Polyfunctional antibodies in viral disease

Detecting, controlling and preventing infections with antiviral antibodies

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CHAPTER
1

Introduction

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Disease-causing viruses repeatedly attack the human body during its lifetime. Our immune system and these viruses fight continuous battles. While the immune system undergoes training and develops memory, viruses mutate, recombine and emerge in new populations. Monumental discoveries and technological advances have led to the eradication of smallpox, while polio, rubella and measles are considered viable targets for eradication[1]. We possess a range of broad-spectrum medicines, some of which may even act against pathogens that remain undiscovered. Recent technological advancements have enabled the production of treatments and vaccines against novel pathogens at unprecedented speed and finesse[2]. However, viral diseases are still prevalent. There is a constant threat of viruses spilling over from animal reservoirs, exemplified by the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Moreover, viruses can re-emerge, as occurred with yellow fever virus and chikungunya virus, or migrate, which is happening for West Nile virus[3]. The emergence of SARS-CoV-2 has exposed our scientific, medical and regulatory shortcomings. It has sparked the establishment of pandemic preparedness programs, but it has also fueled vaccine hesitancy, skepticism about science, and occasionally, social unrest. Viruses are immensely diverse and cause an incompletely known variety of diseases. There is still much to learn about the mechanisms employed by viruses to enhance their effectiveness and evade immune responses. Continued research on viruses, viral disease and antiviral strategies is still incredibly important. We likely understand only a small portion of this arms race between viruses and the host immune system. Additional knowledge will be invaluable to (re-) invent alternative therapies and preventative strategies. This thesis, written in part during the COVID-19 pandemic, describes several novel findings related to antiviral antibodies.

Antiviral immunity

The human body's first line of defense against viruses is comprised of several cellular and biochemical defense mechanisms, collectively called innate immunity. This includes physical and chemical barriers, phagocytic cells, dendritic cells (DCs), natural killer (NK) cells, complement proteins and other blood derived proteins[4]. Adaptive immunity constitutes the next layers of antiviral defense. In contrast to innate immunity, adaptive immunity is a specific response which increases in magnitude upon repeated exposures. Adaptive immunity can be subdivided in cell-mediated immunity and humoral immunity. Innate immunity, cell-mediated immunity and humoral immunity collectively form a coordinated system of host defense against viral challenges[5] (Fig. 1).

The initiation of adaptive T cell immunity requires antigen capture by antigen-presenting cells (APC; such as dendritic cells). After antigen capture, APCs migrate to lymphoid organs to present these antigens to naive T cells leading to activation, proliferation and differentiation[6]. Precursor B cells migrate to lymphoid organs, where the matured naive B

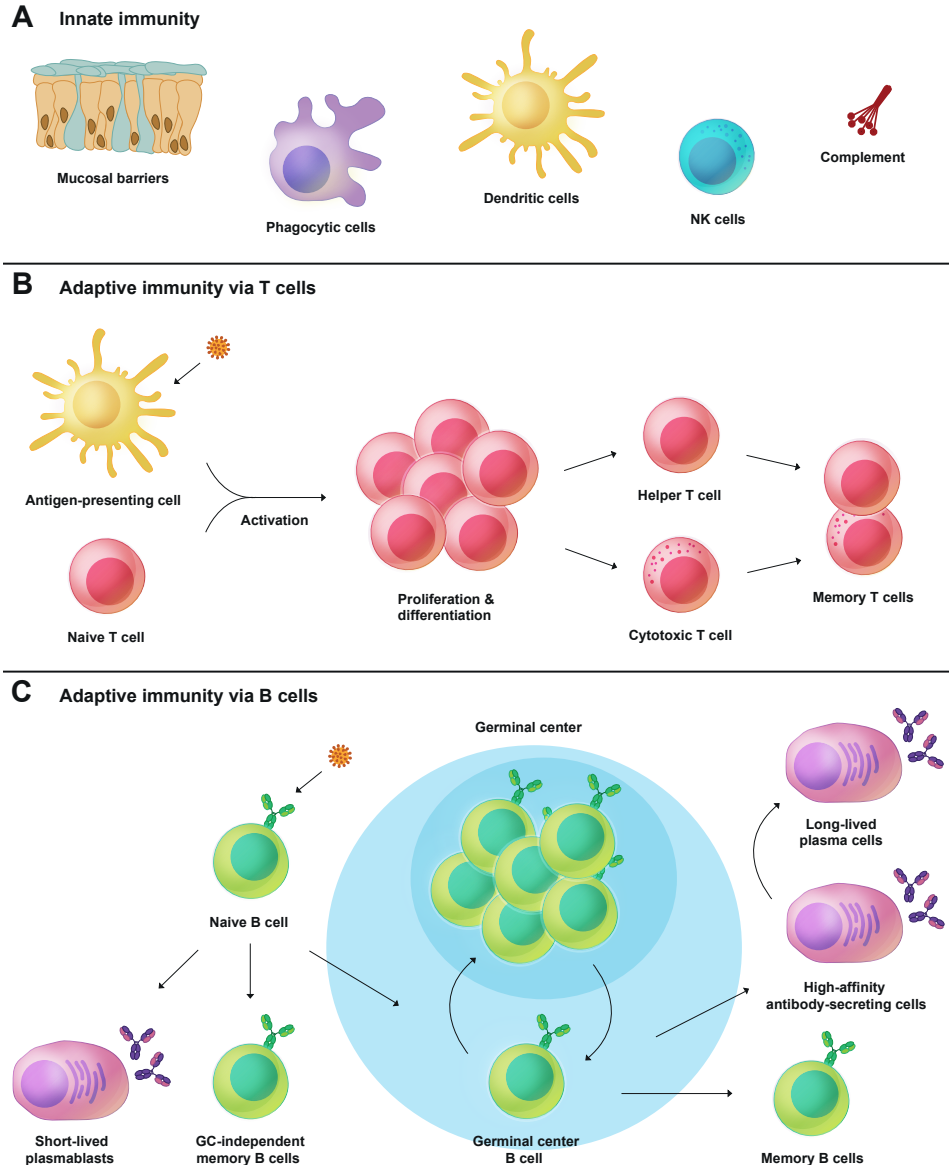

Figure 1. Overview of the mechanisms of innate and adaptive immunity. (**A**) Mucosal barriers, phagocytic cells, dendritic cells, natural killer (NK) cells and complement comprise five components of the innate immune system with relevant effects on viruses. (**B**) Simplified presentation of T cell immunity showing the activation and differentiation of T cells as well as the formation of T cell memory. (**C**) Simplified presentation of B cell immunity including the path generating germinal center (GC)-independent plasma cells and memory cells as well as GC reactions and the resulting formation of high-affinity plasma cells, memory B cells and long-lived plasma cells.

