5 Synthetic Antibodies in Infectious Disease

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Abstract

Rapid spread of microbial resistance and recent outbreaks of viral disease have led to renewed interest in antibody-based therapies for infectious diseases. Synthetic antibody libraries displayed on phage offer unique advantages over traditional immunization-based antibody generation, including full control over library design and selection conditions. The technology has matured beyond natural antibodies and is capable of providing novel insights into infectious disease and can generate novel antibodies that cannot be produced by the natural immune system. This chapter gives an overview of recombinant antibody library technology with an emphasis on our work using a highly successful synthetic single framework Fab library. We demonstrate its utility in targeting viruses and bacterial toxins in five case studies.

Keywords

Synthetic antibody · Fab · Phage display · *in vitro* selection

5.1 Introduction

Already in the late nineteenth century before the chemical nature of antibodies was known, antibody-based immunotherapy for the treatment of bacterial infections was adopted in the form of serum therapy [64]. Serum from immunized animals provided the first effective treatment option against infections with *Clostridium tetani* and *Corynebacterium diphtheriae* [64]. Behring was awarded the Nobel Prize in Physiology and Medicine in 1901 for his work on providing serum-therapy treatment of diphtheria. Although administration of heterologous sera was associated with several side effects, toxicity and variable efficacy, it was widely used because of the lack of alternatives. However, following the introduction of antibiotics in the 1930s, this practice was largely abandoned [17]. Antibiotics have ever since provided a cheap and safe antimicrobial treatment option, which has led to broad and general use in human and veterinary medicine. Due to the low discovery and development rate of

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novel antibiotics and vaccines, modern-day anti-infective therapy faces significant challenges including widespread microbial resistance, development of multi-resistant strains, emergence of new pathogens and infections in immunocompromised patients. Infectious diseases continue to be a leading cause of human mortality and disability worldwide despite the availability of many effective vaccines [27].

Recent advances in antibody engineering hold promise to reintroduce antibody therapy for infectious disease. The high affinity, specificity and flexibility of antibody-based treatments have led to renewed interest in their use in both pre- and post exposure treatment of infections. A key strength of antibody therapeutics is that technologies exist to increase their clinical potential by improving existing properties or endowing them with new activities [16]. Compared to alternative strategies, including probiotics, phage therapy and immune stimulation, antibody therapy has a history of safe use and a high degree of technical feasibility [27]. However, antibody drugs are expensive to develop and manufacture and high doses are typically required. Moreover, the market for a pathogen-specific drug is smaller than for a broad-spectrum antimicrobial agent. Nevertheless, antibodies may still be attractive for commercial development for multidrug resistant microorganisms or new pathogens for which no effective treatments exist. Therapeutic antibodies may also be used in combination with existing antibiotics and have been explored in the form of antibody-antibiotic conjugates [96].

Monoclonal antibody (mAb) therapy has been highly successful in the treatment of cancer and autoimmune diseases. Widespread antibiotic resistance of bacteria as well as frequent emergence of immune-escape mutant viruses have necessitated novel avenues for treatment, thus increasing the interest in pathogen-specific immunotherapies. However, as of November 2016 only four out of over fifty approved therapeutic monoclonal antibodies were for infectious disease indications. mAb development targeting infectious diseases has mainly focused on bacterial toxin-mediated diseases and viral diseases with no available vaccines or effective drugs [110]. Palivizumab (Synagis®), which targets respiratory syncytial virus (RSV) infections in high-risk children, was one of the first therapeutic monoclonal antibodies approved by the United States Food and Drug Administration (USFDA) (in 1998). Raxibacumab (Abthrax®; 2012) and obiltoxaximab (Anthim®; 2016) for the treatment of inhalational anthrax and bezlotoxumab (Zinplava™; 2016), which targets *Clostridium difficile* toxin B, have been approved more recently. Several reviews have reported history and progress in anti-infective mAb development, mostly using natural repertoires [8, 133, 70, 55, 86, 99, 135, 147, 163].

The immune system has evolved to enable humans to produce antibodies to practically all pathogens. Antibodies are produced by B cells of the adaptive immune system and can neutralize and eliminate infectious agents and associated toxins. Antibodies are separated into five classes – IgA, IgD, IgE, IgG and IgM – based on the structure of their constant regions. Because of their high stability, ability to trigger effector functions, favorable pharmacokinetics and ability to be transported to the placenta, antibodies of the IgG class and IgG1 isotype are generally preferred as the basis for new antibody-based therapies. An IgG is a Y-shaped molecule comprised of two heavy chains and two light chains, which associate to form a heterotetramer with two identical antigen-binding sites (Fig. 5.1). Interactions with antigen are mediated by six hypervariable loops, three each on the variable heavy (V_H) and variable light (V_L) domains. The constant region is responsible for initiating effector functions that lead to the removal or destruction of the pathogen or cells harboring the pathogen.

Antibody fragments (Fig. 5.1) have an increasing clinical importance [113] and are commonly used in the engineering of antibody properties. The fragment antigen binding (Fab) is a heterodimer consisting of the light chain and the variable and first constant domains of the heavy chain. A single chain fragment variable (scFv) consists of the light and heavy variable domains connected by a linker. Compared to scFvs, Fabs are generally more stable and binding activity is better retained upon conversion to full-length

