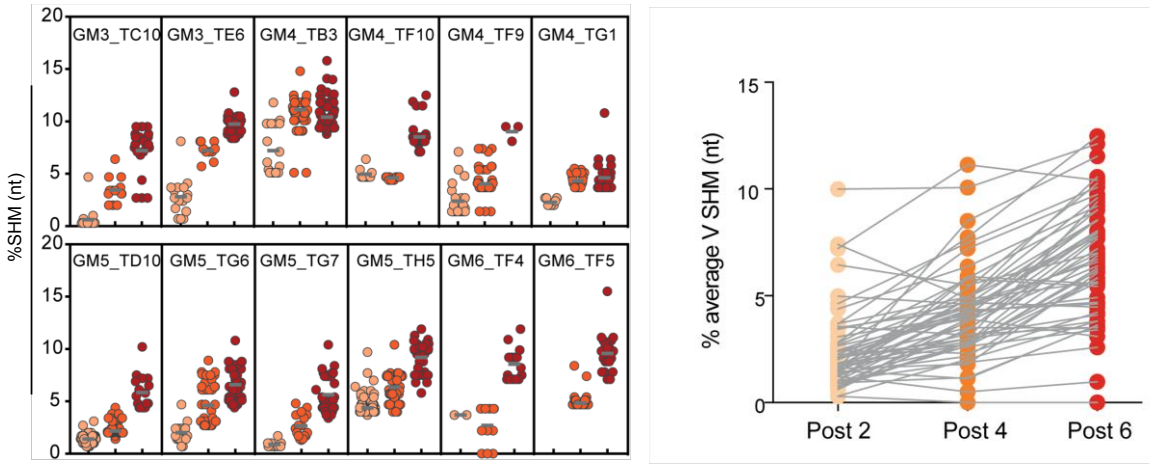

Figure 13. *The left panel shows the gain of SHMs by individual variants within single lineages in blood after every boost. The right panel shows the overall gain of SHMs in all traced lineages in blood after boosting.*

In **paper I**, we showed that the SHM levels of the Env-specific lineages in longitudinal blood samples consistently increased following boosting **(Figure 13**) and was detected throughout the HC CDRs and FRs, primarily FR3, demonstrating that affinity maturation is not just restricted to the HCDRs. Intraclonal analysis of the somatic variants of a potent neutralizing lineage from different compartments established a strong correlation between affinity maturation and neutralization potency (**Figure 14**). However, in the case of this antibody lineage, which targeted a strain-specific epitope, it did not result in an increased breadth.

Clonal variant name	% V SHM (aa)	Neutralization (IC50, µg/ml)	
		IgG	Fab
PB_seq681430_post-1	0	>50	>50
PB_seq24927_post-2	7.3	0.014	2.64
BM_seq138686_post-2	7.4	0.010	0.648
iLN_seq1578500_post-6	15.6	0.004	0.114
SL_seq1387814_post-6	15.6	0.005	0.161
GM9_TH8_post-6	16.7	0.015	0.414
BM_seq362970_post-4	16.7	0.006	1.60
SL_seq192124_post-6	16.7	0.004	0.143
SL_seq213526_post-6	18.8	0.001	0.132
SL_seq597636_post-6	18.8	0.005	0.052
SL_seq898166_post-6	21.9	0.008	0.039
iLN_seq732127_post-6	24	0.002	0.020