cells can encounter antigen. Naive B cells can interact with antigen on APCs, with antigen deposited on follicular dendritic cells, or they can be activated directly by unprocessed circulating antigens. Upon activation, the antigen-experienced B cells undergo clonal expansion and can follow one of two different paths (Fig. 1B). They can differentiate via a rapid extrafollicular response, leading to memory B cells and short-lived plasmablasts. These plasmablasts leave the lymph node and move to the circulation to secrete low-affinity antibodies[7]. Alternatively, activated B cells can move into the follicles of secondary lymphoid organs, interact with T follicular helper cells and form germinal centers. Germinal center reactions lead to B cells that produce antibodies with improved affinity and different functionalities[8]. During affinity maturation, the B cells with the highest affinity are selected while the clones with lower affinity undergo apoptosis. The resulting high-affinity B cells can become antibody secreting cells or memory B cells[9]. When a viral infection is resolved, most of the expanded immune cells undergo apoptosis. However, long-lived memory cells will remain in the circulation: a pool of antigen-specific cells that do not produce antibodies continuously, but can rapidly react to viral antigen re-exposure[6]. Memory cells are crucial for protection against previously encountered viruses. In addition, some plasma cells migrate to the bone marrow to continuously secrete low levels of antibodies in absence of stimulation. These long-lived plasma cells can provide life-long immunity[10]. The type of antigen, the affinity for the antigen, T cell help, prior exposure to the same antigen and the site of activation in the body all influence the quantity and the type of antibodies that are produced by B cells. This thesis will focus on humoral immunity mediated by antibodies (also called immunoglobulins). Antibody production is an essential part of effective antiviral immunity and crucial for protection from viral infections. Furthermore, antibodies form the protective basis for the vast majority of effective vaccines[11].

Formation and structure of antiviral antibodies

Antibodies are composed of two heterodimers of a heavy and light chain, which are linked by a disulfide bond. On one end is the antigen-binding region (Fab) and on the other end is the Fc region. These regions are linked by a flexible hinge (Fig. 2B). The Fab region is extremely variable to allow a nearly infinite amount of specificities. This variability is generated through V(D)J recombination and somatic hypermutation. V(D)J recombination already occurs during the early development of B cells and is therefore antigen independent. Antibody heavy chains are encoded by variable (V), diversity (D) and joining (J) gene segments. D segments recombine with J segments and their product recombines with V segments to yield thousands of possible combinations[12]. Antibody light chains are generated by recombination of V and J segments with hundreds of possible combinations. The result is millions of possible combinations of heavy and light chains. Antibody genes are further diversified by junctional diversity: mutations which are introduced when the gene segments are linked together. Together, this leads to the virtually unlimited pool

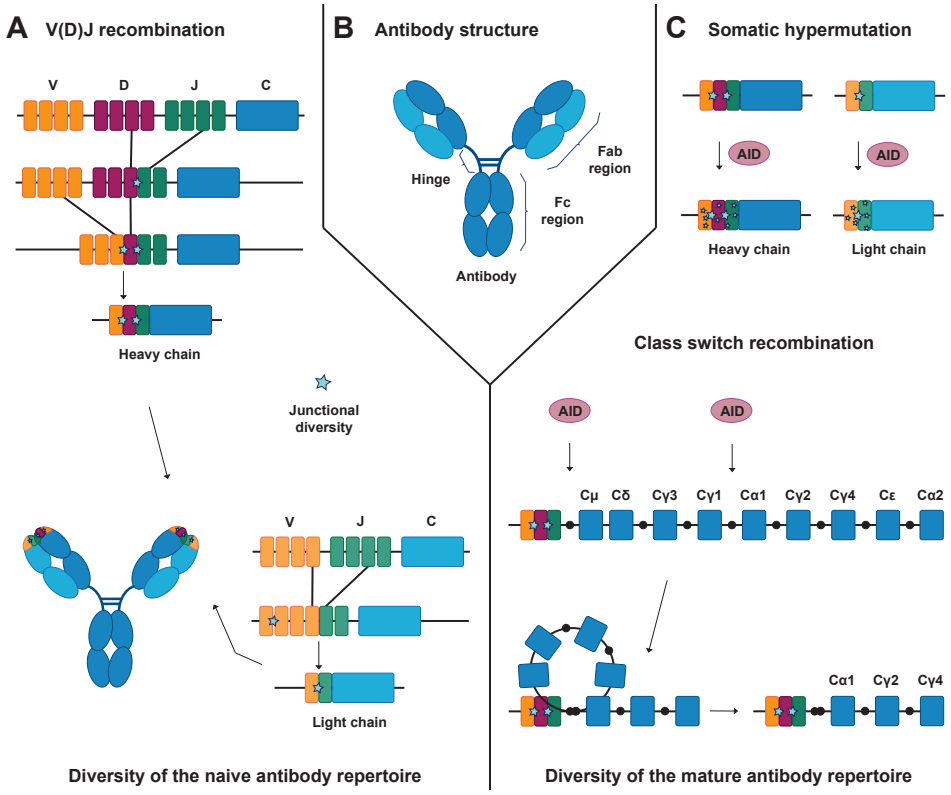

Figure 2. Overview of antibody structure and the mechanisms of the generation of diversity. (**A**) Overview of how the diversity of the naive antibody repertoire is generated. On the top of the panel, the process of VDJ recombination of heavy chains is shown, with first the recombination leading to the joining of a D and J segment followed by the second recombination leading to the joining of this DJ segment to a V segment and finally, the removal of unwanted gene segments to yield the final antibody sequence. On the bottom of the panel, the same process is shown for the light chain, which involves only a single recombination event between a V and J segment. During each recombination event, junctional diversity mutations occur which are indicated by the stars. On the left of the panel, the final antibody product is indicated with the recombined segments highlighted in different colors. (**B**) Structure of an antibody with the Fab region, the Fc region and the hinge region indicated. The light chain is shown in a lighter shade of blue than the heavy chain. (**C**) Overview of how the diversity of the mature antibody repertoire is generated. On the top of the panel, the process of somatic hypermutation (SHM) is shown, where activation-induced cytidine deaminase (AID) introduces mutations in the recombined VDJ gene. Mutations accumulate during iterative rounds of SHM and affinity selection in the germinal center. Mutations are indicated by small stars. On the bottom of the panel, the process of class-switch recombination is shown. AID induces double stranded breaks which lead to excision of unwanted antibody loci and joining of the next-in-line locus which will determine the new antibody class. In the example, the Cα1 locus will lead to the production of IgA1 antibodies. The full organization and order of human antibody loci is shown in the first step, but the sequence is truncated in the next steps.