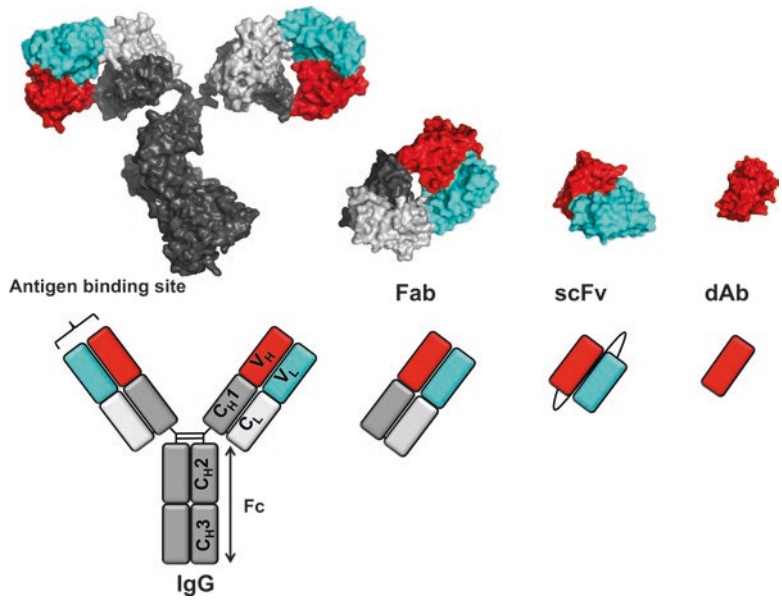

Fig. 5.1 Structure of human IgG and fragments. Constant (C) and variable (V) domains of the heavy and light chains in the heterotetrameric IgG molecule are indicated in the schematic representation. Heavy and light chains are shown in dark or light gray, respectively. V_H is shown in red and V_L in cyan. The following antibody fragments are indicated: Fc (fragment crystallizable), Fab (fragment antigen binding), scFv (single-chain fragment variable) and dAb (domain antibody). Surface representations were generated in PyMOL from PDB 1IGT [62], 2FJF [39], 1P4I [162] and 3B9V [7]

IgG. Single domain formats derived from variable [74, 118]) or constant domains [160, 161] represent the smallest human antibody fragments. The modular architecture of antibodies has been exploited to create a large number of alternative formats with various valences and antigen-binding specificities and has also enabled fusion to other proteins or toxins to further expand functionality.

Compared to small molecule drugs, antibodies have several advantages including high versatility, low toxicity, pathogen specificity, enhancement of immune function and favorable pharmacokinetics. Antibodies can mediate antimicrobial function through several mechanisms including viral neutralization, toxin neutralization, uptake and destruction by phagocytic cells via opsonization, prevention of microbial attachment, lysis through complement-dependent cellular cytotoxicity (CDC) and activation of immune cells to kill infected cells through antibody-dependent cellular cytotoxicity (ADCC) [57]. Pathogen-specific antimicrobial agents have a potential advantage because they are less likely to induce broad resistance among non-targeted microbes and are unlikely to disturb the healthy microbiome of the host. Thus, antibodies may act directly against the pathogen, exert indirect function by neutralizing toxic products of infection or enhance the efficacy of the host immune system [17]. However, drawbacks include high cost, limited usefulness against mixed infections and need for early and precise diagnosis [17].

Immortalizing B cells derived from immunized animals, which is the basis for the hybridoma technology, enables production of large amounts of homogeneous antibodies with defined class, isotype and specificity. The first step towards fully human therapeutic monoclonal antibodies was the production of monoclonal murine antibodies by hybridoma technology (e.g. the anti-CD3 monoclonal murine IgG2 antibody OKT3®, muronomab, approved in 1986 for the prevention of organ transplant rejection). However, low success rate in drug development

due to high immunogenicity and weak interaction with human complement and Fcγ receptors [16] prompted the development of chimeric antibodies, which consist of human constant domains with murine variable regions (e.g. rituximab, Rituxan®; 2006) and humanized antibodies, where mainly the complementarity determining regions (CDRs) are non-human (e.g. omalizumab, Xolair®; 2003). The first fully human antibody (adalimumab, Humira®) was approved in 2002. A human mAb is defined as having variable domains that are entirely derived from human antibody repertoires. Today fully human antibodies can be generated by phage display [107], transgenic mice carrying human antibody genes followed by hybridoma [14, 102], or through direct cloning of immunoglobulin encoding transcripts by single cell PCR [143]. Most antibodies that now enter clinical development are completely human and are derived from phage-display technology or transgenic mice, although several candidates currently under development are chimeric or humanized [114, 127].

Several drawbacks of classical immunization-based methods can be overcome by using *in vitro* selection systems with phage or other display platforms [49]. *In vitro* display physically links an antibody fragment to its encoding DNA and enables screening of libraries containing billions of unique variants. Antigen-binding clones are enriched through rounds of selection and amplification. Following screening of individual clones, the sequences of promising variants are immediately available. Antibody phage display for the generation and selection of antibody libraries displayed in scFv [106] or Fab format [66, 71] revolutionized the field of antibody engineering in the early 1990s. Importantly, *in vitro* display enables full control over the environment where selection takes place and the epitopes that are targeted. Unstable or toxic proteins that are not suitable for immunization can be targeted using appropriate conditions and selections can be designed to deplete antibodies that recognize fusion tags or cross-react with control proteins. Moreover, clones that display cross-species binding can be preferentially isolated by selecting on orthologs of relevance for future testing in animal models of disease or selection can be directed to epitopes of interest by introducing competitors. In contrast, when mining natural immune repertoires *in vivo* it is difficult to raise antibodies against epitopes that are highly conserved across species since antibodies that are reactive to self are eliminated. The transgenic methods suffer from the same restrictions on the target space due to tolerance and the need for immunogenic sequences and epitopes.

