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REVIEW

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Building a better antibody through the Fc: advances and challenges in harnessing antibody Fc effector functions for antiviral protection

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ABSTRACT

Antibodies can provide antiviral protection through neutralization and recruitment of innate effector functions through the Fc domain. While neutralization has long been appreciated for its role in antibody-mediated protection, a growing body of work indicates that the antibody Fc domain also significantly contributes to antiviral protection. Recruitment of innate immune cells such as natural killer cells, neutrophils, monocytes, macrophages, dendritic cells and the complement system by antibodies can lead to direct restriction of viral infection as well as promoting long-term antiviral immunity. Monoclonal antibody therapeutics against viruses are increasingly incorporating Fc-enhancing features to take advantage of the Fc domain, uncovering a surprising breadth of mechanisms through which antibodies can control viral infection. Here, we review the recent advances in our understanding of antibody-mediated innate immune effector functions in protection from viral infection and review the current approaches and challenges to effectively leverage innate immune cells via antibodies.

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Introduction

Antibodies are the effector molecules of the humoral immune system that circulate throughout the body, providing both short-term and long-term immunity against pathogens. The antibody molecule at its most general can be divided into the antigen binding fragment domain (Fab) and the antibody constant domain (Fc). The Fab domain confers the specificity of the antibody and the Fc domain interacts with Fc-receptor bearing innate and adaptive immune cells and effector molecules.

There are many roles that antibodies play in antiviral protection: blocking initial viral infection into host cells via neutralization, limiting dissemination of infection throughout host tissues, killing/clearing infected cells, initiation of inflammation, enhancing antigen presentation, enhancing development of T cell responses, and dampening of inflammation. Thus, while the induction of antibodies specific for a given pathogen is often used as a measure of immunity, the mechanisms through which antibodies can provide protection may differ significantly across pathogens. Simply the induction of an antibody response may be insufficient to protect if those antibodies do not have the specific features needed to provide protection against that pathogen; thus, the ability to provide protection is related to the *quantity* and *quality* of pathogen-specific antibodies.

As antibodies can limit viral infection through multiple mechanisms and cells, antibody-based therapies are increasingly becoming an attractive approach to treat viral infection, especially for viruses with high rates of mortality or cause severe disease. Most of the current approaches to identify and develop new antibodies focus on neutralization as the sole mediator of protection, yet the role of Fc domain is becoming increasingly appreciated as a critical feature of antiviral immunity, and thus, there is a growing interest in identifying approaches to harness and leverage the Fc domain. In this review, we will discuss the role of antibody-mediated induction of innate immune effector functions in protection from viral infection and review the current approaches that are under investigation to effectively leverage innate immune cells via antibodies.

Antibody isotypes and Fc-receptors

Humans have several different antibody isotypes, each serving unique roles in humoral immunity. Immunoglobulin G (IgG) is the most abundant antibody isotype in serum and is further classified into IgG subclasses (IgG1, IgG2, IgG3, and IgG4). The IgG subclasses differ in abundance with IgG1 representing approximately 60% of circulating IgG, IgG2 represent 32%, and IgG3 and IgG4 accounting for the rest. Immunoglobulin A (IgA) is the second most abundant antibody isotype in serum, although when accounting for levels within tissues and in mucosal secretions and saliva, IgA is the most abundant antibody in the body. IgA can be further classified into IgA1 and IgA2 subclasses, which differ mainly in hinge length, and can be produced either as monomeric in the serum or dimeric (two monomers connected by a secretory J-chain) in the mucosa. Immunoglobulin M (IgM) is the largest of the antibody isotypes, present as pentamers or hexamers, and is the first antibody to be generated in a humoral immune response. IgE and IgD are two additional isotypes with roles in allergic reactions and B cell maturation, respectively.

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Antibodies can activate innate immune cells via engagement of type I and type II Fc receptors (FcRs). Human type I FcRs include FcγRs, and FcαR, and type II FcRs include CD23/FcεRII and DC-SIGN[1]. The type I FcRs that engage IgG are the activating receptors FcγR1/CD64, FcγR2A/CD32A, FcγR3A/CD16A, FcγR3B/CD16B and the inhibitory receptor FcγR2B/CD32B; the FcR that binds IgA is FcαR/CD89; and IgE binds to FcεR/CD23. All innate immune cells express FcRs (monocytes, macrophage, neutrophils, dendritic cells, eosinophils, basophils, NK cells) in varying combinations and levels.[2] Moreover, the expression of FcRs is not limited to the classic set of innate immune cells – B cells, platelets,[3] a subset of γδ T cells,[4] and a small subset of resting and activated $CD4^+$ T cells[5,6] also express FcγRs – expanding the repertoire of cells that can respond to antibodies. Importantly, as different cell types express different levels and combinations of FcγRs/FcαRs, the combination of FcR expression, the balance of activating and inhibitory FcγR binding, cell type, and location together confer an incredibly diverse set of innate responses that can collectively shape antiviral immunity and disease (Figure 1).

The functional diversity is further amplified by antibody features that tune the interaction with FcRs: antibody isotype and IgG subclass, antibody glycosylation, and the Fab-epitope. The four human IgG subclasses differ in affinity for FcγRs with IgG3> IgG1> IgG4> IgG2,[7] thus the composition of a polyclonal IgG response can set different thresholds for induction of innate immune effector functions. Antibody glycosylation plays a notable role in further modulating and tuning IgG affinity for Fcγ-receptors in both directions, where fucose-free (afucosylated) glycans enhance affinity for the activating FcγR3A and FcγR3B,[8] and sialyation has been proposed as a mechanism to skew antibody affinity from type I FcRs toward type II FcRs.[9] The role of the Fab in further shaping Fc-FcR interactions is only beginning to be appreciated, whereby affinity of Fab-antigen interactions, distance from target cell membrane, valency of antibody-antigen binding and immune complex size can all impact induction of effector function.[10–13]

Activation of immune cells through type I FcγRs occurs via direct interaction between the FcγR and the Cγ2 domain of the human IgG that have been demonstrated through a number of structural studies (reviewed elsewhere in[14]). Antibody-mediated cross-linking of the activating FcγR induces signaling through a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) domain, ultimately leading to the induction of antibody-dependent effector functions, such as ADCC and ADCP, described in more detail below. Conversely, antibody-mediated cross-linking of the inhibitory FcγR2B acts to inhibit key activating molecules though through the cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) domain, providing a balance to activating FcγRs in innate immune cells. FcγR2B is the only FcγR expressed on B cells and serves to limit the B cell receptor (BCR) signaling and B cell proliferation in the absence of BCR engagement, a process critical to the generation of high affinity antibodies, B cell selection, and tolerance.[15,16]

