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Antibody-mediated protection against Ebola virus

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Abstract

Recent Ebola virus disease epidemics have highlighted the need for effective vaccines and therapeutics to prevent future outbreaks. Antibodies are clearly critical for control of this deadly disease; however, the specific mechanisms of action of protective antibodies have yet to be defined. In this Perspective we discuss the antibody features that correlate with in vivo protection during infection with Ebola virus, based on the results of a systematic and comprehensive study of antibodies directed against this virus. Although neutralization activity mediated by the Fab domains of the antibody is strongly correlated with protection, recruitment of immune effector functions by the Fc domain has also emerged as a complementary, and sometimes alternative, route to protection. For a subset of antibodies, Fc-mediated clearance and killing of infected cells seems to be the main driver of protection after exposure and mirrors observations in vaccination studies. Continued analysis of antibodies that achieve protection partially or wholly through Fc-mediated functions, the precise functions required, the intersection with specificity and the importance of these functions in different animal models is needed to identify and begin to capitalize on Fc-mediated protection in vaccines and therapeutics alike.

Introduction

Monoclonal antibody (mAb) therapeutics represent one of the fastest growing classes of clinically licensed drugs due to their exquisite specificity and tunable precision. Although immunotherapies for oncology and autoimmune disorders are well-established[1], there are fewer clinical applications of immunotherapy for infectious diseases[2,3]. However, due to their rapid discovery process, as well as their remarkable specificity and low toxicity, immunotherapeutics might be particularly valuable for emerging infectious diseases for which no other therapy is yet available[4–7].

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AUTHOR CONTRIBUTIONS
B.M.G. performed the glycan clustering analysis. B.M.G., S.L.S., J.C.M. and E.O.S. prepared the figures. E.O.S., S.L.S., B.M.G., and G.A. analyzed data, drafted and edited the manuscript. Funding was secured by E.O.S. and G.A.

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An antibody molecule links two hypervariable, antigen-binding domains (Fab fragments) via a flexible hinge to a constant region (Fc). After anchoring to a foreign antigen, Fab fragments can mechanically neutralize pathogens by physically inactivating the viral entry machinery through blocking receptor binding, preventing necessary cleavage events, or obstructing conformational changes that drive fusion of the pathogen and host membrane. Following Fab binding, the Fc domain links the target to the innate immune system to signal the presence of a threat and to mediate destruction of the foreign body or unhealthy cell. In the design of an immunotherapeutic agent, both ends of the antibody can be tailored to enhance antigen recognition by the Fab and to drive Fc-mediated immune responses in different directions, from dampened immunopathology ('silent Fc') to enhanced target clearance ('active Fc').

Characterization of Fab-mediated protection can be straightforward through the use measurements of binding stoichiometry and neutralization of authentic viruses or pseudoviruses. Structural analysis of the binding of Fab domains to an antigen can define specific contacts that contribute to antigen recognition and antibody function.

In contrast, measurement and visualization of Fc-mediated mechanisms is complex. Different functions can be orchestrated by Fc receptors (FcRs) expressed on different cells of the immune system, with each cell type able to deploy a unique set of anti-pathogen functions such as cellular cytotoxicity, phagocytosis, direct pathogen killing, and modulation/stimulation of innate and adaptive immunity, which can be further influenced by the tissues or compartments in which they are activated. Indeed, FcRs marshal an incredible variety of anti-pathogen responses[8–10].

Further, these immune-stimulating, Fc–FcR interactions have lower affinity than that of the often nanomolar Fab-antigen binding events, or are more transient than the latter and instead rely on avidity to reach a threshold at which the identified target is marked for destruction. These avid interactions require that antibodies of different subclasses and isotypes compete for immune complex occupancy to collectively regulate binding and activation. Thus polyclonal responses, rather than monoclonal responses, act in synergy to drive interactions involving larger complexes of proteins and cells that result in tiered signaling events that are difficult to directly observe using structural and biophysical techniques, although evolving methods such as correlation light electron microscopy could provide additional visual insights in the future[11,12]. Despite the challenges that accompany study of immune effector function, several examples from oncology and infectious disease have highlighted the importance of Fc activity by showing that the ability of an antibody to affect disease in animal models is dependent on Fc-mediated functions[13–20].

For many infectious disease targets, however, Fc-mediated clearance is or has been overshadowed by Fab-mediated neutralization, which is often the primary means for selecting mAb candidates for immunotherapy. Thus, fewer non-neutralizing, cell-targeting mAbs have been described or evaluated in animal models. Conflicting results in studies concerning filoviruses, which include ebolaviruses and marburgviruses, led us to critically examine the array of other antibody features and variables that together with neutralization correlate with *in vivo* protection. Our recently completed several-year, multidisciplinary

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analysis of antibodies against Ebola virus glycoprotein (GP) generated results that highlight the importance of Fc-mediated effector functions in post-exposure protection from filovirus infection in animal models. Here, we first describe the historical context in which the study began and how the findings relate to other recent work.

A combination of protection-driving variables

One of the first well-characterized mAbs against Ebola virus was KZ52, a human survivor antibody directed against the Ebola virus glycoprotein (GP)[21]. KZ52, initially described in 1999, appeared to possess the required features to be a successful immunotherapeutic against Ebola virus, including neutralization of Ebola virus in cell culture and high-affinity recognition of the receptor-binding core of the Ebola virus GP[22] (Fig. 1a), as well as protection of mice and guinea pigs following challenge with lethal doses of Ebola virus[23]. Nevertheless, passive delivery of KZ52 alone did not protect against Ebola virus infection of nonhuman primates (NHPs), and the animals succumbed to disease. This lack of protection was not due to mutagenic escape: viruses isolated at time of death or euthanasia were sensitive to KZ52 neutralization *in vitro*[24]. Further, treated animals retained 200–400 μg/ml KZ52 in sera at day 4 postinfection, and concentrations were estimated to be more than 100-fold higher than the IC_{90} at time of challenge and for the first few days after exposure.