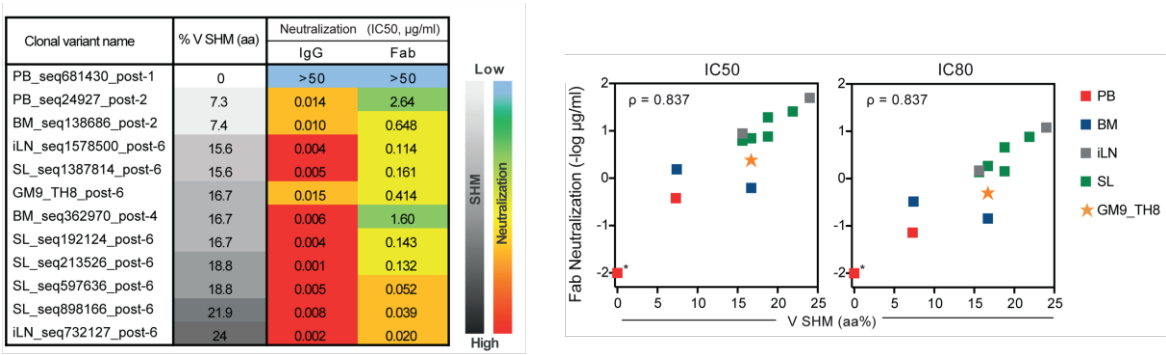

Figure 14. *% SHM (nt) and Neutralization activity of variants **of** Env-specific neutralizing lineage. Correlation between the % SHM (nt) and the neutralization IC50s and IC80s.*

Relatively soon into the COVID19 pandemic, it became clear that also SARS-CoV-2 is a constantly evolving pathogen. The initially developed vaccine candidates were based on the S protein from the ancestral virus, the Wuhan strain. The subsequent emergence of VoCs with mutations in S, and particularly in the RBD, raised concern about if the virus would escape from infection or vaccine-induced antibody responses and thus reducing protective immunity in the population. While there was significant immune escape by the early variants (197,221), it was not until Omicron emerged that this became a major concern. In **paper II,** I isolated a panel of potent, broad, IGHV3-53-using public class of antibodies from an individual seven months after infection with ancestral SARS-CoV-2. Among these we identified CAB-A17, a bNAb whose neutralizing activity extended against most Omicron subvariants (**Figure 15)**.

IC50 (ng/ml)	BA.5 (BA.4)	BA.4.6	BA.2.75	BA.2.10.4	BJ.1	XAW	BA.2.75.2	BA.2.3.20	XBB	BQ.1.1
CAB-A17	7	4	15	9	5	9	202	20	>1000	190
CAB-A18	4	2	10	6	4	11	1282	13	>1000	196
CAB-A49	57	32	>1000	131	28	>1000	>1000	>1000	>1000	>1000
CAB-B37	17	16	>1000	32	10	>1000	>1000	>1000	>1000	>1000
CAB-C19	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
CAB-D16	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
CAB-E24	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
CAB-E30	696	385	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000

Figure 15. *Neutralization activity of IGHV3-53-using antibodies against SARS-CoV-2 Omicron subvariants*

DMS experiments using mutant RBD libraries were used to assess the cross-neutralization efficiency of CAB-A17. F456K was identified to be one of the RBD mutations that afforded the CAB-A17 escape. The assessment of neutralization of activity of CAB-A17 against pseudoviruses with F456K mutation showed that F456K reduced the potency of CAB-A17 by approximately 3-fold. This modest loss of CAB-A17 neutralization activity illustrated its remarkable breadth attained post a single SARS-CoV-2 infection. Most IGHV3-53-using antibodies isolated up to that point were known depend on germline-encoded residues and had very low SHM levels with limited breadth (244,253). CAB-A17 has a total of 14 amino acid mutations in its HC and 10 in its LC, including a deletion in the LC.

To understand the role of SHMs in the cross-neutralization activity, we performed germline (gl) reversion of the CAB-A17 HC. The CAB-A17 with a glHC showed a ten-fold loss in neutralization activity to the ancestral strain and a complete loss of breadth. This result implied that the breadth of CAB-A17 was to a large extent mediated by its HC SHM. To fully dissect the role of affinity maturation, I introduced CAB-A17 HC SHMs individually or in combinations on the glHC backbone and identified four key mutations that together were responsible for the exceptional breadth of this antibody (**Figure 16**).