Engagement of the type II FcRs CD23 and DC-SIGN have, to date, been primarily associated with negative regulation of immune responses, as demonstrated by the anti-inflammatory properties of intravenous immunoglobulin (IVIG).[17,18] An elegant model of IVIG-mediated amelioration of inflammation indicated that sialylated IgG-induced signaling through DC-SIGN in dendritic cells induces a cascade of events that ultimately leads to upregulation of the inhibitory type I FcγR2B on

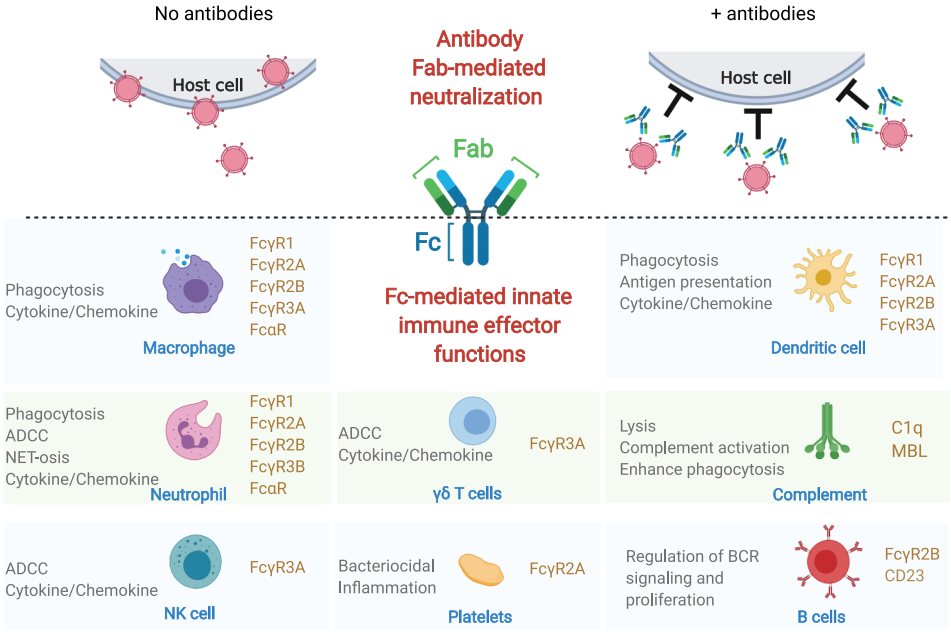

Figure 1. Protection from both ends of the antibody. Schematic representation of how both ends of the antibody can protect host cells from viral infection. On one end (above the dotted lines), antibodies can block virus entry into the cells by Fab-mediated neutralization. On the other end (below the dotted lines), the antibody Fc domain can recruit different Fc receptors (in light brown) and induce a variety of immune responses (in gray) by innate immune cells (names in blue) and complement.

effector cells to limit inflammatory responses.[17,19] Sialylated antibodies have also been proposed to activate CD23-mediated regulation of B cell responses through upregulation of FcγR2B.[20] The mechanisms through which IgG activates type II FcRs is still unclear, as whether IgG interacts directly with CD23 and DC-SIGN has been the subject of debate,[21–23] yet these studies highlight the interplay between type I and type II FcRs in shaping the immune response.

Direct and indirect Fc-mediated innate immune effector functions limit viral infection and dissemination

Direct Fc-mediated innate immune effector functions

ADCC

One of the primary effector functions that can directly limit viral infection is antibody-dependent cellular cytotoxicity (ADCC), which refers to the killing of target cells by innate immune cells. In humans, NK cells and neutrophils are the primary mediators of ADCC and can rapidly kill opsonized infected cells, and several studies demonstrate that FcγR3A-expressing γδ T cells are also mediators of ADCC.[4,24] NK cells in most individuals express a single Fc-receptor, FcγR3A, and thus FcγR3A is often considered as the "ADCC receptor." Measuring ADCC requires target cells that are expressing the pathogen antigen of interest and effector cells, such as primary NK cells or a modified NK cell line, such as NK92 cells expressing FcγR3A/CD16. Ideally, the target cell would be an infected cell or pathogen, yet the use of such target cells has traditionally been a challenge due to high containment requirements of many pathogens, variability between experiments, and are often low-throughput. As an alternative, many laboratories use reporter cell lines that express FcγR3A as an ADCC surrogate, although it should be noted that these assays do not measure cellular cytotoxicity, and thus may not accurately reflect *in vivo* ADCC activity. Similarly, even "true ADCC" assays that measure cellular cytotoxicity *in vitro* may not recapitulate *in vivo* ADCC activity, as seen in the context of HIV, where specific epitopes on the HIV protein gp120 are exposed when using recombinant antigen but are hidden or not exposed in the context of replicating virus.[25,26]

In mice, ADCC is predominantly mediated by macrophage and neutrophils rather than NK cells via the murine-specific FcγRIV,[27] highlighting conserved effector mechanisms but divergence in cell- and FcR-mediators between species. Following activation of immune cells via FcR ligation, the activated cell secretes cytotoxic molecules such as perforin and granzyme toward the target cell, leading to cell death. High-throughput assays using labeled target cells and primary human NK cells to measure ADCC have been described,[28] and NK cell activation but not cellular cytotoxicity can also be measured in the absence of target cells using antigen-coated immunoassay plates.[29,30]

ADCP

Antibody-dependent cellular phagocytosis (ADCP) by phagocytic cells such as monocytes, macrophage, dendritic cells, and neutrophils contribute to clearance of pathogens and infected cells. Following engagement of FcRs on phagocytic cells, the opsonized pathogen/target cell is engulfed into a phagosomal compartment, which then matures, acidifies, and fuses with the lysosome to ultimately kill the pathogen/target cell. Phagocytosis of antigen-coated target cells or beads can be experimentally measured in a number of different cell types,[31,32] and advances in multiplexing analysis allow for simultaneous measurement of phagocytosis of different immune complexes.[33] Similar to the challenges with measuring ADCC, the phagocytosis of labeled target cells or recombinant antigen-coated beads rather than infected cells or pathogens may not accurately reflect *in vivo* activity due to different antigenic targets that may be exposed *in vivo* as well as a myriad of other cellular signals on infected cells that may augment or inhibit phagocytic activity.