Optimism for the prospect of Ebola virus immunotherapy increased when a study showed that polyclonal IgG concentrated from sera of vaccinated and challenged non-human primates (NHPs) could protect naive NHPs from infection with Ebola virus or the related Marburg virus[25]. Subsequently, two separate groups demonstrated that combinations, or “cocktails”, of mAbs could similarly achieve primate protection. One protective cocktail, termed ZMAb[26,27], contains three mAbs[27]: 1H3, which is poorly neutralizing and targets the glycan cap domain of the viral GP that is physically removed from the virion during cell entry, plus mAbs 2G4 and 4G7, which are both neutralizing. Interestingly, the epitopes of 2G4 and 4G7 overlap with that of KZ52[28] on the GP molecule (Fig. 1b), and all three of these antibodies are susceptible to the same escape mutation- a change from Gln to Arg at residue 508[29,30]. The other cocktail, MB-003[31,32] also contains three mAbs: 13C6 against the glycan cap[28,33], and 13F6 and 6D8 against the mucin-like domain[22,34,35] (Fig. 1c). Protection of NHPs observed with MB-003 was thus somewhat surprising, as none of the component antibodies were strong neutralizers and all three antibodies targeted epitopes located on the upper and outer heavily glycosylated regions of GP that are cleaved from the virion during entry[36,37]. The physical removal of these epitopes from virions prior to receptor binding likely explains their poor to no neutralization in cell culture. Their success *in vivo*, however, suggested that their protective capacity could arise instead through Fc-mediated effector functions that tag GP-expressing virions and infected cells for destruction. Notably, whereas MB-003 comprising mAbs expressed in Chinese hamster ovary (CHO) cells offered poor protection to NHPs, MB-003 comprising mAbs produced in a plant-based system involving transgenic *Nicotiana benthamiana* resulted in greater survival of NHPs following Ebola virus infection. MB-003 expressed in these plant cells could also be used at a three-fold lower dose[31,38]. Transgenic *N. benthamiana* attach a truncated mammalian glycan of afucosylated and agalactosylated structure[39], which enhances cell targeting functions[38–42,43–46]. Afucosylated antibodies, in particular, have 50-fold higher affinity for

both FcγRIIIa and FcγRIIIb and increased antibody-dependent cell-mediated cytotoxicity (ADCC) activity[43,44,47–49]. Together, these early findings suggested that neutralization is not the sole predictor of *in vivo* success, and that multiple mechanisms, including cell targeting by Fc, contribute to Ebola virus protection.

In 2014, the six mAbs from the ZMAb and MB-003 cocktails were re-mixed to produce ZMapp, a cocktail that reverted advanced disease and conferred survival to NHPs[50]. ZMapp combined 4G7 and 2G4 from ZMab[50] with 13C6 from MB-003 (Fig. 2a) and differed from the predecessor cocktails in two important ways. All three antibodies were chimerized to attach a human IgG1 Fc to the murine Fab rather than the original murine IgG1 or IgG2a Fcs. Secondly, the component antibodies were produced in *Nicotiniana* with afucosylated/agalatosylated glycans. The change in Fc structure and glycosylation may be linked to the cocktail's success relative to earlier antibodies from which it was derived.

Two novel human antibody therapies have now also been placed on the WHO list of investigational therapies for use in the 2018 Democratic Republic of the Congo (DRC) Ebola virus outbreaks (http://www.who.int/emergencies/ebola/MEURI-Ebola.pdf?ua=1). These include a mAb monotherapy from NIAID (mAb 114, a.k.a. VRC 608)[51] (Fig. 2b) and a three-mAb cocktail from Regeneron (REGN 3470–3471-3479, a.k.a. REGN-EB3)[52] (Fig. 2c). Both mAb 114 and the REGN-EB3 greatly promoted survival of non-human primates when delivered five days post-exposure.

mAb 114 demonstrates both neutralization and Fc-dependent cell-targeting activities *in vitro*[51]. In the VIC study, described in more detail below, all but one of the antibodies in the GP1 head-binding epitope group to which mAb 114 belongs exhibit both neutralization and cell-targeting functions[29]. Thus, the head epitope appears to exist at the physical intersection of those regions of GP that drive neutralization with the regions of GP that exhibit greater activation of phagocytosis and natural killer (NK) cells.

In contrast to the mAb 114 monotherapy, the three mAbs in REGN-EB3 provide complementary activities across the three antibody footprints. One component, REGN 3479, recognizes the conserved GP2 fusion loop and is neutralizing. A second component, REGN 3471, recognizes the outer glycan cap and has cell-targeting functions. The third, REGN 3470, binds the GP1 Head and offers both neutralizing and cell-targeting activities, including FcγRIIIa and other FcγR-related functions[52]. Thus, all three candidate immunotherapeutic treatments for Ebola virus incorporate a mix of neutralizing and cell-targeting functions, and bind to a mixture of neutralizing and cell-targeting epitopes (Fig. 2c). These newer candidate therapies were produced for NHP studies in 293Freestyle cells (mAb 114)[51] and modified CHO cells (Regeneron)[52].

A Comprehensive Study

In 2012, after the first protection results were described, we wondered if the neutralizing, but non-protective KZ52 and the poorly neutralizing, but more protective MB-003 examples were outliers or were typical features of many antibodies. We aimed to better determine, on a more statistically relevant scale, what kinds of antibodies or antibody features correlate

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with protection in animal models of Ebola virus infection. We also wondered what *in vitro* assays would best forecast *in vivo* protection, information which could streamline the research pipeline to identify or rationally design candidate therapeutics more quickly. Cognizant that each research group had their own assays and criteria for identifying, downselecting and producing antibodies, and that we needed to understand the range of antibody behavior, researchers in the filovirus field pooled their intellectual and physical resources into a single collaborative study.

Over 43 academic, industry and government laboratories across five continents united to form the Viral Hemorrhagic Fever Immunotherapeutic Consortium (VIC)[53], supported by a Center of Excellence in Translational Research grant from the National Institute for Allergy and Infectious Disease (NIAID). This consortium performed a large, multidisciplinary study of filovirus antibodies isolated by multiple laboratories and produced by multiple methods. Some participating research laboratories contributed one or a few favorite antibodies (often selected based on neutralization), while other groups contributed a larger array of different samples. All antibodies were code-named to protect the investigators' ability to publish findings from their own antibodies, many of which were not yet described in the literature.

This recently completed project[29] analyzed 171 donated mAbs across a battery of *in vitro* and *in vivo* assays encompassing biochemistry, structural biology, glycan sequencing, neutralization, measurement of immune effector functions, and protection in the mouse model for each antibody in the study pool. Epitopes were determined by ELISA and structural biology at Scripps Research, and by alanine scanning at Integral Molecular. The neutralization activity of each mAb in the panel was measured by three different assays involving: (i) authentic Ebola virus at biosafety level (BSL)-4 (USAMRIID); (ii) a biologically contained Ebola virus (VP30) for which an essential replication complex component, VP30, is expressed in trans at BSL2/3 (University of Wisconsin)[54]; and (iii) at BSL-2 recombinant vesicular stomatitis virus (rVSV) engineered to display Ebola virus GP on its surface (Albert Einstein College of Medicine). For the rVSV assay, the fraction of viral particles left un-neutralized at maximal antibody concentration was also analyzed[55]. In the rVSV and VP30 systems, infected cells were quantified using a reporter gene inserted into the pseudoviral genome. In the authentic Ebola virus assay, infected cells were quantified by detection of KZ52 binding.