of specificities of naive B cells (Fig. 2A). Further diversity is introduced after exposure to antigen. During somatic hypermutation within germinal centers, mutations are introduced by Activation-induced cytidine deaminase (AID) in so-called AID hotspots[13]. Both the heavy and the light chain contain three complementarity-determining regions (CDRs) each. Mutations in these CDRs change the antigen-binding properties of antibodies (Fig. 2C).

During class switching, a particular variable domain is combined with another Fc domain sequence. This results in an antibody with the same antigen specificity but of a different antibody isotype or subtype[12] (Fig. 2C). The Fc region dictates several functional properties of an antibody. Antibody half-life is determined by the affinity of the Fc region for the neonatal Fc receptor (FcRn), which is responsible for keeping antibodies in the circulation. The ability of the Fc region to interact with Fc γ receptors (FcγRs) determines the induction of antibody effector functions. The Fc region also contains a N-linked glycan, with a different composition per antibody class and an important effect on antibody functionality[14]. Next to these antibody classes, which are shared across all individuals, inherited gene polymorphisms cause further diversity between antibody Fc regions in the form of personal allotypes[15]. This allotype diversity also affects the functionality of the antibody response[16]. For some antibodies, the composition of the Fc region also affects the affinity of the Fab region[17].

Isotypes and dynamics of antiviral antibodies

The human antibody isotypes include IgG, IgA, IgM, IgD and IgE. Serum contains on average 75% IgG, 10% IgM, 15% IgA and very low levels of IgD and IgE[12]. IgD is mostly found on the surface of B cells. There is also a secreted form, but its function is not well understood[18]. IgE is associated with allergy and protects from parasites through interaction with the Fc ε receptor[19]. In this thesis, we will focus only on IgG, IgA and IgM, which are known to play important roles in viral disease (Fig. 3A). IgM appears during the early immune response. It is a multimeric antibody (mostly pentameric), has low affinity, and can be produced without T cell help[20]. IgG is produced after B cell class switching. It has high affinity and a long serum half-life, therefore it is generally maintained at high levels for months. There are four subtypes of IgG: IgG1 is the most prevalent (67%), followed by IgG2 (22%), while IgG3 (7%) and IgG4 (4%) are generally present at low concentrations in blood[12]. Since IgG3 is the first IgG subtype on the gene locus, it is produced early in response to viral infection[21]. Compared to other IgG subtypes, most IgG3 allotypes have a shorter half-life due to their lower FcRn affinity. IgG3 also has a longer hinge than other subtypes, leading to increased flexibility[22]. IgA is the main antibody type at the mucosa and it is also present in blood. Systemic IgA plays a key role in the early immune response, however it wanes relatively quickly. IgA can be multimeric or monomeric and has two subtypes: IgA1 and IgA2. IgA in serum is mostly monomeric and generally 90% is IgA1[23].