Monocytes, macrophages, and dendritic cells express high levels of FcγR2A, and as functional blocking of FcγR2A reduces ADCP in monocytes,[31,34] signaling via FcγR2A is often used in reporter cell lines as an ADCP surrogate, although it should be noted that these assays do not actually measure cellular phagocytosis. Moreover, different cell types may use different FcRs to induce phagocytosis. For example, neutrophil phagocytic activity is also mediated by FcαR and FcγRI upon activation.[35] Regardless of which FcR induces phagocytosis, as phagocytic cells are often the first cells at a site of infection, ADCP can be a potent mechanism to rapidly and specifically target opsonized pathogens and infected cells.

ADCD/CDC

Antibody-dependent complement activation and deposition (ADCD) or complement-dependent cytotoxicity (CDC) can enhance neutralization through lysis of infected cells and pathogens via formation of the membrane attack complex. ADCD can also augment phagocytosis through complement receptor CR3/CD11b/Mac-1 on phagocytic cells.[36] Antibodies predominantly activate the complement cascade via C1q and the classical pathway, with IgM and IgG3 having the highest complement-activating activity, followed by IgG1 and IgG2. IgG4 does not activate the complement cascade. ADCD can be measured through deposition of the main central complement component, C3, onto opsonized targets,[37] or through lysis of target cells in the presence of complement.[38]

Together, ADCC, ADCP, and ADCD have been the most commonly studied Fc-dependent mechanisms of viral control, yet a growing body of literature has uncovered the additional ways that antibodies can activate immune cells and induce effector mechanisms. For example, antibody-dependent cellular trogocytosis (ADCT) has emerged as a mechanism leading to infected target cell death whereby the Fc-receptor expressing effector cell, such as neutrophils and monocytes, "nibbles" on an antibody-opsonized target cell.[39,40] The "nibbled" part of the target cell may then be expressed on the surface of the effector cell, distinguishing trogocytosis from phagocytosis,[41] and during this process of "nibbling" and transferring there is significant mechanical disruption to the target cell membrane that can induce necrotic and highly inflammatory cell death, recently termed as trogoptosis.[40] Trogocytosis has been shown to be induced by HIV-specific antibodies[39,41] and SARS-CoV-2 spike-specific antibodies,[42] and plays a critical

role in antibody-mediated killing of cancer cells by neutrophils.[40] The specific role of ADCT controlling viral infection is still unclear, yet these studies have expanded our understanding of the breadth of mechanisms through which antibodies can induce killing of target cells.

Analysis of the role of antibody-mediated activation of FcR-bearing γδ T cells or platelets in infection has been limited to date yet may contribute to anti-pathogen control and disease outcome. For example, the expansion of FcγR3A expressing γδ T cells has been observed in the context of human cytomegalovirus (HCMV) infection[43] and in chronic malaria exposure,[44] and is associated with antibody-mediated restriction of viral replication in HCMV-infected cells. Bactericidal activity of antibody-activated platelets has been observed,[45,46] yet the role of platelets in viral infection has been more commonly associated with pathology. Most recently, hyperactivation of platelets via FcγR2A was observed in severe COVID-19 patients and was associated with cardiac dysfunction markers.[47] Future studies will likely uncover novel mechanisms by which these cells contribute to control or disease.

Indirect Fc-mediated innate immune effector functions

In addition to directly helping to control infection through targeting the pathogen and/or the infected cell, antibodies can also mitigate infection indirectly by inducing cytokine and chemokine secretion from innate immune cells that serves to activate antiviral pathways in uninfected target cells. For example, production of interferon gamma from antibody-activated NK cells and $CD16^{+}$ γδ T cells markedly reduced HCMV replication,[43] and stimulates type II interferon-mediated gene expression in neighboring cells, potentially limiting interferon-sensitive viruses. Activation of the complement cascade via antibodies induces not only lysis of infected cells but also the generation of the chemo-attractants and immunomodulators C3a and C5a resulting in influx of monocytes, macrophage and NK cells.

Sialylated antibodies have been proposed to help develop better humoral immune responses through increased delivery of antigen to antigen-presenting cells and setting higher thresholds for B cell activation, leading to the production of higher affinity antibodies.[20,48] In the context of B cells, the skewing of sialylated antibodies to bind to the type II FcR CD23 on B cells drives increased expression of the FcγR2B, increasing the threshold for B cell proliferation within the germinal center, leading to preferential expansion of high affinity antibodies.[20] Increased delivery of viral antigen to follicular dendritic cells can be enhanced via sialylated antibody-mediated activation of complement,[48] and the formation of immune complexes resulting from ADCC help generate long-term CD8+ T cell immunity via increased presentation by dendritic cells.[49]

As antibodies can limit viral infection through multiple mechanisms and cells, antibody-based therapies are increasingly becoming an attractive approach to treat viral infection. However, given the vast mechanisms that antibodies can engage immune cells, comprehensive approaches to define drivers of antiviral immunity and modulation of disease are critical to effectively and safely use monoclonal antibodies for infectious diseases. Along those lines, profiling approaches such as systems serology that measure qualitative and quantitative features of monoclonal and polyclonal antibodies have helped identify functional correlates of protection against a number of viral infections.[50–53] These correlates may be tested to determine if they are mechanistic correlates through antibody engineering aimed at enhancing/ablating specific Fc effector functions. Thus together, these approaches may help define how specific antibody functions contribute to antiviral immunity.