For immune effector function, we evaluated seven parameters for each of the 171 mAbs: the ability of human and murine phagocytes and neutrophils to engulf particles bearing Ebola virus GP trimers upon incubation with a given mAb (four different measurements); and the ability of each mAb to activate human natural killer cells (three measurements- cell surface expression of CD107a, secretion of the inflammatory chemokine MIP1-β (CCL4) and cytokine interferon γ (IFN-g)[56,57]).

Protection was evaluated in the mouse model for Ebola virus infection at both USAMRIID and the Public Health Agency of Canada (PHAC). To measure protection, groups of 10 mice were infected with mouse-adapted Ebola virus and treated 2 days after infection with a given mAb from the panel. Survival and body weight were monitored for 28 days and the percentage of mice surviving was determined. mAbs that resulted in 6 or more mice

surviving (60%) were deemed protective. Machine learning approaches at Scripps, Ragon, and Los Alamos National Laboratory established correlation networks illustrating how antibody features related to each other and to *in vivo* protection in the mouse model[29].

Several relationships were clear. First, epitopes linked to neutralization of virions in cell culture are predominantly located on the receptor-binding core of the glycoprotein that is retained during virus entry (Fig. 3a). Second, epitopes in the study linked to maximal phagocytic functions are predominantly located in the uppermost regions of GP, including the head, glycan cap and mucin-like domains (Fig. 3b). Antibodies with strong NK cell activity, however, are distributed more broadly across the glycoprotein surface.

Physical clustering of neutralization-linked epitopes on the GP core and phagocytosis-linked epitopes on the upper, heavily glycosylated domains suggests that neutralization functions operate on virion-surface GP, while Fc-mediated phagocytic functions operate on cell-surface GP on infected cells (Fig. 3c). Fc-mediated NK cell functions are spread more broadly. Those mAbs that anchor to the GP core may interfere with steps involved in viral entry: receptor binding, cathepsin cleavage, and fusion[58–61]. In contrast, antibodies bound to glycan cap and mucin-like domain epitopes on virion-surface GP would be removed by cathepsin cleavage of GP in the endosome[37,62–65], but remain on GP molecules displayed on the surface of infected cells. Enhanced phagocytic activity by antibodies bound to these upper regions may result from their greater accessibility to Fc receptors, and/or greater structural flexibility at these epitopes that facilitates multivalent binding, Fc-Fc interactions and immune cell recruitment.

The glycan cap and some GP1 head epitopes are also found on an abundantly secreted dimeric form of the GP, termed sGP. The role of sGP with respect to antibodies remains unclear, but it has been proposed to preferentially adsorb antibody, redirect the antibody response[66] or form immune complexes[33]. Unexpectedly, across the VIC pool, sGP cross-reactivity was neither beneficial nor detrimental to the ability of an antibody to offer protection. Further, those mAbs that cross-react to sGP unexpectedly performed similarly in neutralization assays, whether sGP was present in wild-type amounts (authentic Ebola virus and VP30 Ebola virus) or absent (rVSV). We did note in the VIC study that sGP–cross-reactive antibodies could activate FcR-bearing immune cells. Since sGP is secreted from infected cells, such sGP-containing immune complexes would not directly tag infected cells for destruction, but could form immune complexes that stimulate protection in other ways.

We opted to unblind KZ52 and the murine versions of 13C6, 2G4 and 4G7 to provide reference points. KZ52 is VIC 136, a human IgG1, and was produced for this study in CHO cells[24]. 4G7 and 2G4 in their original murine versions are VIC 18 and 24, murine IgG2a and IgG2b, respectively and were produced in hybridoma culture.

In the VIC study, KZ52, 2G4 and 4G7 all exhibit equivalent neutralization activity, but lower effector function scores than other antibodies analyzed, which included two of seven possible Fc-mediated functions. In contrast, cell-targeting activity for the ZMapp versions of 2G4 and 4G7 was likely improved by the glycan modifications afforded by production in *Nicotiniana*[50].

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Correlates of in vivo protection

In the overall VIC study on these and other antibodies in the field, our expectation from prior work on HIV-1 and other viruses was that neutralization would be a strong forecaster of protection[67–72]. Of the 171 mAbs in the VIC panel, 20 (12%) robustly neutralized in every assay and also conferred strong levels of protection in the mouse model. Measures of neutralization were the strongest univariate predictors of protection in the study (ρ=0.61–0.68). Neutralization alone, however, was an incomplete predictor of *in vivo* success. Eight mAbs in the study (5%) neutralized potently and robustly, but failed to protect *in vivo*. Another nine mAbs in the study (5.4%) neutralized either poorly or not at all, yet did protect *in vivo* (Fig. 4). Of those nine, three offered high levels of *in vivo* protection in the absence of any measurable neutralization in any assay. The remaining six neutralized in some assays, albeit weakly (only 55% reduction in infected cells at 20 μg/ml compared to 99.5% reduction in infected cells at 0.5 μg/ml by more robustly neutralizing samples). For these nine antibodies, the ability to confer post-exposure protection in the mouse model was not predicted by their capacity to demonstrate *in vitro* neutralization.

We note that researcher choice in antibody selection could have biased the composition of the VIC study pool. *In vitro* neutralization, followed by *in vivo* protection in a mouse model are the two most common primary initial selection criteria in filovirus research. Those labs that contributed just a few antibodies tended to contribute neutralizing antibodies. We expect that those neutralizing antibodies already known to succeed in *in vivo* protection would be more likely to be contributed to the study than neutralizing antibodies already known to fail in *in vivo* protection. Such selection processes could amplify apparent correlations of neutralization to protection in our results. Further, if most labs first winnowed antibodies based on *in vitro* neutralization activity, those antibodies that protect without strong neutralization would not have been identified or contributed. As a result, the ~5% population of the VIC that appear to succeed primarily through cell-targeting Fc functions could be an underrepresentation of what is produced in an authentic polyclonal response. In addition, cell-targeting, non-neutralizing antibodies may function better in concert than alone, which is particularly relevant for polyclonal antibody elicited by vaccination or natural infection. Thus, the analysis of single monoclonal antibodies may have further undervalued the therapeutic potential of Fc-mediating antibody.