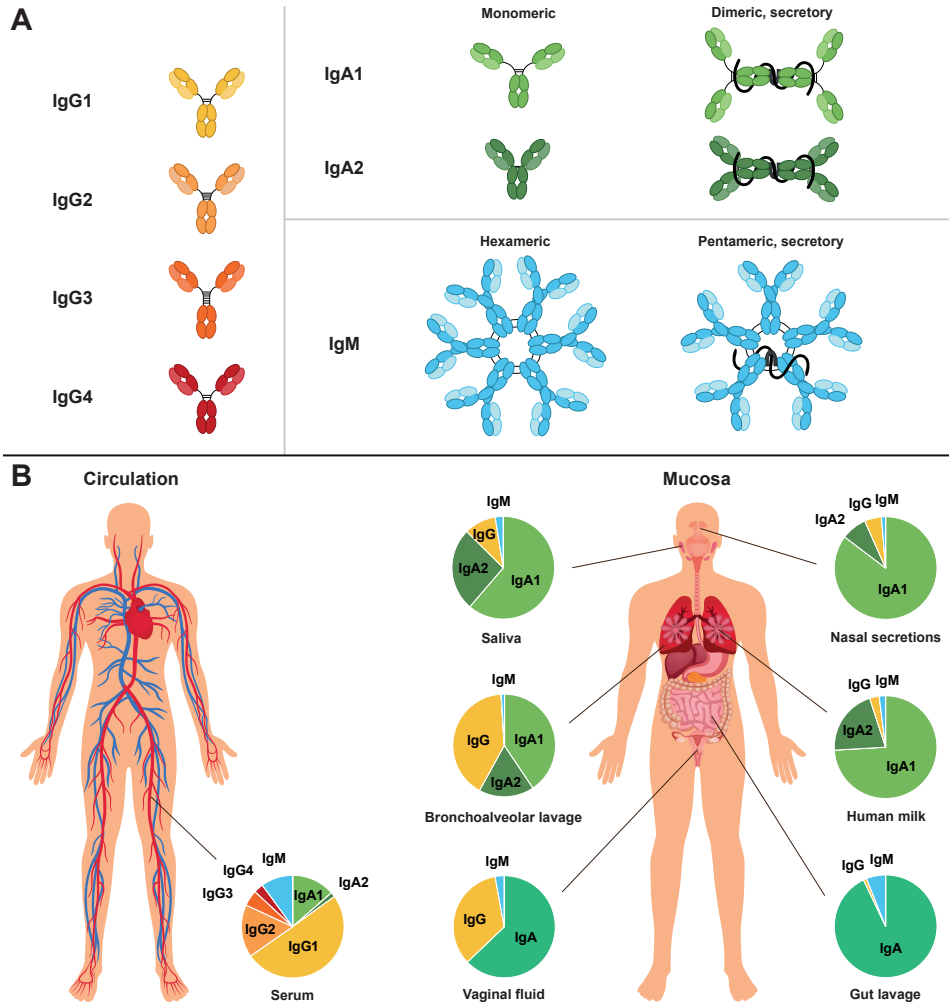

Figure 3. Overview of IgG, IgA and IgM isotypes and subtypes and their localization. (**A**) On the left of the panel, the structure of the four subtypes of IgG is shown. The IgG3 isotype has an elongated hinge region. There are different conformations of the IgG2 hinge possible, but we depicted only the version with four disulfide bonds. On the top right of the panel, the structures of IgA1 and IgA2 are shown in monomeric form and in dimeric form with the secretory component attached. However, non-secreted, dimeric IgA1 and IgG2 also occur. More rarely, IgA can also assemble into a multimeric form. IgA1 has a more extended hinge than IgA2. On the bottom right of the panel, IgM is shown in the hexameric form and in the pentameric form with the secretory component attached. However, non-secreted, pentameric IgM also occurs. (**B**) Overview of different compartments of the body containing antibodies and an overview of the different antibody types that occur in blood and various mucosal secretions. The relative composition of different isotypes and subtypes of antibodies is shown for serum, bronchoalveolar lavage[24,25], human milk[26,27], nasal secretions[28], saliva[29], gut lavage[30] and vaginal fluid[31]. Subfigure B has been created using images of the human body from Freepik.com.

Localization of antiviral antibodies

Mucosal surfaces constitute the main entry site of many infectious viruses. Therefore, local antibodies play a significant role in antiviral defense. Mucosal antibodies can be derived from serum or result from a local antibody response. Mucosa and secretory glands contain more than 80% of all antibody-producing cells in the body. These are mostly IgA-producing cells[32]. IgA is known to act as the first line of defense in the mucosa by coating pathogens and preventing their advance to the cells lining the mucosa. At mucosal surfaces, IgA is commonly present as a multimer (mainly as a dimer) and the IgA2/IgA1 ratio is higher than in serum[23]. Transport of IgA and IgM across the epithelial cells on mucosal surfaces is mediated by the polymeric immunoglobulin receptor (pIgR). After transport, pIgR leaves the secretory component (SC) on the secreted antibody, yielding secretory IgA (sIgA) or secretory IgM (sIgM). The SC can provide protection from proteolytic cleavage, which increases degradation resistance and leads to a longer retention time at mucosal surfaces[33]. IgG does not have a secretory form and is only transferred to the mucosa via transudation, which is a much less efficient process[34].

In the respiratory mucosa, most IgA production occurs in the upper respiratory tract (Fig. 3B). Primed antibody-producing cells preferentially migrate close to the tissue where they have been initially stimulated. Immune cells activated in upper respiratory tissue migrate to proximal lymphoid tissue and matured cells home back to the respiratory tissues (including the upper airway, nasal tissue and salivary glands) to secrete antibodies[35]. Most mucosal plasma cells are considered to be short-lived, however, evidence of a mucosal compartment of long-lived plasma cells is increasing[36]. Moreover, antibody secretion at mucosal surfaces is known to persist for months after infection[37]. Sampling of the respiratory tract can be extremely invasive. However, saliva is an easily collected mucosal secretion which resembles other mucosal sites in terms of antibody isotype distribution. Therefore, saliva is a useful tool to approximate the antibody composition of the upper respiratory mucosa. IgA constitutes the vast majority of saliva antibodies, while saliva IgG is derived from serum through passive diffusion[38]. Ninety-five percent of IgA is produced locally, by cells in the salivary glands, and transported by pIgR[39]. Monomeric IgA in saliva is mostly serum derived[38].

Mucosal antibodies are also abundant in mammary glands of nursing mothers: IgA can be up to 50% of all proteins in colostrum[12]. Antibodies in human milk are mostly produced by plasma cells which home to the mammary glands from other systemic and mucosal sites[40]. Neonates are not yet able to produce their own sIgA and sIgM. Therefore, human milk is the only source of mucosal antibodies during the first months of life[41]. When ingested by the neonate, sIgA, sIgM and IgG provide passive immunity by lining the gut, oral cavity and throat[42]. These antibodies function exclusively in the neonate mucosa, they

are not transferred to blood. Human milk antibodies are an important supplementation to antibodies transferred over the placenta during gestation. Breastfed infants are known to experience less respiratory infections and have a lower mortality risk compared to infants who received formula[43].

Virus neutralization by antibodies

Neutralizing antibodies (NAbs) are a strong correlate of protection for many vaccines, such as for yellow fever, smallpox, measles and SARS-CoV-2[44-47]. Virus neutralization is the independent ability of antibodies to block virus infectivity. This usually occurs by blocking important proteins on the virus surface which prevents interaction of these proteins with their cellular targets[48] (Fig. 4). For many viruses, neutralizing antibodies mainly target the site of the viral glycoprotein containing the receptor binding domain. However, also a variety of virus neutralizing antibodies that bind targets not implicated in receptor binding have been described. This can be mediated through steric hindrance, allosteric hindrance, dissociation of viral proteins, protein subunit shedding, inhibition of membrane fusion or inhibition of viral egress[49-52] (Fig. 4A). Since viral proteins are often metastable, the threshold for triggering conformational changes by antibodies is not very high[50,53]. Neutralization potency can also be affected by the antibody Fc domain composition. For example, for human immunodeficiency virus-1 (HIV-1), IgG3 and IgA were shown to be intrinsically more potently neutralizing than IgG1, likely due to increased flexibility[54-56].