Passive immunity: functional features of effective antiviral immunotherapeutics

High-throughput isolation and identification platforms have accelerated antibody discovery for infectious diseases

There has been an explosive interest in the development of antibody-based therapeutics to treat a range of infectious diseases over the past 10 years, culminating in the recent FDA approval of monoclonal antibody cocktails for Ebola virus and emergency use authorization for SARS-CoV-2 infection.[54,55] Platforms to rapidly identify, clone, and characterize antibodies from B cells isolated from convalescent patients of various diseases and immunization of genetically engineered mice that express human immunoglobulin genes have accelerated the generation of antibody-based therapeutics.[56–58] Coupled with recombinant monoclonal antibody production, these new platforms can rapidly identify hundreds of virus-specific antibodies, sometimes even from a single convalescent donor,[59] that can be screened for neutralization, cross-neutralization, or binding to a specific viral epitope.[60]

These platforms provide the ability to rapidly produce effective therapeutics for use in an outbreak and were put to the test in the SARS-CoV-2 pandemic. Fortunately, these efforts were successful and resulted in the availability of several monoclonal antibodies in clinical evaluation and use within a matter of months. The success of these platforms has prompted efforts to develop panels of monoclonal antibodies against other highly pathogenic viruses with emergent pandemic potential, such as vector-borne viruses including bunyaviruses and alphaviruses.[61–65] Incidence of disease caused by vector-borne viruses threaten human health as the range of the host vector increases and spreads,[66–68] and coupled with high morbidity and mortality, the development of such therapeutics will be critical for the treatment of patients and add to the antiviral toolkit that we have to combat viruses.

While the Fab domains clearly need to be selected based on virus-specificity and neutralization potency, a common strategy to rapidly identify the necessary Fc features to enhance antibody efficacy may further accelerate development of antibody therapeutics. Single B cell sequencing and serum proteomic analysis approaches that are used in the high-throughput antibody discovery platforms enables the identification of the native Fab variable domain and IgG subclass/antibody isotype pairing, providing some insight into the natural function of isolated antibodies.[69] However, whether the same antibody features/effector mechanisms are required to control different

viruses is not clear, and more importantly, it will be critical to determine if enhancement of a specific function may contribute to immunopathology, disease or enhanced infection. The latter has already been demonstrated in the context of respiratory syncytial virus and dengue virus where activation of a subset of innate immune cells via specific FcRs contributes to more severe disease,[70–72] thus highlighting the critical importance of balancing protective and pathologic responses.

Lessons from monoclonal antibody therapeutics against viruses

Whether effector function is required for antibody-mediated protection is still being defined for many viruses, and thus defining and selectively enhancing specific functions required for protection against a given pathogen remains the next frontier for monoclonal antibody therapeutics. However, there are still many lessons to be gleaned from the current body of literature regarding the functional antibody features that contribute to protection (Figure 2). One of the most comprehensive analyses of the role of antibody effector functions has come from the study of monoclonal antibodies against Ebola virus, due in part to the sheer number of antibodies that have been cloned and characterized against this highly pathogenic virus and the collaborative work of the filovirus scientific community.

Lesson 1: enhancing effector function through the Fc can increase therapeutic antibody potency and efficacy

The development of monoclonal antibody therapeutics for Ebola virus stemmed in part from the need to have effective countermeasures for highly pathogenic viruses with high rates of mortality that could be used in the event of a known exposure. The first generation of antibodies that were evaluated for post-exposure prophylaxis included the neutralizing antibody KZ52, isolated from an Ebola virus survivor,[73] along with a handful of additional antibodies isolated from vaccinated mice that were subsequently chimerized to a human IgG1.[74] These antibodies targeted different regions of the Ebola virus glycoprotein, and importantly, cocktails of antibodies that consisted of either neutralizing and non-neutralizing antibodies together or non-neutralizing antibodies alone were able to provide post-exposure protection in non-human primate models[75,76] pointing to a role for Fc-effector function in antibody-mediated protection.

The rapid and widespread emergence of an Ebola virus outbreak in West Africa in 2013 highlighted the need for effective vaccines and therapeutics for treatment of human disease. One of the most attractive therapeutics to treat Ebola infection was monoclonal antibody therapy, due in part from the existing body of work showing post-exposure efficacy in animal models and the established clinical use of monoclonal

