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REVIEW ARTICLE

Engineering Antibodies

Sujan K. Dhar[1] *and Manjula Das*[1,2*]

Abstract | Monoclonal antibodies have emerged as a leading class of therapeutic interventions in the last two decades, particularly for cancer and autoimmune disorders. A number of inventions and improvements including the hybridoma technique and recombinant DNA technology have been instrumental behind the development of recombinant antibodies that have made the commercial production viable. Antibody engineering efforts have resulted in many newer formats like Fab and scFv fragments, Fc-fusion proteins and bispecific antibodies leading to T cell engagers entering the clinical practice. This review explores the engineered antibody landscape, beginning with a historical perspective of technology development, leading to antibodies in current clinical practice, and finally providing a perspective on the future of antibody therapeutics.

1 Introduction

Following the discovery of hybridoma technique, therapeutic monoclonal antibodies have become one of the most important and fastest growing classes of therapeutic agents. This is highlighted in annual publications by commercial entities, in which monoclonal antibodies (mAbs) are consistently among the top ten bestselling drugs. Additionally, the year-wise approval of antibodies by US FDA since the early 1990s has been steadily increasing (Fig. 1).

Out of the current 60 odd FDA-approved therapeutic mAbs, nearly 80% are for various cancer and autoimmune disorder indications. Remaining 20% are made up of other indications like various infections, asthma, metabolic disorder and age-related macular degeneration (AMD). Further, among the many antibodies approved for clinical use, five of them (Rituximab, Infliximab, Trastuzumab, Bevacizumab and Adalimumab) have collectively generated revenues of more than 50% of all monoclonal antibody therapeutics in 2013[1].

Though engineered antibodies play equally important role in the diagnostic and reagent space, here we have concentrated on therapeutic antibodies. We begin by describing the basic structure of an antibody, and then detail the development of engineered antibodies in close to chronological order. Technologies that have been instrumental in the advancements are also discussed. Further, throughout the review, an emphasis is placed on discussing antibodies that are in commercial use.

2 Antibody Structure

Work of Gerald Edelman and Rodney Robert Porter revealed their independent discoveries about the chemical structure of antibodies, which led to them jointly receiving the 1972 Nobel Prize in Physiology or Medicine. Both Edelman and Porter focused on breaking antibodies down into smaller parts. Porter used the enzyme papain to split antibody molecules[2] into three parts—two fragments that bind the antigens (Fab) and a crystallisable fragment (Fc). Edelman focused on breaking the disulphide bridges holding the molecule together[3]. His work revealed that antibodies consist of two pairs of chains—two short 'light' chains and two 'heavy' chains, which are about twice the length of the light chains—held together in a 'Y' shape by disulphide bridges[4].

Their discoveries led to the understanding that antibodies have a complex but well-defined structure. The Fc and the two Fabs are joined by flexible hinge region made of disulphide bonds.

[1] *Beyond Antibody LLP, Bangalore, India.*
[2] *Tumor Immunology Program, Mazumdar Shaw Medical Foundation, Bangalore, India.*
**manjula.msmf@gmail.com*

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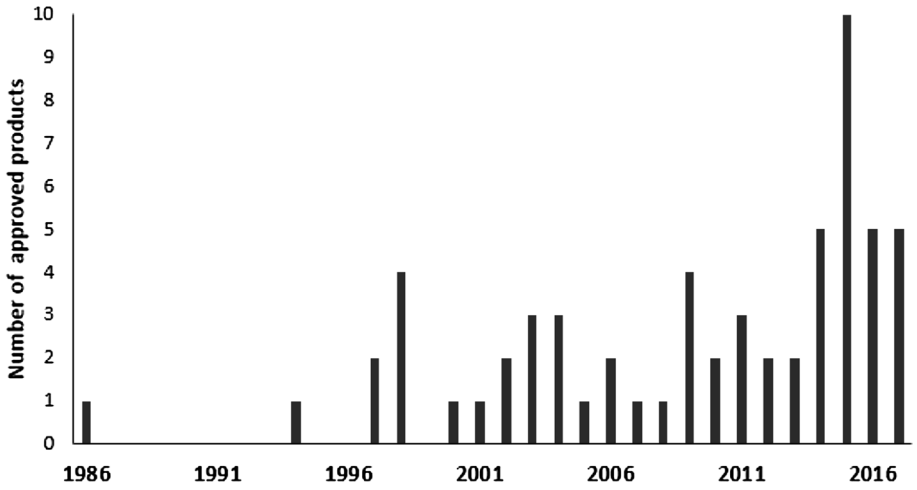

Figure 1: Number of new therapeutic monoclonal antibodies approved by US FDA every year since the first approval in 1986 (note: 2017 data are till October).

Antibodies (immunoglobulins) are broadly divided into five classes (Ig)—IgA, IgD, IgE, IgG and IgM. IgA and IgM are dimeric and pentameric molecules, respectively, while the rest are monomeric. IgG class of antibodies are most abundant and are used widely as therapeutics[5]. IgGs have further subclasses such as IgG1 and IgG2 with finer variations in their structure, which will not be discussed here. To provide a basic understanding of antibody structure, and how to engineer them, we will restrict the discussion of structure to the generic IgG structure.