Antibody-mediated effector functions

Antibodies can also provide protection from viruses via Fc-mediated effector functions (Fig. 4B). Importantly, antibody effector functions can clear virus particles and also lead to killing of infected cells[57]. These functions are mediated by interaction of the antibody Fc region with immune receptors and proteins. Antibody-mediated effector functions can be mediated by neutralizing antibodies or by non-neutralizing antibodies[58]. The structural composition of the Fc region determines the type of functions that are induced. IgG is the isotype that is most well-known to induce effector functions. IgG1 and IgG3 are the more functional IgG subtypes while IgG2 and IgG4 are generally weak at inducing effector functions or may even inhibit these functions[59]. While IgG3 is the most functional IgG subtype, it has a short half-life. This may be an important mechanism to prevent excessive inflammation[22]. The main antiviral functions of IgM are opsonization and complement fixation. IgA can activate complement and also trigger effector functions through the Fc α receptor[23,60]. Binding of IgG to Fc γ receptors (FcγR) on NK cells can initiate antibody-dependent cellular cytotoxicity (ADCC). IgG that interacts with FcγR on phagocytic cells can induce antibody-dependent cellular phagocytosis (ADCP). Antibody binding to C1q protein can induce complement activation which could result opsonization or in antibody-dependent complement-dependent cytotoxicity (ADCDC)[57]. FcγRs and C1q

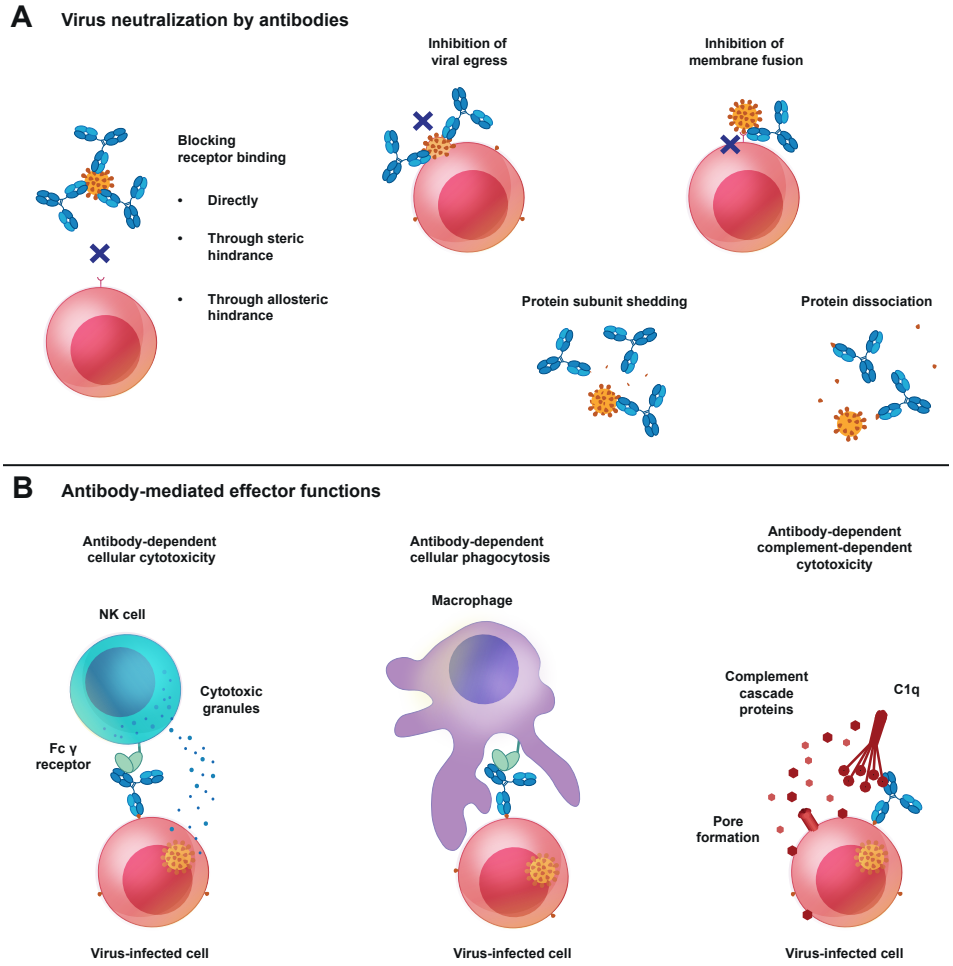

Figure 4. Mechanisms of antibody neutralization and antibody-mediated effector functions. (**A**) Antibodies can neutralize virus particles by blocking the interaction between virus glycoproteins and their receptors. This can be mediated directly, by antibodies targeting the receptor binding domain, or indirectly, by antibodies that target another epitope which results in steric hindrance or allosteric hindrance of receptor binding. Antibodies can also neutralize later in the virus life cycle by targeting essential processes such as membrane fusion or viral egress. Virus particles can also be rendered non-infectious by antibodies that mediate dissociation of trimeric protein or shedding of protein subunits. (**B**) Antibody-dependent cellular cytotoxicity is mediated when the antibody Fc region interacts with Fc γ receptors (FcγR), mainly on natural killer (NK) cells, which release cytotoxins that can kill infected cells. Antibody-dependent cellular phagocytosis is mediated through interaction of the antibody Fc region with FcγRs, for example on macrophages, which can phagocytose virus particles or infected cells. Antibody-dependent complement-dependent cytotoxicity is mediated by interaction of the antibody Fc region with the complement protein C1q. This can lead to activation of the complement cascade, resulting in opsonization of virus particles or infected cells. Cytotoxicity can be mediated by complement via the formation of pores in the membrane of infected cells.

protein need to be engaged by multiple Fc tails (at least two) in close proximity to be properly activated. This ensures that these functions are not induced by free antibody; the antibody needs to be bound to antigen to prevent untargeted effector responses[5]. Antibody-mediated effector functions have been implicated in protection against West Nile virus, influenza virus and HIV-1, among others[61-63]. Furthermore, antibody-mediated effector functions have been associated with control of malaria and reduced HIV-1 disease progression[64,65].