Human recombinant antibody repertoires provide a rich source for mAbs, and they are constructed from collections of human immunoglobulin genes that encode human heavy and light chains and essentially represent an immune system in a test tube. Following immunization, B cells that express a suitable antibody undergo clonal expansion in lymphatic organs. Mutation and selection mechanisms preferentially expand cells that express antibodies with high affinity and specificity for the immunogen. Libraries that sample natural diversity from lymphoid organs or peripheral blood of immunized animals or human subjects that have recovered from infection are referred to as 'immune' repertoires. For example, libraries constructed from human donors have generated neutralizing antibodies to human immunodeficiency virus (HIV) [71] and hepatitis C virus [50]. Since humans cannot be exposed to antigens at will, the available immune repertoires are limited to those induced by infections, vaccinations, autoimmunity and alloimmunity [9]. Naïve libraries are constructed using rearranged V-genes from B cell sources from non-immunized donors and mimic natural repertoire diversity. When natural diversity is incorporated in recombinant libraries, the combinatorial assembly of antibody heavy and light chains creates V_H-V_L combinations that were not part of the natural donor repertoire. In contrast to natural repertoires, synthetic repertoires generally contain artificial sequences that are not encoded in the human genome and cannot be generated by natural gene rearrangements and somatic hypermutation. However, antibodies derived from synthetic repertoires may be indistinguishable from endogenous human mAbs depending on the design. In the first antibody

libraries with synthetic components, a single V_L was combined with a set of rearranged V_H domains in a scFv format [67] and a library based on a human anti-tetanus toxoid mAb with a randomized CDR-H3 was used to select binders to different antigens [5]. Libraries that blend naturally rearranged CDR sequences with synthetic diversity are sometimes referred to as semi-synthetic, for example by grafting of CDRs amplified from donors onto a single framework in combination with synthetic diversity [65]. By contrast, fully synthetic libraries are entirely designed and engineered *in vitro* and contain precisely defined diversity incorporated from synthetic oligonucleotides [136].

Natural antibody paratopes have been optimized for specialized biological functions, and thus, they do not necessarily have optimal affinity, specificity or biophysical properties. Accumulated knowledge about antibody sequence, structure, function, and biophysical and biological properties now allows researchers to design highly sophisticated synthetic libraries to produce antibodies that can rival or surpass natural antibodies in terms of functionality. Synthetic libraries follow many different design strategies that vary in the number of framework regions used and the regions targeted for diversification. Synthetic libraries generally diversify multiple positions known to contribute to antigen recognition by using all or a defined subset of amino acids. The theoretical library diversity typically exceeds the practical limitations of phage display in terms of library transformation and screening. Libraries can mirror natural diversity by randomizing CDR positions at the center of the binding site and using a set of V-gene segments to provide a low level of diversity in peripheral positions [56]. Alternatively, CDR positions known to be involved in binding based on structural information can be diversified in a single framework [137]. To accommodate diverse paratope conformations required for the recognition of various epitopes with different shapes, some library designs utilize several heavy and light chain framework regions. For example, the HuCAL Platinum library [124] uses 49 framework combinations of consensus designed V_H and V_L domains [76] and the Ylanthia library [144] uses 36 fixed V_H-V_L pairs based on natural prevalence, canonical CDR structures, expression yields, stability and aggregation propensity.

The single framework approach offers the advantage that frameworks with high stability and low immunogenicity can be chosen and, by using modular design features, initial clones can rapidly be affinity-matured and reformatted. Frameworks can be optimized for high protein stability and production, and undesired motifs for post-translational modification can be removed. In contrast to inherently complex natural antibodies, this strategy enables the establishment of high-throughput antibody generation procedures. In fact, libraries with single frameworks using both natural and synthetic CDR diversity have been reported to be capable of generating antibodies to diverse antigens and epitopes [139, 90]. Similar studies have demonstrated that heavy chain diversity alone can be sufficient to generate high affinity synthetic antibodies [104, 137]. Using the framework of the humanized antibody Herceptin, Lee et al. found that restricted diversity in CDR-H1 and -H2 combined with high chemical and length diversity in CDR-H3 yielded highly functional Fab libraries [90]. Interestingly, libraries in which diversity was limited to as few as four [36] or two [10, 35] residue types in solvent accessible CDR positions have shown that a very restricted set of amino acids is sufficient to produce diverse and functional antibody paratopes.

These libraries not only demonstrate the versatility of synthetic repertoires, they also provide important insights into molecular recognition. For example, Tyr has been shown to be optimal for mediating favorable antigen contacts whereas Ser and Gly are effective in providing conformational flexibility to allow bulky Tyr residues to achieve optimal contacts [10, 82]. Such cooperation between large and small residues is critical for optimal molecular recognition and also illustrates that chemical diversity can be restricted without compromising function. Notably, similar minimalist strategies can generate functional binding surfaces also on non-antibody scaffolds [81].

These insights have been used in an iterative design process aimed to optimize synthetic anti-