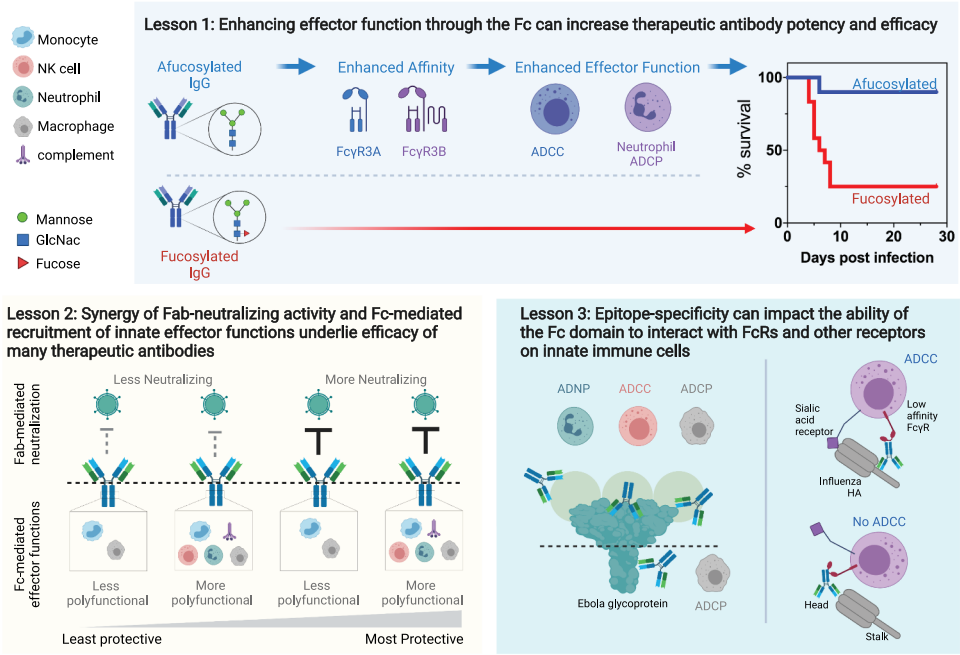

Figure 2. Lessons from monoclonal antibody therapeutics against viruses. *Lesson 1. Enhancing effector function through the Fc can increase therapeutic antibody potency and efficacy*. Afucosylated antibodies (above dashed line), indicated by the lack of fucose on the Fc glycan (red triangle), demonstrate increased affinity for FcγR3A and FcγR3B, which in turn confers elevated levels of effector functions such as ADCC and ADCP compared to fucosylated antibodies (below dashed line). In animal models, afucosylated antibodies (blue line) can show increased protective efficacy compared with fucosylated antibodies (red line).*Lesson 2. Synergy of Fab-neutralizing activity and Fc-mediated recruitment of innate effector functions underlie efficacy of many therapeutic antibodies*. Left to right: antibodies are ordered by protection in animal models, where antibodies that have less neutralizing/no neutralizing activity and low levels of effector functions are less protective than antibodies with less neutralizing/no neutralizing activity but high levels of effector functions. In the context of neutralizing antibodies, increased protective efficacy may be achieved by recruiting more Fc functions and neutralizing antibodies can maximize the efficacy through polyfunctional Fc profiles.*Lesson 3. Epitope-specificity can impact the ability of the Fc domain to interact with FcRs and other receptors on innate immune cells*. In Ebola, antibodies that bind to the top tier of the glycoprotein and/or the secreted glycoprotein (sGP) induce multiple effector functions, such as high levels of ADCC and ADCP; whereas those targeting the base of the glycoprotein were limited to phagocytic functions. In influenza, induction of ADCC requires interaction with the FcγR through the antibody Fc and a sialic acid receptor on the effector cell that binds to the head domain of hemagglutinin (HA). Antibodies that bind to the stalk domain of HA can induce ADCC activity as the sialic acid receptor is able to bind to the head domain of HA, but the interaction with the sialic acid receptor is blocked when an antibody is bound to the head domain, thus preventing ADCC.

antibodies for infectious diseases. Efforts by the scientific community to further the development of monoclonal antibody-based therapies led to the identification of new antibodies that displayed potent neutralizing activity and enhanced effector functionality,[59,77–82] ultimately leading to the approval of an antibody cocktail, Inmazeb, by the FDA for use in humans in October 2020 following clinical efficacy trials carried out in west Africa and in the DRC.[83,84] The sudden abundance of new monoclonal antibodies against Ebola offered an opportunity to systematically analyze and define the Fab and Fc features of protective antibodies against a lethal Ebola virus infection and to date, dozens of antibody panels have been evaluated,[51,59,81,85–88] leading to general conclusions regarding broadly neutralizing epitopes and the role of effector function in protection. Broadly speaking, polyfunctional antibodies are more likely to be protective in animal models with the importance of effector function increasing as neutralization activity decreases.[52] Specifically, the ability to induce multiple innate immune effector functions correlated with protective efficacy for monoclonal antibodies with moderate neutralizing activity, but not for antibodies with potent neutralizing activity,[52] highlighting the synergistic and diverse ways that antibodies can provide protection.

One of the current strategies to enhance effector functionality of monoclonal antibodies is through the production of afucosylated recombinant neutralizing antibodies in glycoengineered cell lines. Afucosylated antibodies demonstrate increased affinity for FcγR3A,[8] thus enhancing FcγR3A-driven effector functions such as ADCC. Production of afucosylated versions of both neutralizing and non-neutralizing Ebola antibodies boosted protective efficacy and antibody potency coincident with enhanced ADCC activity or binding to murine FcRs.[89,90] Beyond viruses, afucosylated cancer antibodies also show increased efficacy against cancer targets,[91,92] highlighting the broader utility of leveraging the Fc domain for therapeutic antibodies. Similarly, several recombinant monoclonal antibodies that encode point mutations within the Fc domain that increase affinity for multiple FcγRs display increased protective efficacy against HIV and influenza, leading to increased survival of challenged animals and accelerated viral clearance from infected tissues.[93,94] However, it must be noted that increasing FcγR3A does not always translate to increased protective efficacy *in vivo* and is monoclonal antibody, and disease/animal model specific. For example, afucosylation of the HIV broadly neutralizing monoclonal antibody b12 did not enhance pre-exposure protection against a mucosal challenge in a rhesus macaque model, despite *in vitro* enhancement of ADCC activity,[95] yet post-exposure therapeutic efficacy against an intraperitoneal challenge was enhanced via increased ADCC/ADCP accompanied by lower viral loads for a highly potent broadly neutralizing HIV antibody, 3BNC117, in a humanized mouse model.[94] Importantly, given recent associations between afucosylated antibodies and disease severity in the context of Dengue virus and SARS-CoV-2,[70,96–98] reducing interaction with FcγR3A may be critical to avoid monoclonal antibody-mediated immunopathology for some viral infections.

Lesson 2: synergy of Fab-neutralizing activity and Fc-mediated recruitment of innate effector functions underlie efficacy of many therapeutic antibodies

Monoclonal antibodies are often categorized into "neutralizing" and "non-neutralizing," with the implication being that the mechanism of protection is either neutralization or induction of effector function. Yet, there is an increasing appreciation for the role of collaboration between Fab and Fc-activities in protection. Ablation of FcγR interactions in HIV-specific broadly neutralizing antibodies resulted in reduced efficacy in non-human primates,[99] and the use of Fc-enhanced variants of broadly neutralizing antibodies displays enhanced viral clearance in humanized mouse models.[94,100] Importantly, it is becoming increasingly clear that the synergy of a neutralizing Fab and functional Fc domain likely underlies the protective efficacy of highly potent antibodies in the context of many different viruses, including Ebola virus,[52,77,87] influenza,[93,101] SARS-CoV-2,[102–106] and alphaviruses.[107,108] Of note, a recent study that was designed to quantify the contribution of effector function to antiviral efficacy of HIV-specific neutralizing monoclonal antibodies found that effector function accounted for up to 39% of antiviral activity across several different animal models.[109] Next generation antibodies for many viruses, including HIV and SARS-CoV-2, are increasingly being selected for potent neutralizing activity and some of these antibodies are able to provide complete prophylactic protection in the absence of Fc-effector functions.[103,110] However, it is important to note that the role of a functional Fc domain for neutralizing antibodies is likely most important in the context of post-exposure therapeutic settings, where infection has already been established, and thus clearing infected cells via Fc-dependent mechanisms in addition to preventing infection of new cells via neutralization is critical for protection. The latter situation was recently demonstrated in the context of SARS-CoV-2 monoclonal antibodies,[111] and highlights that different antiviral mechanisms that antibodies need to harness when administered before or after infection.

In addition to the production of antibodies in glycoengineered cells lines, recent advances in antibody engineering platforms have allowed for the pairing neutralizing Fab domains with highly functional Fc domains and provide an avenue to rapidly develop recombinant monoclonal antibodies with increased potency and directed effector functionality.[112] Thus, these production and engineering strategies, described in more detail below, allow for next-generation monoclonal antibodies that take advantage of both ends of the antibody.