Antibodies against SARS-CoV-2 and other coronaviruses

After its appearance at the end of 2019, SARS-CoV-2 rapidly spread across the world. The resulting coronavirus disease 2019 (COVID-19) pandemic led to major healthcare issues and unprecedented socio-economic losses. At the end of 2022, over 6.7 million deaths had been attributed to the COVID-19 pandemic[66]. Within a year after the publication of the SARS-CoV-2 genome sequence, a rapid response across the globe resulted in the approval of the first effective vaccine and the rollout of mass vaccination campaigns. SARS-CoV-2 is one of the seven coronaviruses known to infect humans (hCoVs). The other hCoVs are SARS-CoV and MERS-CoV, which led to contained epidemics, and the common cold coronaviruses hCoV-OC43, -HKU1, -229E and -NL63, which are endemic and circulate seasonally[67,68]. The hCoV virus genome is internally packaged in nucleocapsid protein. This protein is not present in vaccines since it cannot lead to neutralizing antibodies. Therefore, nucleocapsid-targeting antibody detection has become a popular method to distinguish infection from vaccination[69]. Most SARS-CoV-2 vaccines are based on the Spike protein, which is considered the only target for a protective antibody response against hCoVs[70]. The Spike protein is a trimeric class I fusion protein that mediates cell entry. It consists of a membrane-distal S1 domain, which contains the receptor binding domain (RBD), and a membrane-proximal S2 domain, which contains the fusion peptide (Fig. 5)[71]. Many neutralizing antibodies bind to the RBD, which has also been the target site of many mutations across SARS-CoV-2 variants[72,73]. The S2 domain is more conserved and S2-targeting antibodies are more often cross-reactive[74]. HCoV infection induces antibodies that can cross-react with other hCoVs. Seroconversion studies have shown that seasonal hCoV infection may provide short-term protection from closely related seasonal hCoVs[75]. Although antibodies induced by seasonal hCoV infection may influence the incidence, course and severity of SARS-CoV-2 infection, they rarely cross-neutralize[76-78]. Since its original appearance, many variants of SARS-CoV-2 have emerged, often with reduced vulnerability to vaccine-induced neutralizing antibodies[79]. Moreover, protective neutralizing antibodies induced by vaccination or infection wane quickly. It is currently estimated that a yearly vaccination may be necessary to maintain protection, at least in vulnerable populations[80].

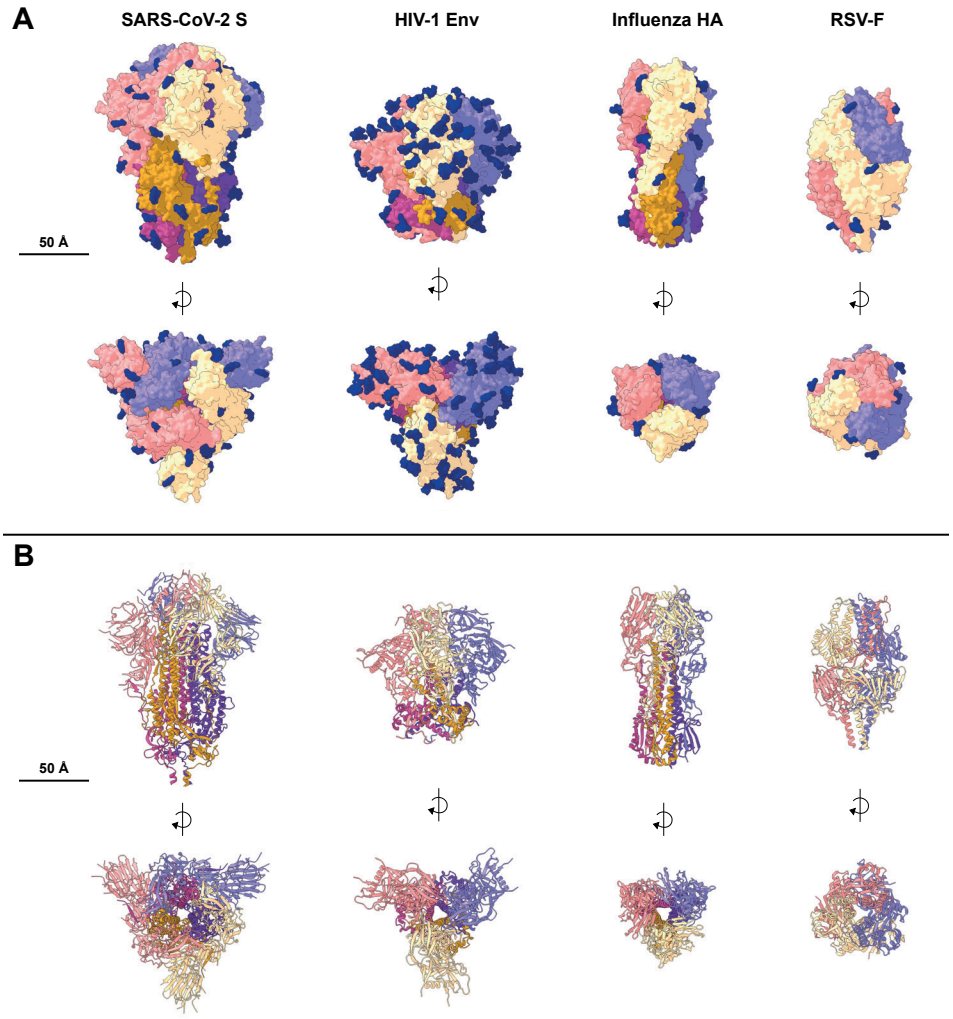

Figure 5. Structure of the main antigen of SARS-CoV-2, HIV-1, influenza virus and RSV. From left to right: the spike protein (S) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the envelope protein (Env) of human immunodeficiency virus-1 (HIV-1), the hemagglutinin protein (HA) of influenza virus and the fusion protein (F) of Respiratory syncytial virus (RSV). For each class I fusion protein, the individual protomers are shown in different colors. Side views and top views are included. For SARS-CoV-2 S, the S2 domains are shown in darker colors than the S1 domains. For HIV-1 Env, the gp41 subunits are shown in darker colors than the gp120 subunits. For influenza virus HA, the HA2 domains are shown in darker colors than the HA1 domains. (**A**) Density maps with glycans indicated in dark blue. Only the core N-acetyl-glucosamine groups are shown on the structure, therefore this only provides an indication of the abundance of glycosylation sites, not of potential differences in glycan composition between viral proteins. (**B**) Ribbon structures. Glycans are not included in this panel. Structures were visualized in UCSF ChimeraX v.1.6.1.[81] using Protein Data Bank IDs 6VXX, 5FYK, 4FNK and 7LVW.