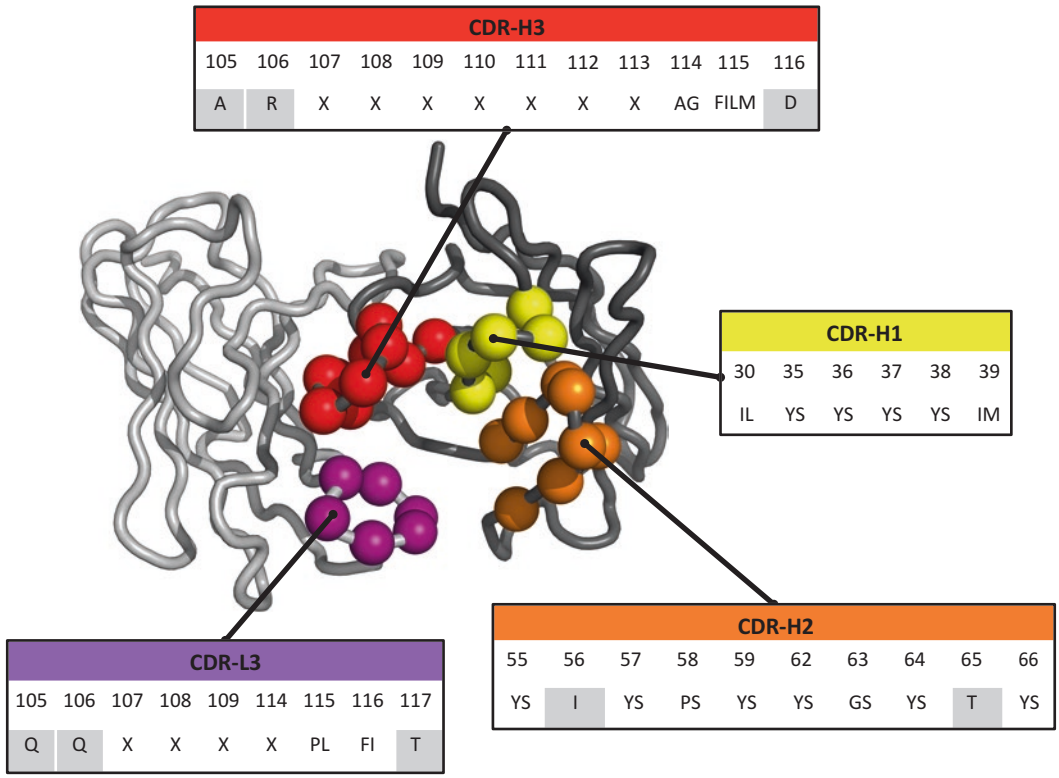

Fig. 5.2 Design of synthetic Fab Library F. The backbones of the heavy and light chains are shown as dark or light gray tubes, respectively. Diversified CDR positions are shown as spheres colored as follows: CDR-H1 (yellow), CDR-H2 (orange), CDR-H3 (red), CDR-L3 (purple). The figure was redrawn from Persson et al. [122] using PDB entry 1FVC [30] as a template. CDR positions shaded in gray were not varied and X indicates a mixture of nine amino acids (Y, S, G, A, F, W, H, P or V in a molar ratio of 5:4:4:2:1:1:1:1:1). Replacing the positions denoted by X with 1–17 or 3–7 degenerate codons, respectively, varied the lengths of CDR-H3 and CDR-L3

body library design using a minimalist design as a starting point [34]. Designs that include limited diversity in buried non-paratope positions and bias CDR diversity in favor of Tyr/Ser/Gly augmented with small quantities of other residues can further enhance functionality by allowing higher conformational flexibility and shape complementarity of CDR loops, respectively [34]. A current highly successful Fab library referred to as "Library F" (Fig. 5.2) is based on a very stable and well-expressed human Fab scaffold and contains restricted diversity in four of the six CDRs. Randomization in CDR-L3 and -H3 is biased towards Tyr, Ser and Gly with smaller amounts of other amino acids whereas CDR-H1 and -H2 contain binary diversity dominated by Tyr/Ser [122]. The amino acid distribution applied in CDR-L3 and CDR-H3 was designed to favor residues that are commonly found at natural protein-protein interfaces [82]. The trinucleotide phosphoramidite method, or trinucleotide-directed mutagenesis (TRIM), was applied to tailor the amino acid diversity. It uses a pre-synthesized set of trinucleotide codons for the synthesis of diversified CDRs, which is a significant technological development over simple degenerate codon synthesis. The gene encoding the heavy chain of the Fab is fused to gene III and the light chain is co-expressed using a phagemid system [42]. The Fabs assemble in the periplasm of *E. coli* and are subsequently displayed in a bivalent form on the phage coat [91]). Library F has been successfully used for high-throughput antibody generation [68, 112]. Moreover, this approach can generate antibodies with properties that cannot be achieved by immunization such as

recognition of specific protein conformations [41, 117, 128] and targeting of neo-epitopes on protein complexes [87]. Interestingly, cases where CDR-L3, which has equivalent chemical diversity as CDR-H3 in Library F, dominates antigen binding have also been found, which demonstrates that synthetic antibody function is not constrained by the same rules as natural antibodies [1, 122]. In summary, the synthetic approach has now evolved beyond simple mimicry of natural antibody repertoires and offers access to antibody specificities that would be unattainable using conventional methods [12].

In the second part of this chapter, we highlight case studies where synthetic antibody libraries combined with phage display technology have been employed to develop monoclonal antibodies targeting bacterial and viral pathogens, with an emphasis on antibody development using the fully synthetic Library F. In some cases, the properties of the generated antibodies could not be easily obtained with traditional immunization approaches or from libraries based on natural immune repertories. With the advance of synthetic antibody engineering, this *in vitro* antibody discovery approach will complement the current toolset of anti-infective antibody discovery, will open up new avenues for anti-infective antibody development, and will further our understanding of disease pathogenesis and vaccine-induced immunity.

5.2 Case Studies

5.2.1 Human Immunodeficiency Virus Type 1 (HIV-1)

HIV-1 is an enveloped retrovirus that was identified to be the causative agent of Acquired ImmunoDeficiency Syndrome (AIDS). HIV-1 infection requires fusion between the viral and host cell membranes, which is facilitated by the virus envelope glycoproteins gp120 and gp41. These two proteins form hexameric spikes at the viral surface, with each spike composed of three monomers of membrane-anchored gp41 associated with free gp120 [88, 119] (Fig. 5.3a). Viral entry is mediated by specific interaction of gp120 with the cell surface receptor CD4 and the chemokine co-receptors CCR5 or CXCR4 on CD4+ T cells and macrophages. Following co-receptor binding, gp41 undergoes a conformational change that results in insertion of the fusion peptide into the cell membrane, creating a hairpin loop intermediate that finally forms a highly stable six-helix bundle that facilitates membrane fusion [88, 119]. The two glycoproteins gp120 and gp41 represent the principal targets for the humoral response. In addition, CD4 and co-receptors CCR5 and CXCR4 are possible targets for therapeutic interventions. Phage display technology has been intensively used for decades to explore the epitope landscape recognized by HIV-1-specific antibodies, and thereby provided valuable insights about immunodominant and neutralizing epitopes and early vaccine strategies. Current development of mAb therapies against HIV-1 is heavily dependent on single B cell sorting or reverse transcriptase PCR techniques and the screening of *in vitro*-activated B cells [23].