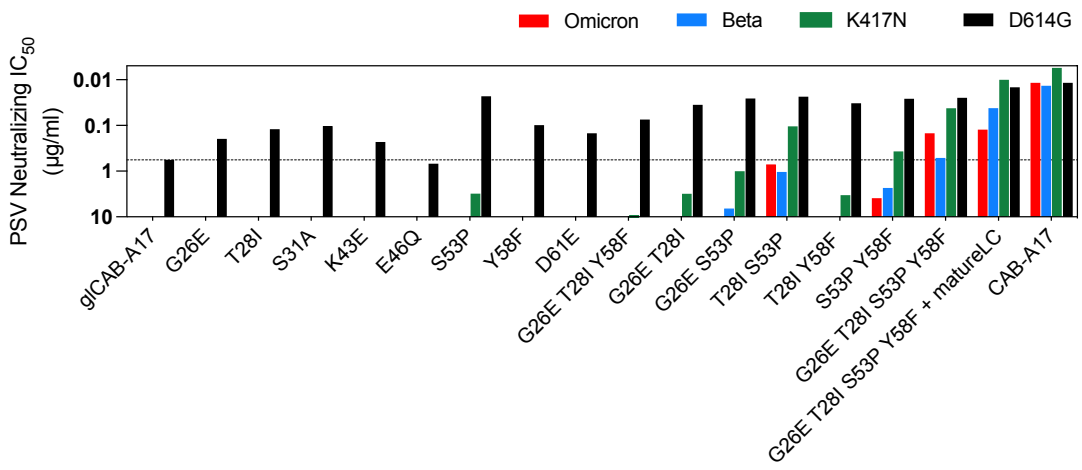

Figure 16. *Neutralization IC50s of CAB-A17 germline and mutated mAbs against SARS-CoV-2 variants.*

Resolution of the molecular interactions between CAB-A17 and the ancestral S through cryo-EM showed that the identified amino acid substitutions G26E, T28I, S53P, Y58F in the HCDR1 and HCDR2 made critical interactions with the RBD. These results together firmly established the importance of the affinity maturation process in improving the antibody such that it retained activity even against future VoCs. The role of affinity maturation in acquisition of additional breadth and potency is also revisited in **papers III and IV**, where germline reversions clearly reduced the neutralization IC50s. In **paper IV**, germline reversion of a potent mAb, CAB-F52, illustrated the critical role of SHMs in gaining breadth against the delta variant.

Based on our studies, the answer to the question posed in the title of this section is yes for most antibodies and depends on the target epitope.

5.3 Functional impact of IGHV allelic polymorphisms

Antibodies accommodate pronounced and subtle differences to cover a large array of antigenic epitopes. Affinity maturation has been addressed in all papers of this thesis and the results have been discussed in the previous section. In addition to this, individuals may have different starting capacities to bind a given antigen due to differences in their naïve B cell repertoires. These differences are partly due to stochastic events that occur during the V(D)J recombination, resulting in antibodies with different CDR3s in different people. However, they may also be due to the presence of different V, D or J germline alleles in different persons. The functional significance of germline-encoded polymorphisms in IGHV genes has been discussed above for Influenza virus antibodies. During the COVID19 pandemic, several papers reported the over-representation of IGHV3-53, IGHV3-66, IGHV1-69, IGHV3-30, IGHV3-30-3, IGHV5-51 genes in the S-specific antibodies, which re-stimulated questions about the role of IGHV germline-encoded residues in S-specific responses. Several structures of S-specific mAbs that use the above

genes reveal important interactions made by germline-encoded motifs, including some covered in the literature review section. In **Paper III** and **paper IV** presented in this thesis, we explored the biological consequences of human IGHV germline variation by combining state-of-the-art immunogenetics, mAb isolation, functional assays and structural biology. One gene that is of particular interest is IGHV1-69 gene, which has the highest number of allelic variants of all IGHV genes and is often duplicated in individuals, which means that many persons have more than two alleles. Alignment of the different IGHV1-69 alleles demonstrates that of the six polymorphic amino acid residues, four are present around the HCDR1 and HCDR2 regions, suggesting that they may influence the function of some IGHV1-69-using antibodies (**Figure 17**) (227,237).