Importantly, the performance of antibodies for which neutralization did not adequately forecast protection could be linked to the presence or absence of NK cell and other Fc-mediated functions. All antibodies in the VIC study that neutralize well, but fail to protect *in vivo,* register zero to low scores of NK cell activity (Fig. 4). Meanwhile, all antibodies in the study that neutralize weakly or not at all, but do achieve *in vivo* protection, register high positive scores in natural killer and/or phagocytic functions. The VIC study suggests that evaluating and enhancing NK functions should be key goals of immunotherapeutic efforts.

Recent work in the research field further emphasizes the importance of Fc-mediated cell-targeting functions. Notably, in several studies of Ebola virus vaccines, total binding antibody, rather than neutralizing antibody, is the strongest correlate of protection[73–75]. Total binding antibody, by definition, would include the fraction that operates via cell-targeted Fc

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functions. Another study of three novel mAbs against Ebola virus found that protection in the mouse model correlated with their ability to confer ADCC, not with their neutralization capacity[76]. These antibodies were functional only in wild-type mice bearing NK cells, but not in NK cell-deficient mice. The additional link to enhanced NK cell activity corroborates evidence from natural infection: fatal cases of Ebola virus disease are associated with lower NK cell function than survivable cases[77].

In the comprehensive VIC study and in other recent work, we thus note a spectrum of antibody behavior. At one end of the spectrum are those antibodies that achieve protection by effector mechanisms alone. Nearly all of these mAbs target the upper and outer regions of the viral glycoprotein, and their physical position may facilitate antibody access to FcR-bearing cells as well as promote multivalent binding, and decoration of infected cells, circulating virions, or even secreted forms of sGP for immune activation. At the other end of the spectrum are potent neutralizing antibodies that protect in the absence of effector activity. These antibodies may achieve protection via Fab-mediated mechanical inactivation of the entry glycoprotein alone. Many of these antibodies bind to the GP core, in the middle to lower (membrane-proximal) tiers of the structure. Some of these epitopes may preclude access to effector cells and thus engineering to enhance effector function may or may not improve their performance. In the middle of the spectrum are antibodies that exhibit a variety of features, with a range of neutralization behavior and effector function strength.

Our immune effector assays were performed in a high-throughput standardized format to characterize the immune functions of all 171 VIC panel mAbs. Since the GP trimer display on beads may differ from that on cells, assays using GP-transfected or Ebola virus-infected cells are now in progress. Nonetheless, results from these high-throughput screens go beyond mere antibody affinity for GP to correlate with experimental protection in the mouse model. Moreover, epitopes that recruit an array of effector functions often have moderate GP affinity, whereas those antibodies that lack apparent effector function and protect via mechanical neutralization tend to have higher GP affinity.

A major limitation of the VIC study is that the *in vivo* protection results were obtained in the BALB/c mouse model. The apparent importance of effector functions of vaccines and immunotherapies tested in humans and NHPs, however, suggests that some of these general findings in the mouse model will translate to higher animals. However, the degree to which protection in the BALB/c mouse model compares to protection in larger animal models like guinea pigs, ferrets or NHPs, or even alternative mouse models such as those expressing human Fc receptors[78–81] awaits systematic study. Fc receptor-mediated mechanisms of protection among animal models are expected to differ since guinea pigs, NHPs and humans have evolved analogous, but often discrete Fc–FcR systems that have varying affinity for different antibody-FcR combinations. Indeed, mice lack both Fcγ RIIA and Fcγ RIIIB receptors, the latter being important for neutrophil activation in humans[82]. Mechanical neutralization solely due to Fab binding, which requires only a strict biophysical interaction between virus and antibody to confer protection, likely translates more faithfully from cell culture to rodents and to primates, perhaps reinforcing initial downselection via neutralization. In other words, antibodies that function primarily by physical, Fab-mediated

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blockade may have been easier to identify because they do not require immune functionality, which is, as yet, more difficult to recapitulate *in vitro* and across animal models.

The VIC study findings strongly suggest that we must also consider immune effector function in development of immunotherapeutics and capitalize on the full repertoire of immunological options at our disposal. We need to develop better bridging methods to study antibodies, for use in humans, across rodent and other non-human species used for *in vivo* evaluation. Given the potential differences among animal models and the effector functions themselves, a systematic comparison of different antibodies, that achieve protection by different mechanisms, across the standard animal models in use in filovirus research is needed. Such analyses would contribute to the understanding of the relative factors that contribute to protection in each animal model, as well as the role of immune effector function. Our previous expectation prompted the assumption that immunotherapies should focus on neutralizing antibodies. However, the existence of a class of antibodies that likely achieve protection via their Fc alone indicates that complementary or alternate routes to protection are under-appreciated and insufficiently understood. Identification of antibodies that mediate protection through immune effector function will facilitate dissection of the specific Fc features and geometry of Fc recognition that lead to protection, and will illuminate Fc engineering strategies that could improve protection by these and neutralizing antibodies alike.

Relevance of glycan modifications on antibodies

A common assumption regarding antibody production is that all *in vitro*-produced antibodies will be glycosylated similarly, and will faithfully represent the profiles that may be generated *in vivo*. However, more than two decades of monoclonal therapeutic research has pointed to major differences in glycosylation among recombinant antibodies produced within 293T- or across CHO-based or hybridoma-based expression systems, all of which likely do not reflect the original glycan profile that may have been present on the original antibody. Differences in culture conditions, cell quality, medium content, and even transfection conditions result in substantial changes in glycan content observed even within the same production laboratory[83–85]. Across the VIC mAb panel, we observed significant heterogeneity in glycan content across the 19 glycan structures that we measured[29,86], even among the 94 human IgG1 mAbs produced in traditional mammalian cell lines. We wondered, across the VIC pool, how glycan structure influenced induction of different effector functions.