Synthetic antibody approaches have been applied to existing anti-HIV-1 antibodies in order to increase their binding affinity and potency, as well as to broaden strain reactivity. For instance, a "CDR walking" strategy was adopted to improve a human anti-gp120 antibody directed to the CD4-binding site [6]. Residues in the CDR-H1 of the Fab were randomized for isolation of improved CDR-H1 variants, and next, the same approach was applied to the CDR-H3 using newly identified CDR-H1 variants as templates. The best clone exhibited eightfold improvement in affinity. Virus neutralization studies with laboratory isolates demonstrated a 54-fold improvement for the highest affinity clone. Moreover, neutralization studies with primary clinical isolates indicate that the improved Fab gained a broader neutralizing profile than the parent. By using a similar approach, this group improved the affinity of the same parental clone 420-fold for HIV-1 gp120 [159]. In addition to improving existing antibodies, various synthetic antibody libraries have been utilized to identify HIV-1 inhibitors binding to viral targets as well as host

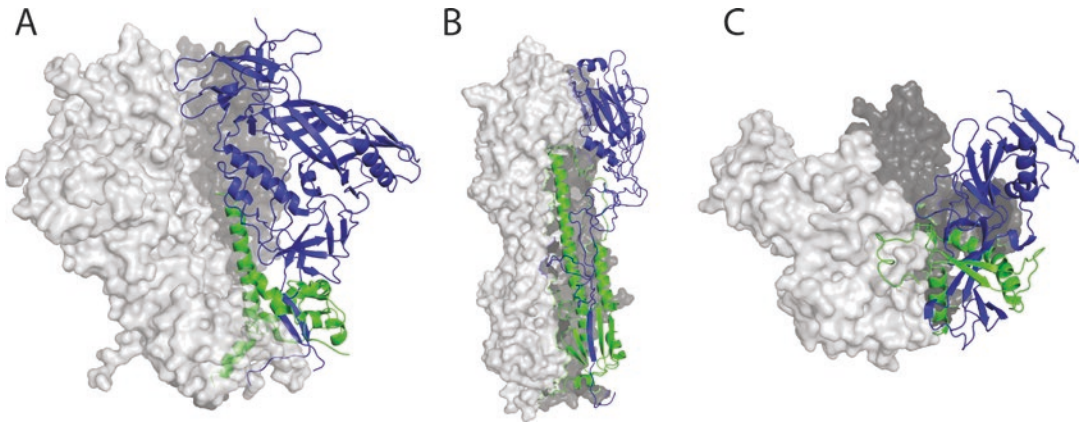

Fig. 5.3 Pre-fusion structure representation of (**a**) HIV-1 gp120-gp41 (PDB entry: 5I8H) [83], (**b**) Influenza virus HA1-HA2 (PDB entry: 4HMG) [151] and (**c**) EBOV GP1-GP2 spike (PDB entry: 3CSY) [93] ectodomains. The homotrimeric structures are depicted with one monomer shown as a colored cartoon and the other two shown as gray surfaces. The two subunits in cartoon representation are colored as following: (**a**) gp120 (blue) and gp41 (green), (**b**) HA1 (blue) and HA2 (green), (**c**) GP1 (blue) and GP2 (green). In EBOV GP, the bulky mucin-like domains that shield GP1 are not shown

receptors. By using a designed protein (5-helix) that mimics the conformation of the fusion intermediate, the Lai group identified highly specific antibodies from a minimalist phage display Fab library with binary diversity (Tyr/Ser) at selected combining sites [101]. The identified Fabs were highly specific for the HIV-1 epitope and comparable in affinity to a known scFv fragment derived from a natural antibody repertoire that targets the same region, demonstrating that minimalist synthetic antibody libraries have the potential to develop antibodies targeting HIV-1. Moreover, neutralizing human Fabs targeting gp41 derived from the synthetic HuCAL Gold library [76] have been described [61]. Some of these synthetic antibodies have been affinity matured [60] and structurally characterized [58, 59], showcasing that neutralizing antibodies with similar sequence signature and targeting epitope to those isolated from naïve or immune human antibody libraries could be obtained from synthetic antibody libraries, and more importantly, could be optimized through synthetic antibody engineering. Variable and constant domain antibody libraries have also generated promising anti-HIV binders using natural [21] or synthetic [52, 157] CDR diversity. Owing to their smaller sizes, these domain antibodies may target hidden conserved epitopes or spatially restricted regions that are not accessible to larger antibodies, therefore exhibiting unique application in viral neutralization.

5.2.2 Influenza Virus

Pandemic influenza remains a threat to global health given the absence of a universal vaccine and the emergence of new strains due to its high antigenic variability and rapid antigenic drift. Influenza viruses are a family of RNA viruses that cause respiratory tract infection in vertebrates, including birds, humans and other mammals. Influenza A viruses, the type most closely associated with human infections, are classified based on structural and antigenic characteristics of the two major viral glycoproteins, hemagglutinin (HA) and neuraminidase (NA) [40]. The HA molecule (Fig. 5.3b) mediates attachment to sialic acid on the host cell surface and subsequent fusion of the viral and host membranes. The NA molecule facilitates the release of newly budding viral particles from the host cell by cleaving sialic acid residues from host and viral proteins [40, 138]. As is the case with HIV-1 envelope glycoproteins, both HA and NA proteins display a high

degree of antigenic diversity and are prone to evolve mutations to evade host immune surveillance. However, the HA stem domain, which is involved in the pH-dependent endosomal membrane fusion, is a more conserved region in the viral glycoprotein [11, 23].

Similar to anti-HIV-1 mAb discovery, attempts to discover broadly neutralizing mAbs against influenza have been focused on single B cell sorting and *in vitro*-activated B cell screening [25, 38]. Given the genetic diversity and rapid evolution of escape species, antibody therapy targeting the most conserved HA stem epitopes is a promising strategy for development of broad-spectrum protection against influenza viruses. Phage display technology has been applied to discover neutralizing mAbs from naïve- and immune antibody libraries constructed from infected and vaccinated donors [73, 140, 142]. Broadly neutralizing mAbs isolated from these libraries generally target the conserved stem domain and execute their neutralizing function by inhibiting endosomal membrane fusion. Moreover, the IGHV1–69 germline immunoglobulin gene (Fig. 5.4) is frequently used for influenza HA stem targeting broadly neutralizing antibodies [120, 140].