Lesson 3: epitope-specificity can impact the ability of the Fc domain to interact with FcRs and other receptors on innate immune cells

With respect to IgG, there are two well-known features of the Fc that modulate effector function: IgG subclass and glycosylation. A majority of monoclonal antibodies are produced as a human IgG1 and are increasingly being analyzed for glycosylation or produced in cell lines to generate antibodies with homogenous glycosylation. Yet, differences in functional activity have still been noted between antibodies despite identical

subclass and glycosylation,[52] and these differences may be attributed to the antigenic epitope targeted by the antibody Fab domain. The epitope-skewing of effector function has been observed in the context of Ebola virus,[11,113] influenza,[12,114] and HIV,[115,116] adding another feature that can modulate the effector potential of antibodies.

In the context of Ebola virus, antibodies targeting different regions of the glycoprotein differ in the ability to recruit specific innate effector functions. For example, antibodies that bind to the top tier of the glycoprotein and/or the secreted glycoprotein (sGP) on average induce multiple effector functions, including high levels of ADCC and ADCP,[11] whereas those targeting the base of the glycoprotein were more limited in functional activity, recruiting phagocytic functions but not ADCC, despite identical IgG subclass and glycosylation.[52] One possible reason for this difference may be that binding to epitopes that are closer to the viral/cellular membrane constrain the Fc domain from engaging with FcγRs. In addition, another hypothesis is that the differences in FcR/cytoskeletal architecture and interactions between NK cells and monocytes/macrophages may further limit interactions between constrained Fc domains with FcγRs and may explain why potentially constrained Fcs can still induce ADCP but not ADCC.[10] As different epitopes are linked with differential induction of effector functions, the inclusion of antibodies that target different regions of the Ebola glycoprotein appears to be critical to the success and efficacy of monoclonal antibody cocktails. Specifically, the inclusion of polyfunctional antibodies targeting the glycan cap/sGP in addition to neutralizing antibodies are important in both the two-antibody version of the ZMapp cocktail,[117] and in the three antibody Inmazeb cocktail,[80] indicating the complementary and synergistic combinations of antibodies.

Epitope-directed effector function has also been noted in the context of influenza, where antibodies that bind to the stalk domain of hemagglutinin (HA) are able to induce ADCC activity whereas neutralizing antibodies that bind to the head domain do not.[12,118] The mechanism underlying this epitope specificity is that the induction of effector function against influenza requires not only engagement of the FcR but also interaction between a sialic acid-bearing receptor on the effector cell and the head domain of the HA, which may be occluded by neutralizing head-binding antibodies.[114] Interestingly, this two-contact requirement was restricted to low-affinity FcγRs, such as FcγR3A and FcγR2A, as HA head-binding antibodies can still induce activation via the high affinity FcγRI.[119] Thus, the anti-HA head antibodies likely selectively recruit effector function from cells expressing FcγRI, which include activated phagocytic cells (neutrophils, dendritic cells, monocytes/macrophages), but not NK cells.

FcR polymorphisms and FcR and innate immune cell dynamics during infection may impact therapeutic antibody efficacy and mechanisms of protection

Polymorphisms in the low affinity FcγRs, FcγR2A and FcγR3A, have been shown to alter IgG affinity for the respective FcγR. For example, a histidine at amino acid position 131 (H131) in FcγR2A notably increases human IgG2 binding over arginine (R131),[7] and valine at position 176 (V176) in FcγR3A increasing human IgG1 affinity over phenylalanine (F176),[7] leading to elevated levels of ADCP and ADCC, respectively.[120] These and other known FcR genetic polymorphisms can impact the efficacy of monoclonal antibodies in the context of cancer therapeutics.[121] Moreover, recent evidence suggests that polymorphism within FcγR2C, which is only expressed in approximately 20% of individuals,[122,123] was associated with decreased risk of HIV infection in the moderately protective RV144 vaccine trial,[124] yet was associated with infection risk following the non-protective Adenovirus 5-vectored HIV clinical vaccine trial.[125] Together these studies highlight that FcR variation between individuals is an additional factor that should be taken into account when considering antibody-based therapeutics.

While assays for *in vitro* characterization of antibody effector function have been established and have predictive value for *in vivo* protection, there remains the question of how infection impacts FcR expression and innate immune cell function that are critical to consider particularly in the context of post-exposure therapeutic antibodies. The effector function potential of antibodies is typically evaluated *in vitro* one function at a time using resting innate cells from healthy human donors or in cell culture, but infection itself may modulate abundance of FcR-expressing cells, FcR expression, and activation of innate cells, thereby altering the repertoire of cells that antibodies can leverage during infection. For example, activation of neutrophils leads to upregulation of the high affinity FcγR, FcγRI, which enables enhanced phagocytosis and ADCC activity mediated by monomeric antibodies.[126,127] In addition, *in vitro* polarized macrophages show distinct impacts on phagocytosis and reactive oxygen species (ROS) depending on the mechanism of polarization. Specifically, macrophages polarized by IFNγ (typically considered an M1-polarizing cytokine) displayed induction of higher levels of ROS despite lower phagocytic activity following antibody-mediated phagocytosis compared with macrophages polarized by IL-10 (typically considered an M2-polarizing cytokine),[128] indicating that the downstream indirect antibody-effector functions can be modulated by different cytokine milieus.

Developing a fever is the most common symptom of infection by any pathogen and is thought to aid the immune system in combating infection.[129] Beyond impacting the stability and infectious potential of the pathogen itself, fever and thermal stress have been shown to increase recruitment of both innate and adaptive immune cells to sites of infection[130,131] and enhance a broad range of anti-pathogen activities in innate immune cells, including respiratory burst, antigen processing, and phagocytosis.[132–134] Moreover, viral envelope structural dynamics are impacted by temperature, allowing for antibody access to temporarily exposed epitopes during viral breathing,[135] which may be further enhanced during fever. Thus, the efficacy of antibodies to mediate both neutralization and induce innate immune effector functions may be altered in the setting of fever.