We used an unbiased hierarchical clustering algorithm to group antibodies by glycan content alone, resulting in 17 different clusters (Fig. 5a). As a comparison, we also used an unsupervised principal component analysis of glycan content (Fig. 5b), which also separated the antibodies into these 17 clusters, or 17 distinct glycan profiles. To visualize the average glycan profile of the antibodies within each cluster, we generated a heat map that depicts the glycan content of each cluster: average total galactose content (divided into G0, G1, and G2), fucose, bisecting GlcNAc, and sialic acid (Fig. 5c). Next, to determine if the different clusters resulted in different functional antibody profiles, we graphed the average effector functional response of each cluster, as high, medium or low/no activity (Fig. 5d). Notably,

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several clusters were characterized by high functionality (clusters 1, 3, 11, and 12), whereas others were characterized by low activity (cluster 7) or limited functional diversity (clusters 4, 5, 9, 10, 13, 17). Of note, the highly functional clusters (1, 3, 11 and 12) represent different glycan profiles: afucosylated/agalactosylated (clusters 1 and 3) and mono-galactosylated, bisected glycan profiles (clusters 11 and 12). Both been independently associated with enhanced effector functions[87–89]. In contrast, the low/limited functionality clusters were predominantly characterized by higher content of fucose in combination with sialic acid (clusters 7, 8, 9), suggesting that these glycans may hinder effector functionality. While afucsoylation was clearly linked to enhanced FcR functions, additional glycan changes were also linked to both unique functional and polyfunctional profiles. These known, and perhaps other, unexplored glycan profiles naturally and actively leverage Fc receptors to drive enhanced functionality. Thus, the selection of a production cell line and evaluation of the resulting antibody glycans should be considered in order to maximize functional activity by therapeutic antibodies. Systematic and comprehensive mapping of the individual Fc-glycan–functional profiles may provide a roadmap to improve protection in next-generation therapeutic design.

Strategy going forward

Overall, the finding that a relatively high number of anti-Ebola GP antibodies can confer protection either solely or significantly through effector mechanisms was unexpected. Moreover, the number of effector-driven antibodies in the VIC panel suggests that lack of neutralization does not necessarily indicate a lack of protective activity. The antibodies that strongly drive such effector functions, together with control antibodies that do not, will provide focused sets for further study. These study sets will allow dissection of which immune effector functions are important, and determination of how the importance of particular functions differs by epitope recognized (e.g., upper vs. lower on the viral antigen), and if the significance of effector function is retained in other animal models.

How should protective antibodies be identified in the first place? In a rapid selection process intended to identify potential therapeutic candidates quickly, the VIC study indicates that cell culture neutralization is indeed an effective downselection mechanism. Therefore, if time only permits one assay, neutralization alone would likely provide an effective rough cut, and would likely identify features that can translate from *in vitro* to *in vivo* studies. Indeed, if we had selected among the VIC pool based on neutralization alone, we would have selected 60% of the protective antibodies.

However, if we initially downselected the VIC pool based instead on Fc polyfunctionality (i.e., sum of the scores for the seven separate immune effector measures), we similarly would have selected about 60% of the protective antibodies. A two-pronged selection approach, using both neutralization and Fc function, would have predicted 96% of the protective antibodies.

The ability of an antibody to promote killing of infected cells should be actively sought in antibody discovery programs, rather than being simply a parallel feature during *in vivo* testing. We find, in particular, that NK effector function is likely the feature that tips the

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balance in favor of greater likelihood of protection. Screens for NK activity and substitutions to enhance NK activity may be particularly effective in identifying and enhancing protective activity. A better understanding of how to evaluate large panels of antibodies for Fc-mediated protection could reduce the number of animals required for eventual *in vivo* experimentation, and support the three Rs of *in vivo* research: Replacement, Reduction and Refinement[90].

Although Fc-mediated protection is more challenging to understand *in vitro* and may not readily translate from cell culture to different animal species, these functions can be engineered using well-characterized point mutations that affect FcR affinity and the type of glycan modification[8,10,91]. Substitutions in the Fc such as L234A/L235A[92], G236R/L236R[93,94] and deletion of the glycosylation sequon at N297[95,96], for example, diminish effector functions. Modification of the glycan linked to N297 can enhance functions. Reduced fucosylation and addition of bisecting GlcNAc addition improve ADCC[91,97], and several afucosylated therapeutic antibodies have been approved or in human clinical trials for oncology applications[98–101]. Amino acid substitutions in the Fc, such as S239D/I332E[102], E345R[103] and others[8,102,104–106], increase Fc affinity for FcγRIIa, FcγRIIIa and complement as well as enhance cell targeting. Multiple antibodies bearing these substitutions have also advanced to clinical trials for oncology[107–112]. Substitutions such as S267E, H268F and S324T, which improve affinity for complement component C1q, and substitutions like E345K or E340G, which enhance IgG hexamerization[103,113], stimulate complement-dependent cytotoxicity. Other substitutions, such as M252Y/S254T/T256E[114] or M428L/N434S[115], extend antibody half-life. Engineered antibodies against *Staphylococcus aureus* alpha-toxin[116] and HIV-1[117] bearing these substitutions have been evaluated in clinical trials. In contrast to the well-characterized Fc modifications, Fab-mediated neutralization is difficult to engineer, and there are no universal point mutations that can confer neutralizing activity to a non-neutralizing antibody. Attachment of an engineered Fc, however, to a potently neutralizing Fab could improve *in vivo* performance by improving targeting and destruction of infected cells so they do not continue to be viral factories, and by improving potential bioactivity at lower antibody concentrations, required for long-term protection and control. Which Fc modifications are ideal, and whether the optimal modifications differ according epitope on Ebola virus GP are still unclear.

Information gathered from studying these antibodies will help characterize which specific features beyond neutralization increase *in vivo* efficacy of antibodies, and will provide roadmaps for rational engineering and improvement of immunotherapeutics. A greater understanding of the features of these non-neutralizing, yet highly protective samples may also provide strategies to identify such antibodies in the sera of vaccinees. Such studies could be broadly applicable as recent work suggests that induction of cell-targeting antibodies is desirable against a variety of pathogens that threaten human health[118–122], particularly when neutralizing antibodies are difficult to elicit.