Synthetic antibody strategies have also been applied to explore specific immunoglobulin signatures associated with broad neutralization [3]. For example, a semi-synthetic library heavily biased towards the germline IGHV1–69 sequence was designed and used in phage display selection. The results revealed that hetero-subtypic binding and neutralizing activity of IGHV1–69 based mAbs is conveyed by a critical amino acid triad consisting of a pair of anchor residues in CDR-H2 and a properly positioned CDR-H3 Tyr, and as few as two V-segment substitutions, one of which is localized in CDR-H2 [3]. In a more recent study, CDR sequence preference in the heavy chain of a broadly neutralizing antibody (F10) was systematically investigated [145]. F10 is derived from IGHV1–69 and also targets the conserved HA stem epitope. In this study, one

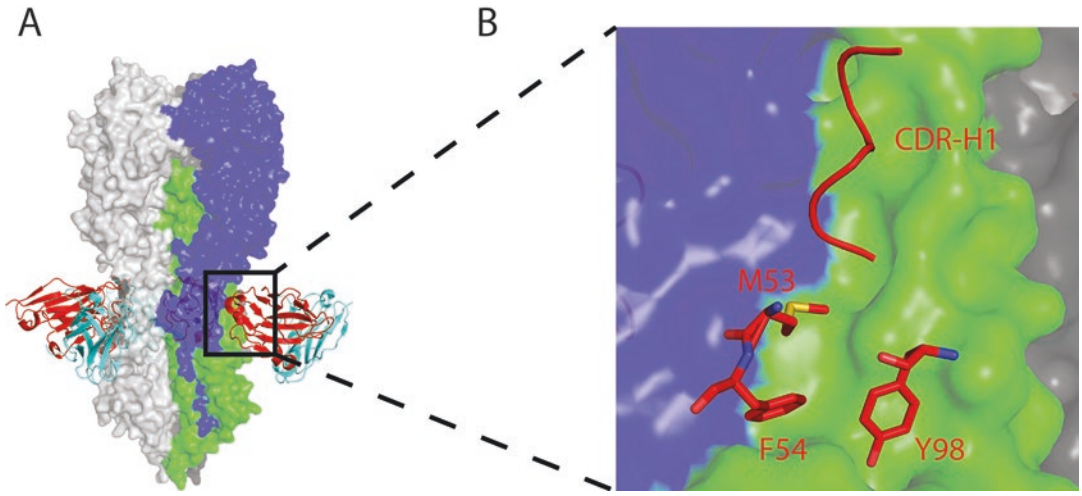

Fig. 5.4 Structural basis of germline IGHV1–69 encoded broadly neutralizing Abs against influenza A viruses. (**a**) Structure of the H5 homotrimer bound to scFv F10 (PDB entry: 3FKU) [140]. The H5 homotrimer is shown in surface-shaded view and color-coded as in Fig. 5.3. The F10 scFvs are shown as cartoons with VHs colored in red and VLs colored in cyan. (**b**) Close-up of the H5 binding interface showing H5 trimer as surface-shaded view, with the presence of signature paratope residues from F10. F10 binds to the highly conserved membrane-proximal patch on the HA stem region mainly through the germline-encoded Met53 (or Ile53 in other Abs), Phe54 in CDR-H2 and Tyr98 in CDR-H3. CDR-H1 normally interacts with a membrane-distal patch of the HA stem region through diverse configurations among IGHV1-69 encoded broadly neutralizing Abs against influenza A viruses, and in the case of F10, CDR-H1 adopts a noncanonical conformation that contacts HA with extensive intermolecular hydrogen bonding and hydrophobic interactions

library for each of the three heavy chain CDRs was constructed by completely randomizing certain CDR residues. The three libraries were screened against HA antigens from two influenza isolates. The results were used to guide the design of a fourth synthetic antibody library, from which clones with up to sevenfold higher neutralizing potency compared with the parent F10 were identified. These studies, aided by synthetic antibody approaches, help further our understanding of the structural requirements that enable a precursor IGHV1–69 germline based Ab to become a potent HA stem-directed cross-neutralizing antibody as well as the potential to identify HA stem domain targeting antibodies with higher neutralizing potency.

It has been suggested that the IGHV1–69 germline has evolved to intercept viruses in their acute phase in an SOS response before the immune system gets time to mount a more effective response by somatic hypermutation [97, 98]. Interestingly, the versatility of the human antibody repertoire is demonstrated by the identification of antibodies to the same epitope that use a different heavy chain gene (VH3-30) using phage display selection from a Fab library derived from a healthy donor [156]. Remarkably, computational protein design has been used to generate synthetic proteins *de novo* that bind to this conserved epitope [37], which have subsequently been optimized using insights from next-generation sequencing [153] and have been shown to provide *in vivo* protection in mice independently of a host immune response [78].

5.2.3 Bacterial Toxins

Although monoclonal antibodies have had limited success in treating bacterial diseases, promising outcomes have been reported in treatment of bacterial toxin-mediated diseases. Bacterial toxins are a class of highly conserved virulence factors, which play an essential role in pathogenicity. Toxin neutralization provides the potential to intervene in the pathology of the disease, alleviate host symptoms caused by infection, and may allow the host immune system to clear the bacterial infection more effectively [8]. Indeed, this strategy underlies the USFDA approval of raxibacumab (Abthrax™; 2012) and bezlotoxumab (Zinplava™; 2016), which target anthrax toxin and *Clostridium difficile* toxin B, respectively. An anticipated advantage of this approach is that it is unlikely to lead to resistance, since the antigen is separated from the toxin-producing organism, and shows that antibody therapy can effectively and safely ward off infection by targeting a bacterial toxin [110].