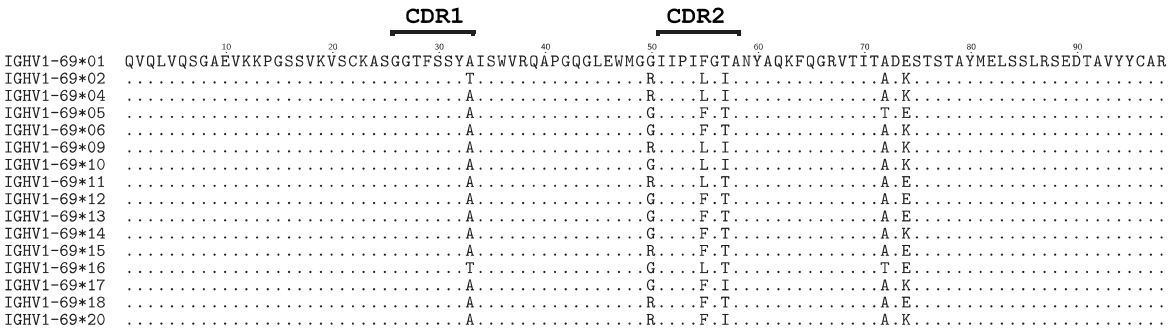

Figure 17*. An amino acid alignment of IGHV1-69 alleles showing the polymorphic residues.*

Further, IGHV1-69 is one of the most used genes in the human B cell repertoire, consistent with its frequent use in antiviral antibodies. In **paper III**, we investigated the role of the polymorphic amino acid residues of IGHV1-69 allelic variants in SARS-CoV-2 RBD interactions. A potent infection-induced IGHV1-69-using antibody, CAB-I47, was isolated from one of the study participants (SP14).

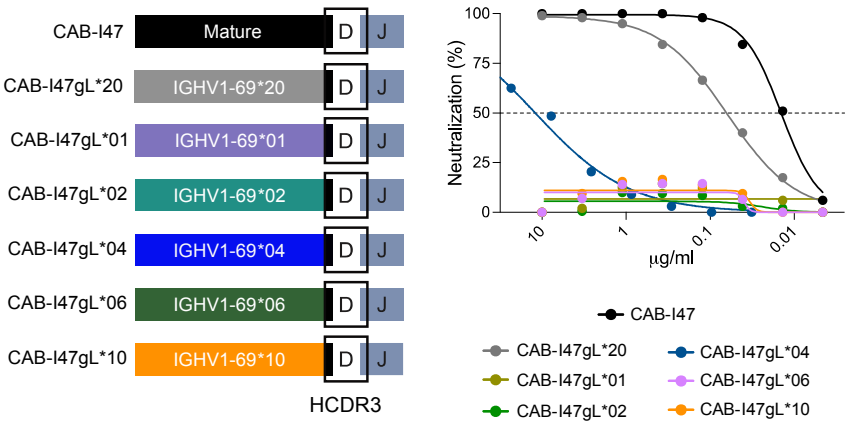

Figure 18. *The left panel shows the design of CAB-I47 germline HCs. The right panel shows the neutralization activity of the germline swapped CAB-I47 mAbs compared to the mature antibody.*

Prior to mAb isolation, genotyping of SP14 by IgDiscover was performed to determine the IGHV1-69 allele content. SP14 had three IGHV1-69 alleles – IGHV1-69*01, IGHV1-69*02, IGHV1-69*20 and CAB-I47 utilized IGHV1-69*20. Germline reversion (see methods) of CAB-I47 resulted in retainment of its neutralization activity albeit with a 10-fold reduction in IC50. To investigate if the IGHV1-69*20 allele was required for this neutralizing activity observed in the absence of SHM, we replaced the IGHV-region of CAB-I47 with several other commonly used IGHV1-69 alleles, expressed them as mAbs with the mature LC, and tested their neutralization activities. We found that all allele-swapped CAB-I47 germline mAbs completely lost neutralization activities, except the antibody that used IGHV1-69*04, which differs from IGHV1-69*20 by only one amino acid, F55L (**Figure 18**).

The results demonstrated a strong reliance of CAB-I47 on IGHV1-69*20 allele to show neutralization activity. Introduction of this single point mutation F55L in the HCDR2 of the mature CAB-I47 reduced the neutralization IC50 by more than 10-fold. A high-resolution structure of the CAB-I47 Fab bound to S from the ancestral virus revealed that a second germline-encoded, polymorphic residue formed critical contact with the epitope, R50. While F55 was shown to dock into a hydrophobic pocket in RBD, R50 interacted with E484 of the RBD through hydrogen bonds and a salt bridge, one of the strongest protein-protein interactions (**Figure 19**). When we introduced the R50G mutation in the mature CAB-I47, it completely knocked out both binding and neutralizing activity, demonstrating the importance of a single germline-encoded, polymorphic motif. E484K was one of the earliest S mutations shown to afford escape of neutralizing antibodies recognizing the E484 residue. It is likely that high prevalence of CAB-I47-like antibodies exerted pressure the virus to mutate this residue.