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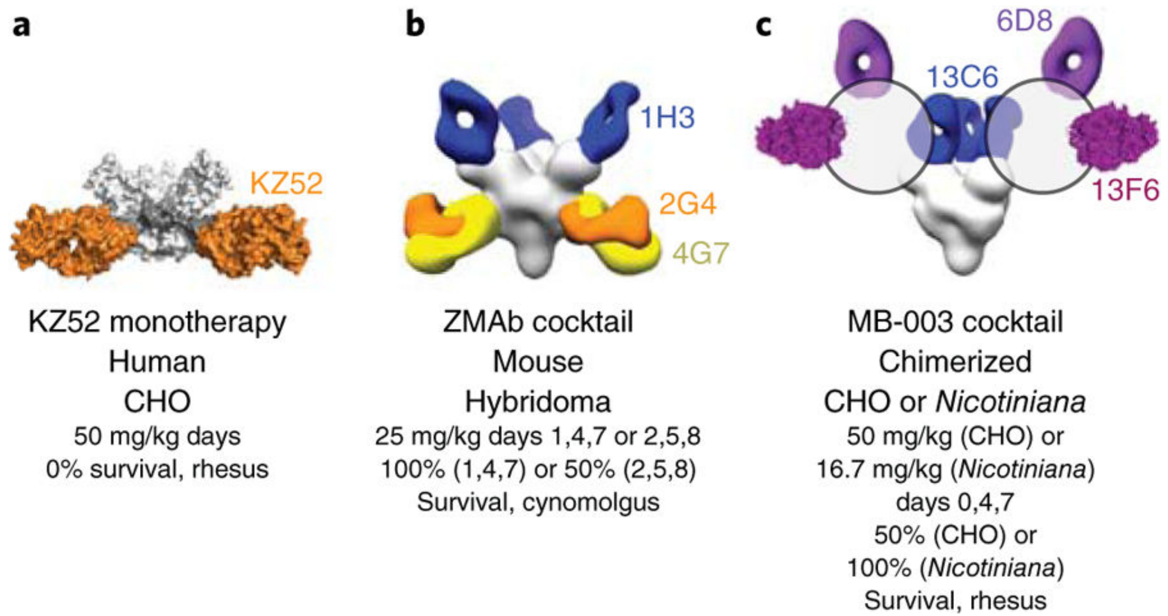

Figure 1.

Evaluation of early antibody treatments evaluated for their protection of NHPs. In Figures 1–2, all mAbs that bind the GP core remaining after cathepsin cleavage (termed GPcl) are colored orange or yellow. All head- and glycan cap-binding mAbs are colored blue or purple. **(a)** Crystal structure of Ebola virus GP (grey) in complex with KZ52[22] (orange). KZ52 was produced in CHO cells for evaluation in rhesus macaques at 50 mg/kg one day prior and four days after viral challenge[24]. **(b)** Superimposed negative stain EM structures of antibodies contained in the ZMAb cocktail[28] (2G4, orange; 4G7 yellow; 1H3 blue) in complex with Ebola virus GP (grey). ZMAb antibodies were produced in murine hybridoma culture for evaluation in cynomolgus macaques at 25 mg/kg on days 1, 4 and 7 (100% survival) or 2, 5, and 8 after challenge (50% survival)[27]. **(c)** Model of the MB-003 cocktail made from the negative stain EM structure of the 13C6-GP complex[28] and the crystal structure of 13F6 (purple molecular surface) bound to with its mucin-like domain linear epitope[123] (white ball-and-stick). MB-003 antibodies were produced in CHO or *Nicotiniana* cells and evaluated in rhesus macaques at 50 mg/kg (CHO cell-produced, 50% survival) or 16.7 mg/kg (*Nicotiniana*-produced, 100% survival) at 1 hour and 4 and 7 days after challenge[31]. Delivery of *Nicotiniana*-produced MB-003 at later time points (5, 7, and 10 days after challenge) resulted in 43% survival[32]. No high-resolution structural information yet exists for the mucin-like domain or MB-003 component 6D8. These have been modeled with grey circles and a purple Fab fragment, respectively. The relative positions of the two mucin-binding antibodies 13F6 and 6D8 are as yet unknown.

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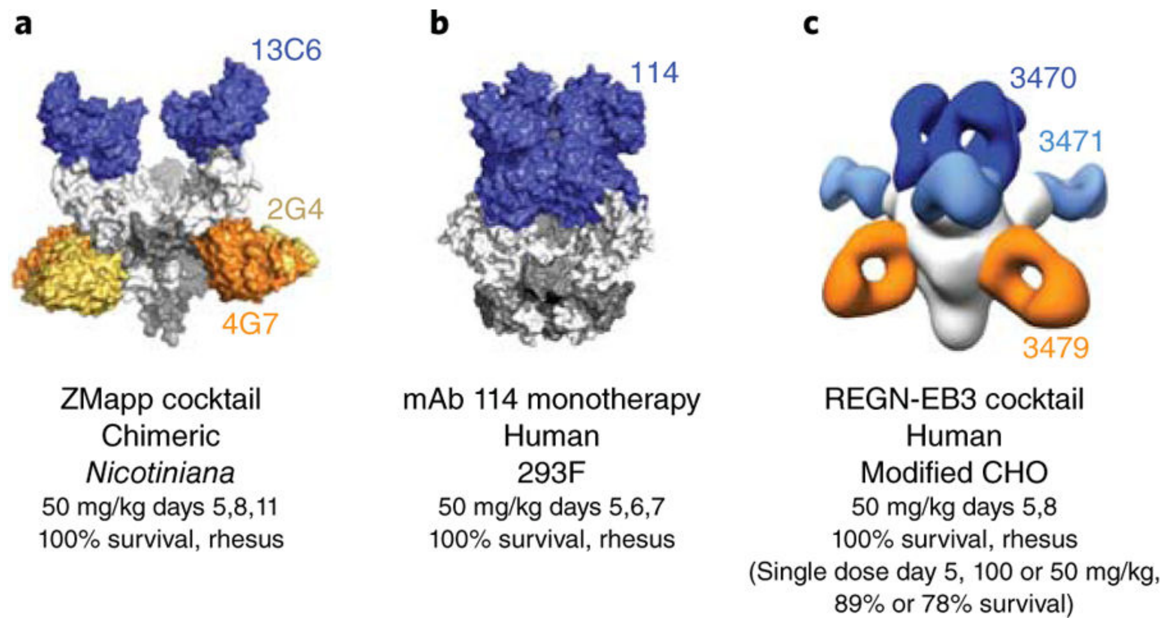

Figure 2.

Antibody treatments approved for evaluation in outbreaks of Ebola virus in 2018. **(a)** Superimposed CryoEM structures of the ZMapp antibodies 2G4 (green), 4G7 (yellow) and 13C6[33] (blue). Only the variable domains (Fv) could be built into cryoEM maps. ZMapp antibodies were produced in *Nicotiniana* for evaluation in rhesus macaques at 50 mg/kg on days 5, 8 and 11 after challenge[50]. ZMapp was also evaluated in humans in 2014[7]. **(c)** Crystal structure of mAb 114[124] (blue) in complex with Ebola virus GP, and its dosage information in rhesus macaques at 50 mg/kg on days [51]. **(D)** REGN-EB3 containing antibodies 3470, 3471, and 3479 visualized by negative stain EM and dosage information in rhesus macaques[52]. REGN-3B3 was evaluated in three-dose and single-dose regimens.