Recently, our synthetic Fab Library F was applied to develop mAbs targeting bacterial toxins and virulence factors. By selection against the receptor-binding component of *Clostridium difficile* transferase (CDT), a binary toxin also produced by this bacterium, a panel of synthetic antibodies was identified and demonstrated to functionally neutralize CDT cytotoxicity in cell-based assays [155]. In addition, Library F was utilized to identify neutralizing antibodies against staphyloccoccal enterotoxin B (SEB), a potent bacterial superantigen that may induce massive release of inflammatory cytokines leading to toxic shock and multiorgan failure [72]. Through synthetic antibody engineering, antibodies with sub-nanomolar antigen binding affinity were developed from initial clones, and the lead candidates demonstrated full protective efficacy from lethal SEB challenge in a mouse model. Promising synthetic antibodies to the same antigen have also been isolated [89] from the HuCAL library [76]. Moreover, Library F was recently used to isolate antibodies that neutralize Shiga Stx2 toxin *in vitro* [103].

5.2.4 Ebola Viruses

Ebola virus (EBOV) and Sudan virus (SUDV) are the most pathogenic species among the ebolaviruses, a major genre of the enveloped negative-sense RNA viruses of the filovirus family that cause severe hemorrhagic fever (Lee and Saphire [32, 85, 94, 95, 108]). The most recent 2014 Ebola virus outbreak in Western Africa highlights the urgent need for effective pre- and post-exposure treatments for ebolavirus infec-

tions. Unfortunately, there is currently no approved treatment or vaccine for these infections [109]. There are quite a few mono-specific therapies or vaccines at different stages of development, including post-exposure vaccines [33, 44, 45, 31, 43], small molecule inhibitors [26, 149], siRNA-based therapeutics [46, 47], and mAbs [132, 154]. Notably, an experimental ebolavirus-specific mAb cocktail, Zmapp [126], was used compassionately in several patients during the 2014 outbreak, demonstrating that antibody therapy holds significant promise and that the synergistic effect conferred by targeting more than one epitope may be the key to success [111, 126]. Subsequent studies have demonstrated that use of two antibodies [125] or only one [24] may be sufficient and that novel approaches using bispecific antibodies offer a promising alternative strategy [150].

Viral attachment to host cells is mediated by a glycoprotein termed GP, which is the only protein expressed on the envelope surface [131] (Fig. 5.3c). The mature filovirus GP is a trimer of three disulfide-linked GP1-GP2 heterodimers, generated by endoproteolytic cleavage of the precursor polypeptide by furin during virus assembly ([93]; Lee and Saphire [94, 95, 28]. GP1 is responsible for viral adhesion to host cells, whereas GP2 mediates fusion of the viral membrane with cellular endosomal membranes during cell entry. GP1 contains a head domain harboring a receptor-binding region, which is capped by a glycan and a heavily glycosylated mucin-like domain [93]; the base of GP1 interacts extensively with GP2 and clamps it in its prefusion conformation. The mucin-like domains and glycan caps are cleaved from the viral surface GP by host cathepsins in the endosome once filoviruses enter cells [13, 18]. This cleavage renders GP accessible for Niemann Pick C1 (NPC1) receptor binding [15, 26]. After enzymatic cleavage and receptor binding, the GP2 subunit disengages from its GP1 clamp and undergoes a series of irreversible conformational changes to form a six-helix bundle that drives the fusion of virus and host membranes [63, 105, 152].

Although encouraging progress has been made recently, the precise timing of endosomal proteolytic cleavage and concomitant exact structural changes in GP remain to be further investigated. Reagents targeting fusion intermediates of the EBOV GP will not only be greatly helpful to dissect GP intermediates in the fusion pathway, but also for potential use as immunotherapeutics or diagnostics. Natural human Ebola virus antibodies are a limited source for antibodies targeting proteolytically cleaved GP as the epitopes on the cleaved GP are normally inaccessible to the host immune system. In contrast, synthetic antibody libraries are more suitable since the repertoire is unbiased and *in vitro* display enables selection pressure directly against target epitopes inaccessible during immunization. In this regard, we and others have made encouraging attempts in developing mAbs targeting viral epitopes from synthetic antibody libraries. By using uncleaved (GP_{UNCL}) and proteolytically cleaved (GP_{CL}) forms of GP from EBOV as antigens, we successfully identified novel mAbs capable of distinguishing between GP_{UNCL} and GP_{CL} [79]. Two Fabs identified from Library F bound selectively to GP_{UNCL} or GP_{CL}, respectively. Neutralization assays with GP-containing pseudotyped viruses indicated that these antibodies inhibited GP_{CL} or GP_{UNCL}-mediated viral entry with specificity that matched their recognition profiles. This indicates that epitopes on GP_{CL} could be targeted for viral neutralization by antibodies, and these antibodies may also be useful tools in dissecting intermediates of EBOV entry. Hence, this study clearly demonstrates that highly specific functional antibodies, which have orthogonal recognition and neutralization profiles for distinct viral membrane fusion intermediates, could be obtained through *in vitro* synthetic antibody engineering. This illustrates a clear advantage of recombinant antibody development against viral targets for which limited sources of human antibodies exist in nature.

In addition to developing antibodies targeting EBOV fusion intermediates using *de novo* selection from synthetic antibody libraries, this technology can be used for humanization of non-human antibodies, which may evoke immune responses leading to increasing rates of antibody clearance and ultimately hampered efficacy if

administrated in humans [2, 130]. We recently applied structure-guided synthetic antibody engineering to humanize a SUDV-specific antibody 16F6 [20]. 16F6 is a murine Ab identified by mouse immunization [28], which limits its therapeutic utility. In search of an appropriate antibody template for 16F6 humanization, we found that 16F6 and YADS1, a humanized vascular endothelial growth factor (VEGF)-specific synthetic Ab derived from the common VH3-23 germline gene [36], have high sequence homology in the framework regions and strong structural homology of framework segments leading into the CDR loops. This analysis suggested that the YADS1 scaffold might be suitable for incorporation of 16F6-like recognition to generate anti-SUDV antibodies bearing a human framework. We then designed and constructed a 16F6 humanization library based on a chimeric template where CDR segments from 16F6 were grafted onto the YADS1 scaffold with tailored mutations introduced in selected framework positions. The 16F6 humanization library was screened against soluble GP_{SUDV} protein and a total of 17 unique clones were identified. These antibodies were produced as IgG1 molecules and their neutralizing efficacy was tested in a pseudotype virus infection model. The most potent candidates demonstrated efficacy at levels on par with murine 16F6 and had no activity against EBOV, which indicates that they maintained the specificity profile of the murine 16F6 [20]. Two clones, E10 and F4, were further shown to potently inhibit authentic SUDV and confer protection in mice from lethal SUDV challenge at similar levels as murine 16F6. These antibodies represent promising immunotherapeutic candidates for treatment of SUDV infection.