Antibodies against HIV

Discovered in the 1980s as the causative agent of acquired immunodeficiency syndrome (AIDS), HIV-1 has led to a global epidemic. In 2022, there were approximately 1.3 million new HIV-1 infections and an estimated 630,000 people died from AIDS-related illnesses (https://www.unaids.org/). Despite over 40 years of intensive research, there is no effective vaccine or cure for this virus. Fortunately for the approximately 39 million people living with HIV-1, there is effective antiretroviral therapy (ART). When taken consistently, ART results in undetectable viral loads and prevents virus transmission[82]. However, ART can lead to long-term organ toxicity and HIV-1 viruses can evolve escape mutations[83,84]. Other factors contributing to the continuing spread of HIV-1 include poor access to therapy in some populations and unawareness of HIV-1 positivity. Therefore, the need for a vaccine or cure is evident. HIV-1 contains a single viral protein on its surface, the envelope glycoprotein (Env). HIV-1 Env is sparsely distributed and the only possible target of neutralizing antibodies[85]. It is a trimeric class I fusion protein consisting of three gp120 domains on top of three membrane-proximal gp41 domains[86] (Fig. 5). Neutralizing antibodies generally target the native, pre-fusion conformation of HIV-1 Env. An important target for neutralizing antibodies is the CD4 (receptor) binding site, since blocking this site prevents cell entry[87]. However, this epitope is difficult to access and shielded by the dense glycan shield of the Env protein[88,89]. HIV-1 Env contains six other known epitopes that can be targets of broadly-reactive neutralizing antibodies: variable loops 1-2, the variable loop 3 glycan patch, the gp120-gp41 interface, the fusion peptide, the silent face and the membrane proximal external region[90]. Interestingly, glycans are also involved in the epitopes of several NAbs[91]. The immense genetic variety in HIV-1 Env protein sequences is a major hurdle for the development of a protective vaccine. Antibodies induced by a single strain are generally not protective against other strains of HIV-1[92]. There are four groups of HIV-1 and group M is responsible for the global epidemic. Group M consists of 9 subtypes, each containing many different strains. Even in a single individual, different viral quasispecies are commonly found due to viral evolution caused by chronic infection[93]. During untreated HIV-1 infection, 10-30% of individuals develop broadly neutralizing antibodies (bnAbs) as their immune system chases behind the rapidly mutating virus over the course of years[94,95]. These bnAbs fail to eliminate the continuously mutating virus in the infected individual. However, they can protect from infection and delay viral rebound when passively administered to others[96-98]. Therefore, eliciting potent bnAbs is a major goal for the development of an eventual HIV-1 vaccine.

Antibodies against influenza virus and RSV

With distinct and overlapping winter seasonality, Respiratory syncytial virus (RSV) and influenza virus cause substantial morbidity and mortality. While healthy adults are minimally affected, both viruses are known to cause critical disease in young children,

the elderly and immunocompromised individuals. In the elderly, the mortality rate of RSV and influenza virus is comparable. In young children, seasonal influenza is less likely to be deadly while RSV can be life-threatening in the first two years of life[99,100].

RSV contains three viral membrane proteins: small hydrophobic protein (SH), glycoprotein (G) and fusion protein (F)[101]. Both G and F are essential for viral entry in cells. Since the F protein is more conserved and more immunogenic, this is the target of virtually all antiviral strategies under development for RSV[102] (Fig. 5). RSV has two antigenic subtypes; A and B, which can be further divided in different clades. While RSV poses a significant health burden, there is no cure and supportive care is still the best medical response. RSV is known to employ several immune-modulatory mechanisms that disrupt the development of immunity. Because of this, protection from re-infection is limited despite initially high antibody titers[103]. After a vaccine trial in the 1960s that failed because the vaccine aggravated disease, progress towards a RSV vaccine has been slow. However, in 2023, two vaccines against RSV were approved by the United States Food and Drug Administration (FDA)[104]. Both are subunit vaccines based on the pre-fusion stabilized F protein and both vaccines are effective against RSV subtypes A and B[105,106]. Furthermore, a monoclonal antibody injection was recently approved for prophylactic use in infants[107,108].

Influenza virus contains three viral membrane proteins: hemagglutinin (HA), neuraminidase (NA) and matrix protein 2 (MP2). HA is required for cell entry, while NA is implicated in release of virus particles[109,110]. The HA protein consists of a stem region and a head region (Fig. 5). While the stem is more conserved, the head is immunodominant and contains the receptor binding domain[111]. There are four species of influenza virus, but only influenza A and influenza B are generally problematic in humans. Influenza virus strains can mutate, leading to substantial antigenic drift and escape from immune responses[112]. Because of this, the strong strain-specific immunity elicited by infection is often irrelevant. Various vaccines are available against influenza virus. These vaccines are periodically updated with specific strains that warrant protection in the upcoming season, as determined by the World Health Organization (WHO)[113]. However, it remains difficult to accurately predict future strains. As a result, the current vaccines are far from optimal despite the frequent updates[114]. For decades, there has been considerable effort to generate an universal influenza vaccine that would be protective against many different influenza viruses. Currently, several clinical studies for universal influenza vaccines are ongoing[115]. Beside antigenic drift, antigenic shifts also occur. An antigenic shift is a major recombination event which can lead to new viruses with pandemic potential that are dangerous for people of all ages. Influenza has caused four pandemics since the beginning of the 20th century due to antigenic shifts[116].

Antiviral antibodies to detect, control and prevent infections

Antibodies can play multiple roles in antiviral strategies. They can be used as markers for viral infections and, with sufficient quantity and specificity, they can prevent infections from occurring. When infection does take place, antibodies can control or induce clearance of viral infections. The role of antiviral antibodies in prevention, control and clearance is also not one-dimensional, and a polyfunctional antibody response is likely a desirable response.

The first step towards countering viral disease is obtaining knowledge on the occurrence of infections. Antibodies are attractive biomarkers because they are usually plentiful in serum and can be easily detected. Antibody-based diagnosis is standard practice for HIV-1, hepatitis viruses and flaviviruses, among others. Moreover, antibodies possess a variety of properties that can provide additional information on the infection course and severity, as has been demonstrated for HIV-1 and SARS-CoV-2[117,118]. When enough information is available, it may even be possible to predict disease course by studying temporaneous antibody responses. This can be highly informative when considering treatment options. Finally, antibody assays are widely applied and the most cost-effective approach for immune surveillance. Immune surveillance allows tracking of infection- and vaccine-mediated population immunity to different viruses. This can identify areas where interventions are needed[119]. Moreover, our continuously evolving knowledge on the roles of different antibody features such as type, localization and specificity can provide opportunities for additionally informative surveillance in the future.