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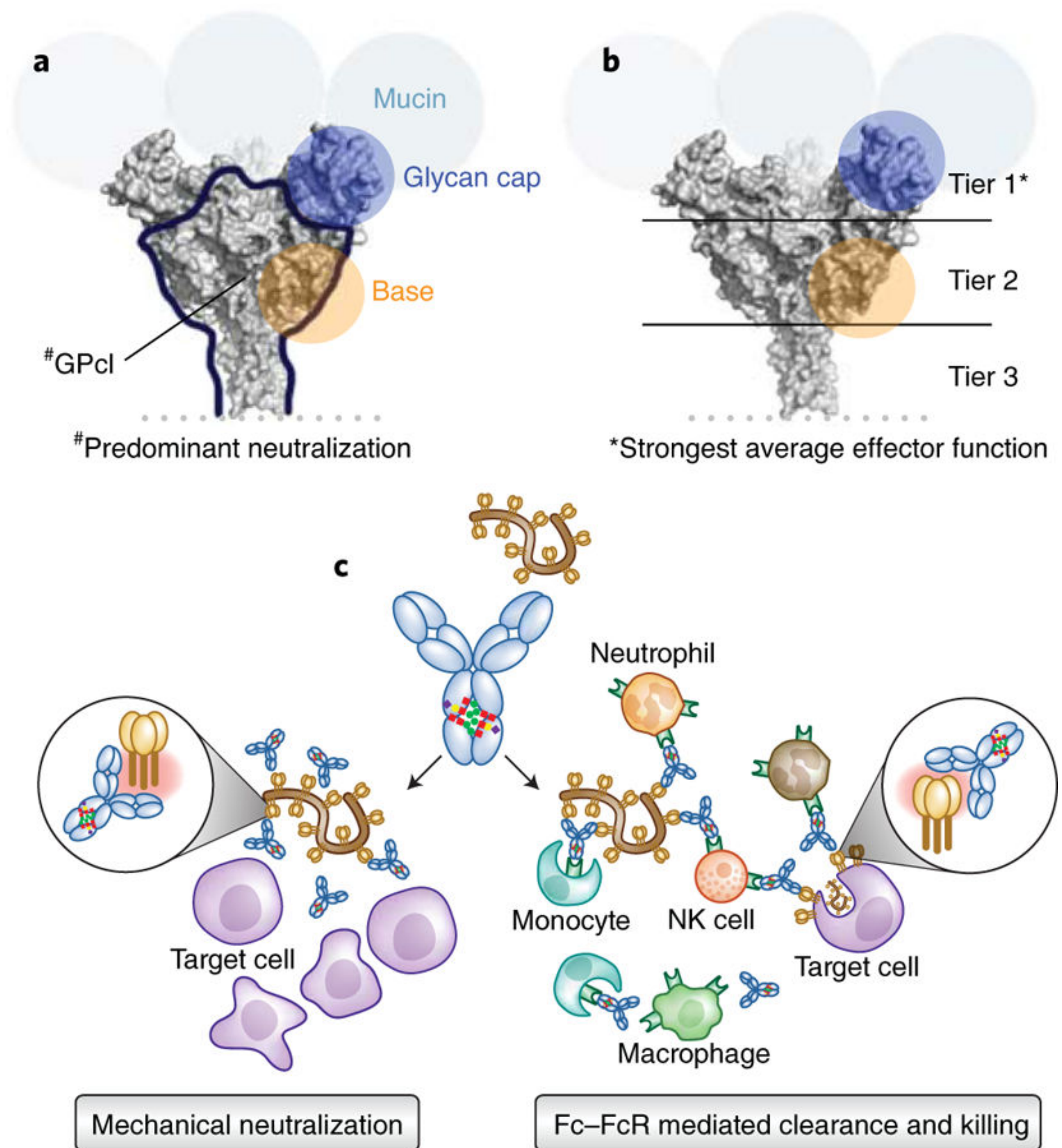

Figure 3.

Structure, epitopes of Ebola virus GP, and antibody functions. **(a)** Ebola virus GP is cleaved in the endosome to remove the glycan cap and mucin like domains. The remaining GP core, outlined in black, is termed GPcl and is competent for receptor binding. Antibodies against GPcl are much more likely to be neutralizing[29]. The base region of GP that encompasses the epitopes of KZ52, 2G4 and 4G7, is indicated by the orange circle. The glycan cap, including the 13C6 and 3471 epitopes, is deleted by enzymatic cleavage of GP and is not retained on GPcl. Light blue circles represent the mucin-like domain of GP (light blue), which is disordered in higher resolution structures. The viral membrane is indicated by a grey dotted line. **(b)** Antibodies against epitopes in the upper tier of GP, Tier 1, exhibit stronger effector functions on average than antibodies against the middle and lower regions of GP, as measured by immune 'polyfunctionality'[29]. This greater sum of Fc-mediated immune functions in Tier 1 is driven by measures of phagocytosis. Epitopes of antibodies with strong

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NK activities occur across the GP[29]. **(c)** Mechanical neutralization and effector function protection mediated by antibodies. At left, IgG antibodies are shown anchoring to viral GP (blue) on Ebola virus (yellow). By anchoring to the GP, neutralizing antibodies prevent viral entry into potential target cells (purple). At right, IgG antibodies are illustrated as anchoring to viral GPs on Ebola virus (yellow) or an infected cell (purple). Through GP anchoring, the antibody Fc couples to neutrophils, monocytes, macrophages, NK cells and other immune mediators to mediate destruction and clearance of the virus and infected cells. Neutralization and effector-mediated clearance are independent functions and may occur on the same or different antibodies.