5.2.5 Engineering of Antibodies Recognizing PML-Specific Mutants in Polyomavirus JC (JCV)

Polyomavirus JC (JCV) is a highly prevalent human pathogen believed to be the causative agent of progressive multifocal leukoencephalopathy (PML), a rare but frequently fatal brain disease that afflicts a small fraction of the infected population with compromised immune system, including HIV patients and transplantation recipients on immunosuppressive therapy [54, 77, 123]. No specific therapy is available for PML, and the primary treatment relies on reconstitution of the patient's own immune response [75, 141]. Currently, interest focuses on developing diagnostic tools that could identify patients with higher risk of PML, thus reducing the risks associated with immunomodulatory therapy [146]. Although the usefulness of such diagnostics is under debate, some progress has been made so far [53, 84, 129].

Recent studies on the major capsid viral protein 1 (VP1), the key component of JCV pathogenesis, provide valuable insights into potential PML-specific biomarkers that may be essential for risk stratification of PML development [115, 116, 141]. A comprehensive analysis of JCV VP1 sequences isolated from both PML patients and healthy individuals indicated that a subset of PML-specific mutations in JCV VP1 sequences, such as leucine to phenylalanine mutation at position 55 (L55F) and S269F, might favor PML onset [141]. Therefore, early diagnosis of these PML-specific mutations may help identifying patients at high risk of PML. Monoclonal antibodies are powerful reagents in analysis of the viral protein mutant, owing to their exquisite epitope specificity. However, mutant specific mAbs with high specificity and affinity are difficult to obtain through traditional animal immunization and hybridoma technology, as mAbs cannot be directed to desired epitopes particularly in the extreme case of a single point mutation. However, *in vitro* selection can direct selection pressure to the conformational epitope centered at the mutated residues. Moreover, phage display-based antibody engineering can be readily applied to improve initial antibodies with desired properties. In searching for reagents recognizing PML-specific mutations potentially useful in diagnostics, we recently developed antibodies that specifically target the L55F mutation on JCV VP1 through phage selection and synthetic antibody engineering [19]. From synthetic antibody

Library F, antibody fragment GC058 with preferential recognition of L55F mutant over the wild type VP1 protein was initially isolated. Homolog-scanning combinatorial mutagenesis was applied to GC058 to analyze the functional contributions of individual side chains of selected CDR loops to antigen recognition. Based on the results from homolog-scanning analysis, a next generation library was constructed for specificity optimization, which yielded antibodies with much higher specificity against the L55F mutant compared with the parental GC058 [19]. This iterative process of developing antibodies specific for mutant proteins showcases the great potential of synthetic antibody engineering, which enables precise control of targeting desired epitopes.

5.3 Summary and Future Outlook

The majority of anti-infective therapeutics on the market and in development are small molecules. However, there is now a nascent pipeline of biological agents in development [70]. Antibody therapy is a promising strategy to meet current challenges of increasing multidrug resistance, inability to treat immunocompromised patients, risk of bioterrorism and new emerging diseases. Interest in antibody therapy for infectious disease has experienced resurgence and technological developments in the manufacturing of therapeutic antibodies will hopefully reduce the production cost and enable broader utilization. However, the transition from traditional broad spectrum targeting to precision medicine requires a deeper understanding of bacterial targets and pathogenesis as well as humoral antibody responses. Since the first in-depth characterization of an antibody library by deep sequencing [51], next-generation sequencing now enables identification of high frequency native VH:VL pairs in immunized subjects [48, 69, 92]. This strategy was recently applied to identify and express antibodies targeting ebolaviruses [148]. Insights from deep sequencing will likely play a central role in future efforts to design targeted synthetic antibody libraries and vaccines. Moreover, sequencing of many microbial genomes has yielded several new potential targets. However, limited biochemical knowledge makes it difficult to develop novel therapies. As an example of the complexity of infectious disease, the recent Zika virus epidemic took place in Dengue virus endemic areas where the population has acquired antibodies to this related virus. Recent studies indicate that pre-existing dengue antibodies may cross-react with Zika and thereby enhance Zika infection. It has been hypothesized that Zika virus entry through Fcγ receptors on macrophages and monocytes, facilitated by cross-reactive non-neutralizing antibodies, is responsible for this phenomenon [29].

We believe that synthetic antibody repertoires hold particular promise to provide novel insights in infectious disease and generate novel antibodies that cannot be produced by the natural immune system. Optimal implementation of synthetic antibody phage technology requires creation of large, high quality, highly functional libraries followed by functional screening. Binding sites and frameworks can be precisely tailored and the entire process can be performed in a controlled fashion. Synthetic strategies can utilize stable, highly expressed, fully human frameworks and design principles that are easily converted between formats, which will hopefully translate into higher success in the drug discovery process. Moreover, focused synthetic libraries can be designed to target a specific type of epitope by using variable regions encoding combinations of canonical structures that resemble the structural features of antibodies that bind the desired class of ligands. For example, synthetic antibody libraries specialized in peptide binding [22], hapten binding [121], carobohydrate binding [134] or binding to phosphorylated amino acids [80] have been generated. Another interesting development of synthetic antibody libraries is incorporation of an expanded genetic code in the directed evolution of proteins with specific properties. For instance, a synthetic scFv library with unnatural amino acid diversity in CDR-H3 was used to select a binder to gp120 that contained a sulfotyrosine [100]. Additional exciting progress includes phenotypic screens using cells harboring antibody libraries [158] and continuous *in vitro* evolution systems [4].

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