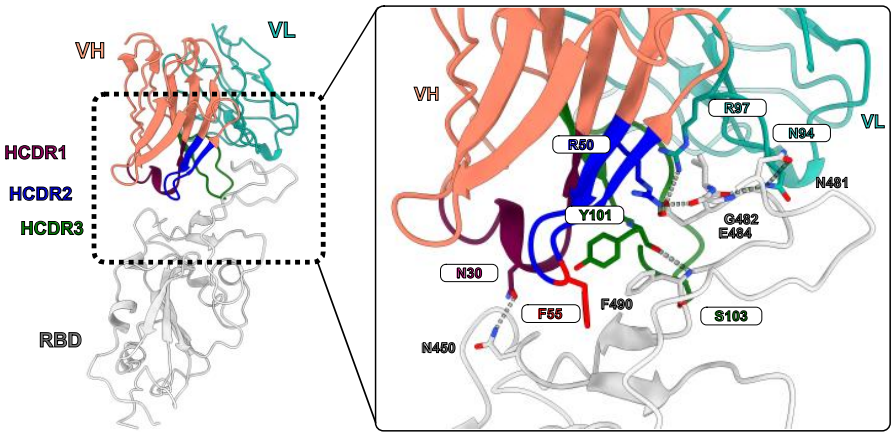

Figure 19. *Interaction between the amino acid residues of CAB-I47 Fab and the RBD. R50 highlighted in blue and the F55 highlighted in red.*

To show the existence of similar IGHV1-69*20-dependent mAbs across donors carrying this allele, we isolated two other IGHV1-69*20-using mAbs from a different donor and

we also identified ten IGHV1-69*20-using mAb sequences from CoV-Ab-Dab, a database of antibodies assembled from various independent studies. This indicates that IGHV1-69*20 antibodies are commonly elicited in individuals carrying this allele. The genotyping and haplotyping of the cohort included in this study clearly demonstrated the high allelic and copy-number variation observed for IGHV1-69 gene. These results together illustrate the influence of germline-encoded polymorphic residues on antibody functions and stresses on the important of determining the IGHV allele content to define what is germline-encoded polymorphisms and what is SHM.

IGHV3-30-3 is yet another gene family that is highly used by S-specific neutralizing antibodies and is often found to be deleted among individuals. Further, IGHV3-30, IGHV3-33, IGHV3-30-3, IGHV3-30-5 region of the IgH locus is particularly subjected extensive allelic, structural and copy number variation as discussed before. This group of genes are highly similar, and alleles of different genes can even be identical to each other at the nucleotide level and hence can only be differentiated by their chromosomal position (**Figure 20**).

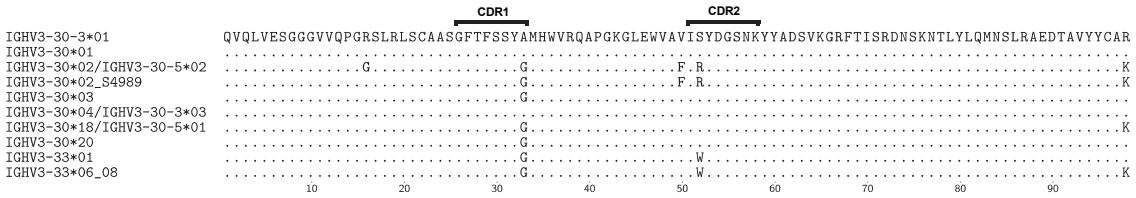

Figure 20. *An amino acid alignment of IGHV3-30, IGHV3-30-3 and IGHV3-33 alleles showing the polymorphic residues.*