Finally, as innate immune cells are often targets of viral infection, the loss of FcR-bearing cells impacts the efficacy of therapeutic antibodies. Ebola virus infection, for example, results in loss of non-classical FcγR+ monocytes early during infection which ultimately recovers,[136] and tracks with survival.[137] Thus, use of therapeutic antibodies that can

harness multiple different innate immune cells may be beneficial depending on the stage of infection the antibody is administered.

Convalescent plasma: polyclonal therapeutics

Passive transfer of polyclonal antibodies from convalescent individuals into patients with acute infection has long been an attractive possible alternative therapy to monoclonal antibodies. However, the utility of convalescent plasma in treatment of viral infection remains unclear. Early studies in the context of Ebola virus showed promise,[138] yet follow-up studies have yielded variable results,[139–142] but ultimately indicate that the efficacy of Ebola virus disease (EVD) convalescent plasma is correlated to antibody titers and high levels of neutralizing antibodies.[143] A majority of human survivors of EVD develop neutralizing antibodies, yet EVD survivors differ in the ability to recruit innate immune effector functions,[144] raising the possibility that some convalescent donor plasma may lack effector functionality, despite having neutralizing activity. Thus, a strategy aimed at screening donor plasma for at least one effector function in addition to neutralization may increase the utility and efficacy of convalescent plasma in the treatment of patients.

Passive transfer of convalescent plasma is being explored as a therapy for SARS-CoV-2,[145] yet to date, the correlates of convalescent plasma efficacy have yet to be defined. Interestingly, analysis of COVID convalescent donors demonstrated that only a small fraction of donors had polyfunctional antiviral activities,[146] further highlighting the need for comprehensive screening of donors prior to infusion.

As an alternative to donor-based convalescent plasma, production of human polyclonal antibodies in trans-chromosomal (Tc) cattle may offer a controlled source of polyclonal neutralizing and functional antibodies for use in patients with a number of different viral infections.[147–149] These genetically modified cattle express human immunoglobulin genes to produce human virus-specific antibodies following immunization with a viral antigen, and passive transfer of the Tc-derived antibodies into non-human primates were protective against Ebola virus, coincident with high levels of neutralizing activity and recruitment of effector function.[150]

Functional features of vaccine-mediated immunity

The development of vaccines against infectious diseases remains the most effective defense against localized and widespread incidence of disease. The induction of a polyclonal antibody response against a given pathogen via vaccination can target multiple protective epitopes that can limit viruses at multiple steps of infection and dissemination. Correlates analysis of vaccines against both bacterial and viral pathogens indicate that the ability to recruit innate immune effector function may be a critical component of vaccine-mediated protection,[151–153] and the functional humoral response can be shaped by vaccine vectors, adjuvants, and vaccine regimens.[154]

In the context of HIV vaccines, the induction of broadly neutralizing antibodies via vaccination has long been elusive. However, the modestly successful HIV trial RV144 induced ADCC-recruiting antibodies that were identified as correlates of protection.[152] In natural HIV infection, elevated levels of effector function-inducing antibodies have been found in elite and viremic controller individuals, suggesting that functional antibodies may play a role in natural control of HIV.[155,156] Thus, identifying strategies to boost the induction of functional antibodies has been of great interest to the HIV vaccine community.

Different prime-boosting strategies have been shown to impact IgG subclass selection which in turn affects the ability of antibodies to recruit effector functions. Specifically, repeated protein boosting regimens, such as those used in the non-protective VAX-003 trial, forced class-switching to IgG2 and IgG4, leading to less functional antibodies,[89,157–159] in contrast to the elevated IgG3 levels observed in RV144 linked to increased effector functions induced by a canarypox-vectored prime and two protein boosts.[157] However, subsequent boosts of uninfected RV144 trial participants with either vector or the protein alone failed to recall IgG3 responses, although functional IgG1 responses were recalled by the protein boost alone,[160] highlighting differences between the composition of the primary immune responses and long-term boosts.

Different viral vectors have been evaluated for the ability to skew induction of functional antibodies. Adenoviral vectors have shown promise across several different pathogens, and antibodies induced by the Adenovirus serotype 26 (Ad26) vector are polyfunctional in the context of both HIV and SARS-CoV-2.[153,161,162] The protective efficacy of the vesicular stomatitis virus (VSV)-vectored Ebola vaccine, Ervebo, is linked to the induction of antibodies,[163] and the functional activity of vaccine-induced antibodies is skewed toward ADCC rather than ADCP.[164] Interesting, while the vaccine is highly effective in non-human primates and in humans,[165,166] the functional activity of vaccine-induced antibodies is reduced when compared to natural survivors of EVD,[164] suggesting that the activity of vaccine-induced antibodies is sufficient for protection. Of note, vaccine-induced IgM is posited to play a critical role in neutralizing Ebola,[167] and thus, ADCD activation by IgM may further contribute to vaccine-mediated protection, yet to date, has not been reported.

Finally, vaccine adjuvants are used to boost magnitude, durability, and quality of the immune response, often acting through stimulation of innate immunity and antigen uptake by antigen presenting cells, and increasing evidence suggests that different adjuvants may qualitatively impact antibody function. For example, MF59 induced more ADCC-activating antibodies compared with alum in the context of influenza vaccination;[168] a toll-like receptor (TLR)-7 stimulating adjuvant induced production of antibodies with high ADCD activity; and a TLR-3 stimulating adjuvant induced polyfunctional antibodies.[168,169]

Approaches to modify effector function in monoclonal antibodies

Isotypes and IgG subclasses

Human IgG3 binds with the highest affinity to FcγRs, followed by IgG1, IgG4, and then IgG2.[7] Despite the higher affinity to FcγRs conferred by IgG3 and association with increased

pathogen control in natural and vaccine-mediated protection, virtually all monoclonal antibody therapeutics have been generated as human IgG1. Concerns regarding potential immunogenicity of different human IgG3 allotypes, short half-life, stability and purification methods have long hampered the investment in IgG3-based therapeutics.[170] However, advances in engineering have increased the stability and manufacturability of recombinant IgG3,[171,172] which may open the door to the development of IgG3-therapeutic antibodies with high levels of effector function for a range of infectious diseases.