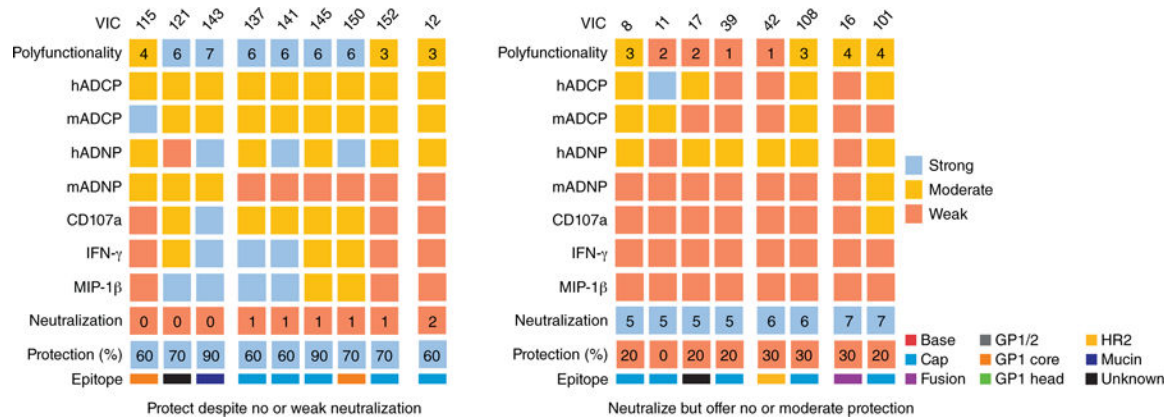

Figure 4.

Outliers in the VIC study. At left, nine antibodies protect 60% of mice, yet offer little to no neutralization (neutralization score of 0 (no neutralization measured at all in any assay) to 1 (weak neutralization measured in one assay, no neutralization measured in the other three) or 2 (strong neutralization in only one assay). Seven of these nine recognize Tier 1 or unknown epitopes. At right are eight antibodies that protect 30% of mice, despite relatively strong neutralization scores of 5–7. These antibodies typically neutralize at least moderately, and often potently, in all four measures, and recognize Tier 2 and 3 epitopes. In parentheses are the average polyfunctionality, neutralization score and protection value for the protective/poorly neutralizing group at left and the neutralizing/poorly protective group on the right. Each antibody in the panel is labelled atop a vertical column of boxes representing polyfunctionality, immune effector functions scored, protection and overall neutralization. For clarity, each measure is indicated by a colored box rather than an absolute value. Blue, yellow and red boxes indicate strong, moderate and weak scores, respectively. Polyfunctionality is a sum of yes/no answers for each of the seven effector functions measured including markers of both phagocytosis and natural killer cell function. For polyfunctionality, one point was given for strong or moderate activity on each of the seven readouts, with a maximum polyfunctionality score of 7. Antibody neutralization scores were calculated with 2, 1 or 0 points given for strong, moderate and weak/no activity on the four neutralization readouts, for a maximum score of 8. Epitopes of each antibody are indicated at the bottom with the color code at right.

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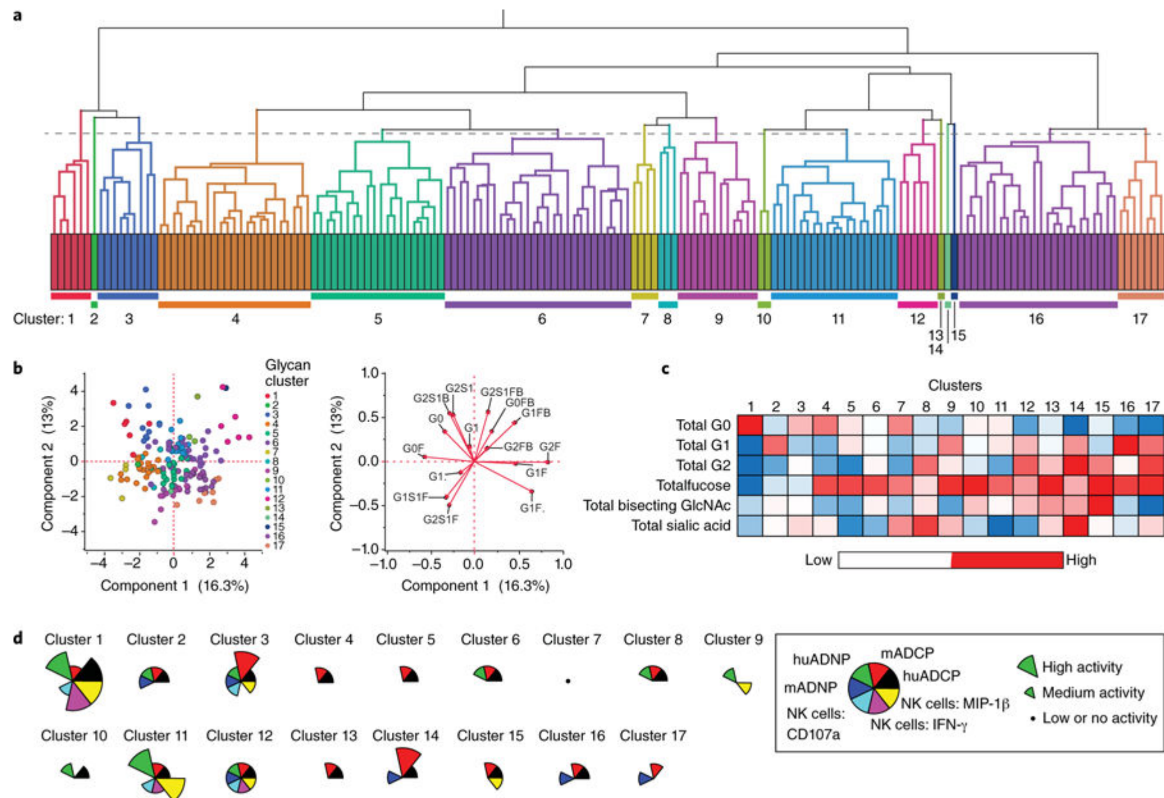

Figure 5.

Specific glycan profiles are linked to distinct functional activity. **(a)** Hierarchical clustering of VIC mAbs using Fc glycan content revealed 17 clusters of antibodies within the panel. The dashed line across the dendogram indicates the cut-off used to define the clusters. Each cluster is represented by a different color, and cluster number is listed below each cluster. **(b)** Unsupervised principal component analysis of Fc glycan content of VIC mAbs. The mAbs are color-coded according to the clusters identified in (A) in the plot at left, and the loading plot showing the glycan features driving separation of the mAbs is illustrated at right. **(c)** Heatmap of the average total galactose (G0, G1, or G2), fucose, bisecting GlcNAc, or sialic acid content of the mAbs within each cluster. Dark blue represents the row minimum and red represents the row maximum. **(d)** The functional activity of mAbs within each cluster was averaged for each effector function measured, and categorized into high, medium, or low/negligible based on assay cutoffs. Each wedge is color coded by effector function, and the size of the wedge indicates the magnitude of response.

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