As a follow-up to **paper III**, we examined an ultrapotent (IC50 0.001) RBD-specific mAb, CAB-F52, that utilized IGHV3-30-3 gene. Inspection of the CoV-Ab-Dab confirmed the prevalence of IGHV3-30-3-using antibodies across individuals. Mature CAB-F52 HC contained six amino acid substitutions compared to its germline configuration. Upon HC germline-reversion, CAB-F52 retained its neutralization activity and most of its breadth. Genotypes and haplotypes of the cohort showed 4 out of 10 people lacked IGHV3-30-3. When we examined the IgM sequences carrying IGHV3-30 group of genes, the individuals with the IGHV3-30-3 deletion contained significantly lower number of naïve HC with using the IGHV3-30 group of genes compared those without the deletion (**Figure 21**). This could mean that the people with the deletion have a lower possibility of engaging these BCR during an ongoing immune response.

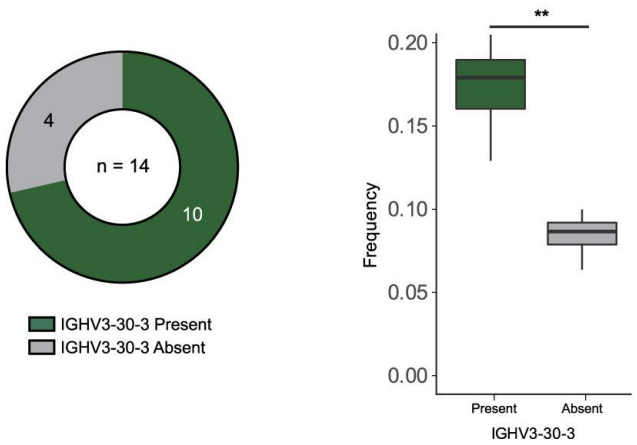

Figure 21. *A pie chart showing how many of the individuals in this cohort lacked IGHV3-30-3 on both chromosomes (gray). The right panel shows the combined frequencies of IGHV3-30 and IGHV3-30-3 reads in IgM repertoire data.*

Due to the high similarity among these genes, we investigated if they could replace each other. We made germline allele-swaps of CAB-F52 by replacing the IGHV-region with selected IGHV3-30/33 alleles. Swapping of CAB-F52 VH-region with IGHV3-30 germline alleles did not affect the neutralizing activity of CAB-F52 demonstrating functional redundancy, at least for this antibody (**Figure 22**). Whereas, when the CAB-F52 IGHV-region was replaced with the IGHV3-33 alleles, it resulted in a complete loss of neutralization activity, probably because of the presence a tryptophan at position 52, which is not present in the IGHV3-30 and IGHV3-30-3 alleles. Together, **papers III and IV** demonstrate that single polymorphisms in antibody germline genes can make a functional difference.

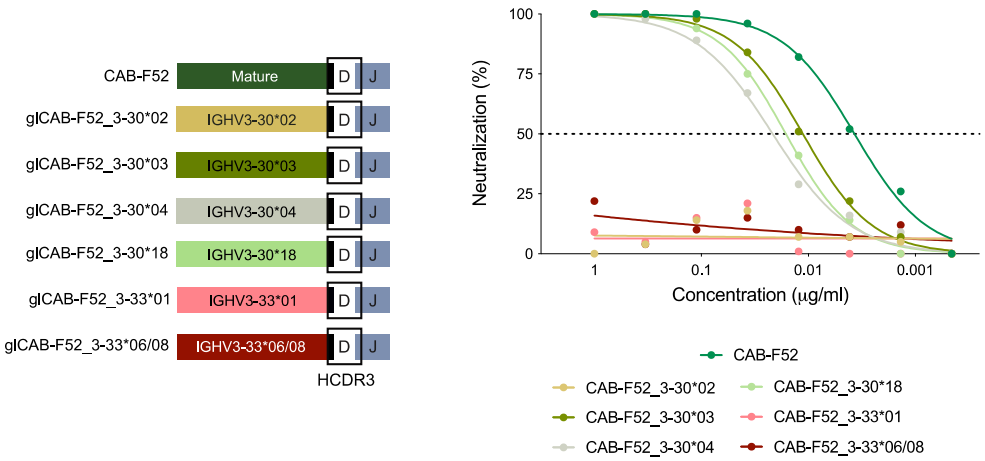

Figure 22. *The left panel shows the design of CAB-F52 germline HCs. The right panel shows the neutralization activity of the germline swapped CAB-F52 mAbs compared to the mature antibody.*