There has been increased interest in the development of IgA-based prophylactics and therapeutics due to the enrichment of IgA at mucosal sites, which are common sites of entry for many pathogens.[173] Recent reports that IgA can neutralize SARS-CoV-2 with elevated potency compared to IgG1[174,175] have added to the growing interest in utilizing IgA-based therapeutics against viral infection. FcαR (CD89), and IgA-mediated activation of neutrophils results in phagocytosis, ADCC, and production of neutrophil extracellular traps against viruses such as Ebola virus and HIV.[176] Additional unique properties of IgA may further contribute to antiviral efficacy, especially for influenza virus. The conserved cytoplasmic tail of IgA that allows for the dimerization of two IgA molecules has blocks influenza virus entry via competition between binding to the sialic acid receptors that influenza uses as an entry receptor via sialylated glycans on the IgA tail.[177]

Glyco-engineering

IgG are glycosylated with biantennary glycan structures at a single site on each heavy chain of the Fc domain, and afucosylated glycans increase IgG affinity for FcγR3A by 100-fold,[8] resulting in elevated ADCC activity, but does not enhance binding to other FcγRs receptors. As a result, no major effect on monocyte/macrophage FcγR2A-mediated phagocytosis has been observed. Production of afucosylated recombinant antibodies in mammalian cell lines that lack the fucosyltransferase FUT8 or in *Nicotiana benthamiana* plants has shown to increase potency and efficacy of Ebola monoclonal antibodies.[76,89,90,178] Novel approaches such as adenovirus-associated virus delivery of short-hairpin RNAs to transiently knock down FUT8 with simultaneous expression of broadly neutralizing HIV antibodies offers an exciting new opportunity to deliver and sustain high levels of afucosylated antibodies *in vivo*.[179]

Agalactosylated antibodies, a well-established biomarker of rheumatoid arthritis[180] and HIV,[181] have been linked to increased phagocytic activity induced by HIV-specific antibodies[182] yet has also been shown to reduce complement-mediated inflammation via FcγRIIb.[183] As the role of antibody galactose in effector function has yet to be fully resolved, to date there has been little movement in the development of galactosylated antibody therapeutics but could represent a new avenue to modify phagocytosis and complement.

Point mutations

Mutational screens and co-crystal structure analysis of antibodies in complex with FcRs have identified critical amino acids in both the hinge and constant domains of the antibody Fc domain that alter binding affinity to FcγRs,[34,184–192] complement,[184,188,193–195] and/or the neonatal Fc-receptor (FcRn) to increase antibody half-life.[196–200] Engineering these mutations into monoclonal antibodies has allowed for the analysis of the role of FcR-mediated activation in infection, dissection of mechanisms, and highlighted potential opportunities to enhance efficacy of monoclonal therapeutics.

Several approaches have been developed to take advantage of these mutations, and the most recent platform, Rationally Engineered and Functionally Optimized Monoclonal antibodies (REFORM), utilizes a high-throughput cloning approach to rapidly generate Fc-variants, and a systems serology approach to profile effector functions across the panel of antibodies that then can be tested *in vivo* to determine the impact of specific effector profiles on antibody efficacy.[112] Using this approach in the context of Ebola virus revealed the role of antibody-dependent complement activation and moderate not maximal ADCC activity in combination with neutralizing activity in complete protection against disease and death in an animal model, highlighting that combinations and levels of induction of effector functions may be key to more precise and highly effective antibodies.[112] A similar approach based on the SARS-CoV-2 specific non-neutralizing antibody CR3022 demonstrated that while enhancing Fc effector function led to reduced viral loads, increased lung pathology and weight loss were observed,[201] further highlighting the critical need to balance protective and pathologic functions of antibodies and warranting the need to carefully select induction of specific effector functions.

Conversely, some viral diseases can be enhanced by Fc-dependent mechanisms, and thus strategies to ablate Fc-FcR interactions may be needed. For example, increased antibody function is associated with more severe disease in the context of Dengue virus infection,[70] and cross-reactive Dengue virus antibodies have been demonstrated to enhance Zika virus, a related flavivirus, infection.[202,203] Engineering of mutations to ablate effector functions in neutralizing antibodies such as L234A/L235A (LALA) or the glycosylation site N297, which disrupts Fc-FcR interactions, have been shown to block infection without increasing the risk of antibody-dependent enhancement of infection and disease.[202,204]

Translating the role of antibody effector function between animal models

It is worthwhile briefly discussing the challenges of translating the role of effector functions in controlling viral infection between different animal models and humans. There are significant differences in expression of FcRs across different species, often complicating the analysis of mechanism of action of therapeutic antibodies. Some innate functions, such as antibody-dependent complement activation, can be readily translated across species as the complement pathway is highly conserved,[205] but other functions are more difficult to translate. Mice express a unique FcγR, FcγRIV, which mediates ADCC in mice and is expressed on macrophage and neutrophils but not NK cells,[27] and non-human primates have multiple FcR polymorphisms that can modulate induction of effector functions.[206,207] Chimerized antibodies composed of human Fab domains with species-specific

subclasses is one approach to determining if effector function enhances protective efficacy,[208] but still does not provide direct translation to efficacy in humans. Transgenic expression of human FcRs in mice is another alternative to evaluate human IgG in mouse models,[187] yet is still in the setting of a mouse innate immune system and mechanisms of antibody-mediated protection may not directly translate to humans. However, these approaches have been fundamental in demonstrating the vast repertoire of functions that antibodies can mediate beyond neutralization.

Moving forward, development of bridging analyses to help translate findings between the most commonly used animal models for viral infection (mice, non-human primates, and guinea pigs) and humans are needed. Comparative analysis of human antibody binding to recombinant FcRs from other species has provided a preliminary framework for these bridging analyses,[112,209] but additional comparative analyses using innate immune cells from different animal model species are needed to fully develop cross-species maps to define mechanistic translatability across model systems.

Concluding remarks

Our understanding of the repertoire of antiviral effector functions that can be deployed by antibodies is constantly expanding and offers the opportunity to harness the antibody Fc domain to enhance efficacy and potency of monoclonal antibody therapeutics against viral infection. Despite the advances in experimental assays, production techniques, and antibody engineering technology that allow for modulation of antibody effector functions, there are still many unknowns that are critical to examine in order to safely and effectively direct innate functions. Specifically, defining the role of specific effector functions in antiviral control/modulation of infection and the impact of infection on expression of FcRs on innate cells will be critical next steps. Advances in antibody engineering technology together with a deeper understanding of the antibody and innate features that regulate antibody Fc-effector function will undoubtedly lead to the development of highly effective next generation antibody therapeutics and vaccines against viral infection in the future.

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