Antibodies are known to control viral infections by mediating clearance of virus particles and killing of infected cells. Antibodies can be passively administered to substitute or supplement an effective antibody response. This was shown to be effective for the control of Ebola and SARS-CoV-2 infection[120,121]. Antibody therapy is also being considered for HIV-1, and progress is being made to overcome the challenge of achieving prolonged viral suppression[122]. However, there is still much we can learn from mechanisms employed by our humoral immune system to naturally control and clear viral infections. Factors underlying either effective viral control or disease progression are still incompletely understood and new insights on these factors may lead to the development of better antibody-based treatment options.

Finally, antibodies also play an important role in protection against viral infection[123]. At sufficient dose, specificity, and precise timing, passively administered antibodies can also protect from viral infection[124]. However, this approach still lacks large-scale feasibility in many settings. Prophylactic antibody treatment is currently mainly attractive in specific scenarios of high infection risk with associated high mortality or morbidity[125]. Antibodies

also indirectly play an important role in the design of protective vaccines, with the recently developed RSV and SARS-CoV-2 vaccines are prime examples[126]. Structure-based vaccine design has become a popular strategy where the vaccine antigen is designed with the induction of a specific antibody profile in mind. For HIV-1, this strategy is of considerable importance in ongoing research. Sequential immunization schedules are being tailored to take B cells by the hand and lead them precisely on the path towards development of the most effective (potent and broad) antibody response[127]. Increased knowledge on protection by antibodies is vital for the success of these strategies. As our knowledge on the many antiviral roles of antibodies grows, so will our toolset to fight viral disease, with the hope that one day even HIV-1 will no longer be a threat to human health.

Scope of the thesis

The research in this thesis explores the many roles and functionalities of antiviral antibodies. In the first chapters of this thesis, we focus primarily on the role of antibodies as markers for respiratory virus infections and for population immunity screening. First, we measured different types of antibodies systemically and locally to clarify the value of studying mucosal antibodies in children. In **chapter 2**, we show an additional role for saliva antibodies in the detection of SARS-CoV-2 infection in children. Our outcomes demonstrate that a combination of serum and saliva testing provides the most complete overview of children with a SARS-CoV-2-specific antibody response. In **chapter 3**, we studied the prevalence of SARS-CoV-2-specific antibodies in children during a period of much higher infection prevalence. In this setting, we could clarify that most children that were positive for SARS-CoV-2 antibodies in serum were also positive for SARS-CoV-2 antibodies in saliva. Therefore, this chapter demonstrates that saliva antibody testing could be an attractive alternative for serum antibody testing to alleviate the need for venipuncture. Then, we broadened the scope to other human coronaviruses, influenza virus and RSV to study antibody levels after a period of reduced immune stimulation. **Chapter 4** reveals that antibody levels to other respiratory viruses such as RSV decreased during the restrictive measures of the COVID-19 pandemic. This antibody waning was apparent in human milk, suggesting that young infants may have received less antibody-based protection from respiratory infections due to the COVID-19 pandemic restrictions.

Antibodies can play an important role in the control or clearance of viral infections and we sought to investigate which antibodies are optimally equipped to mediate this. In the next chapters of this thesis, we investigated the functionality and breadth of HIV-1 specific antibodies during natural infection and of SARS-CoV-2-specific antibodies after infection and vaccination. Additional information on the mechanisms of viral control during natural HIV-1 infection, which is achieved only in some individuals, could lead to valuable insights. In **chapter 5**, we clarify which HIV-1 antibody features are associated with naturally

delayed disease progression. In this chapter, we present a profile of antibody features and functions to aim for in vaccine design. Moreover, this profile illustrates targets for future antiviral therapy and cure strategies. Both HIV-1 and SARS-CoV-2 consist of many different strains and variants, although the extent of this diversity differs substantially. Therefore, the breadth of the antibody response is an important factor contributing to viral control and protection from infection. In **chapter 6**, we explored whether SARS-CoV-2 antibodies might confer cross-protection against other hCoVs. Accordingly, we determined the levels of antibodies that are cross-reactive with all other hCoVs after SARS-CoV-2 infection and vaccination. Our demonstration of detectable cross-reactivity, which is mainly targeting the S2 domain, provides a basis for further research towards a pan-coronavirus vaccine.

Even for highly effective vaccines, it remains important to investigate to what extent antibodies are induced in different populations as well as factors such as antibody functionality, localization, and breadth. Therefore, in the next chapters of this thesis, we investigated how antibodies develop after vaccination against SARS-CoV-2 and HIV-1 and how we can steer the antibody response by vaccine design. While vaccines against SARS-CoV-2 have proven to be very effective, they are not equally immunogenic in all individuals in the population. It is vital to keep monitoring immune responses to new vaccines, especially for individuals with a compromised immune system. **Chapter 7** describes the immunogenicity of SARS-CoV-2 vaccination in people living with well-controlled HIV-1. In this chapter we demonstrate that both B and T-cell immunity are comparable between people with HIV-1 and controls. In **chapter 8,** we describe the waning of the immune response following two SARS-CoV-2 vaccinations. We observed that individuals with inborn errors of immunity do have a similar waning speed of B and T-cell responses, but that lower initial responses in some cohorts coincide with low immune levels six months later. Furthermore, individuals without an antibody response did not benefit from an additional third vaccine.

Even though current SARS-CoV-2 vaccines are highly effective, their efficacy was shown to be clearly reduced for novel SARS-CoV-2 variants that emerged over the past three years. Therefore, investigating ways to broaden vaccine-induced immunity is very relevant. **Chapter 9** describes the breadth of the antibody response induced by a virosome-based vaccine presenting the SARS-CoV-2 beta variant Spike. This vaccine actually resulted in a more narrow antibody response compared to the ancestral Spike, demonstrating that informed immunogen choices are necessary to achieve desirable vaccine breadth. Next to quantity and breadth of the antibody response, the functionality of the antibody response is also an important aspect to consider in vaccine design. After earlier demonstration that antibody effector functions are positively associated with natural HIV-1 control in chapter 5, we investigated the extent to which effector function-mediating antibodies are

elicited by vaccination. In **chapter 10,** we demonstrate the induction of several antibody-mediated effector functions by an HIV-1 Env trimer vaccine in a human clinical trial. One of the major findings was the observation of sex-specific differences in the magnitude of these responses. This chapter also shows that antibody-mediated effector functions can be predicted in animal models and that vaccine administration methods and adjuvant choice can modulate the functionality of the elicited antibodies.

Finally, in **chapter 11,** the results presented in this thesis, their implications and the remaining challenges are discussed.

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