6 CONCLUSIONS

The results described in this thesis add to our current understanding about antibody elicitation and maturation. Main conclusions include:

- HIV-1 Env-induced antibody responses elicited by soluble Env trimers in adjuvant are highly polyclonal are disseminated into blood, BM, spleen and lymph node. Boosting promotes affinity maturation of the majority of traced lineages **(paper I).**
- Infection with the ancestral SARS-CoV-2 elicits both public and private antibodies. Affinity maturation allows some lineages to develop into broadly neutralizing antibodies with activity against of highly divergent variants such as Omicron **(paper II, paper III and paper IV).**
- The IgH locus accomodates exceptional allelic, strucutural and copy number variation giving rise to significant inter-individual IGHV germline diversity, which influences the types of antibodies elicited **(paper III, paper IV)**

7 POINTS OF PERSPECTIVE

Despite the early success of many empirically developed vaccines, our understanding of vaccine immunology remained limited, and for long time vaccinology and immunology remained two separate fields. During the past decades, the emergence of HIV-1 and other challenging pathogens that pose major human health risks has stimulated extraordinary advancements in vaccine immunology bringing together the two fields. These advances are in part responsible for the swift response to the SARS-CoV-2 pandemic with several new vaccine platforms gaining approval. Virus neutralizing capability largely determines the quality of protective antibody responses and remains the most important goal of vaccines. To fully understand the specificities of neutralizing antibodies, the characterization of monoclonal antibodies has become more common. Such efforts have two broad applications – to guide the design of improved vaccines and to develop therapeutics. The results presented in this thesis add to both these areas.

HIV-1 is highly pathogenic virus that undergoes continuous evolution. The surface Env glycoprotein is highly adapted to resist recognition by neutralizing antibodies through conformational and glycan shielding. Years of research has resulted in the design of soluble Env immunogens that retain native-like conformation. These contain multiple stabilizing mutations, such as the NFL trimers used in this thesis, to increase the chance that the immune system is exposed to relevant epitopes. Future efforts focusing on sequential administration of heterologous Env trimers, or attempts to induce certain classes of neutralizing antibodies, so-called germline-targeting approaches, will demonstrate if it is possible to target conserved epitopes and ultimately induce broadly neutralizing antibodies.

In contrast, when SARS-CoV-2 emerged it was a highly neutralization-sensitive virus. Because it was new to humans, it had not yet evolved ways to escape antibody recognition and consequently, it was easily targeted by the immune system. The emergence of VoCs is stepwise changing this, resulting in a demand for updated vaccines. However, since it does not cause chronic infection in healthy individuals, it is not under long-term immune pressure in each host as is HIV-1. Tools such as DMS and a better understanding of human immune responses at a population level can help the development of vaccines that are more universally protective. Understanding antibody affinity maturation trajectories and characterizing human antibody germline genes will play important roles in outlining antigen-antibody interactions that can influence vaccine design.

8 ACKNOWLEDGEMENTS

My Ph.D journey has been an extraordinary learning experience. It has helped me a great deal in understanding science and life better. I am extremely grateful for all of it. This journey wouldn't have been so without some great people I met along the way.

I would like to start by thanking my supervisor **Gunilla Karlsson Hedestam** for being the most integral part of my Ph.D. Thank you for guiding me in the best possible way. Your positivity and enthusiasm for science is simply marvellous and has been a huge motivating factor for me throughout. Thank you for challenging me when needed, helping me navigate through the ups and downs, and for just always being there. You have created an incredibly beautiful environment for aspiring minds to thrive and learn. I am glad to have experienced the world of science from you. Besides being the excellent scientist that you are, you have been a generous, kind human being, even when you did not need to be. I cannot thank you enough for that. I take great pride in being your student and hope to have your continued guidance even after my time at KI.

My co-supervisor **Karin Loré,** thank you for your kind guidance and presence during this journey. Thank you for giving me one of my first collaborative projects and for great scientific questions and discussions. You have always taken the time to be there whenever I needed expert advice and I am grateful for that.

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