IgG Structure Typical structure of an IgG molecule is shown schematically in Fig. 2. Briefly, it consists of two identical heavy polypeptide chains each of molecular weight 50 kDa or more

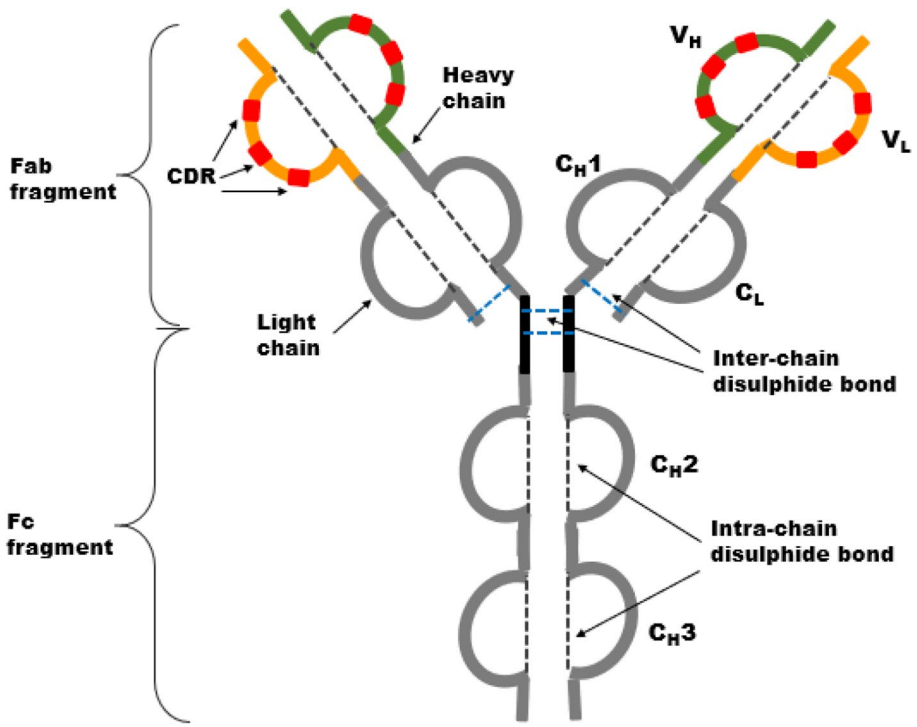

Figure 2: Structure of an IgG molecule. Hypervariable regions (CDR) in V_H and V_L are shown as red bands.

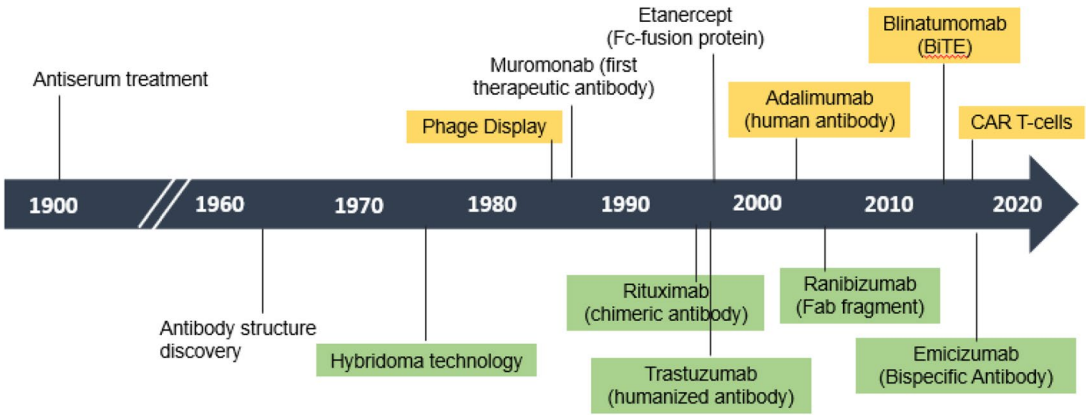

Figure 3: Brief timeline of therapeutic antibodies (hybridoma-derived antibodies shaded in green and phage display antibodies in gold).

and two identical light polypeptide chains each of molecular weight 25 kDa, adding up to a little more than 150 kDa molecular weight for the entire IgG[6]. Different chains are held together by disulphide bonds between the cysteine residues—two such inter-chain bonds in the hinge region hold the heavy chains, and two other bonds attach each of the light chains to adjacent heavy chains. In addition, there are intra-chain disulfide bonds in both heavy and light chains that provide stability to the entire antibody structure. Positions of disulfide bonds are fixed for each subclass of IgG molecule.

The first ~ 110 amino acids in the NH_3-terminal of both heavy and light chains are called variable regions (marked as V_H and V_L in Fig. 2) as the amino acid sequence in this region varies across antibodies. This variation provides the specificity of an antibody to a particular antigen[7]. The variation is further restricted to smaller stretches of sequences in this region called Complementarity Determining Regions (CDR) or hypervariable regions (shown as small bands in the variable region in Fig. 2). The Fc fragment is constant across antibodies of a particular species and has the appropriate sequences to bind to antibody receptors that initiate downstream signaling in cells. To summarize, about 5% variation in primary structure of an antibody imparts the capability to specifically bind to a single protein out of ~ 19,000 possible targets[8]. The variable regions along with a small stretch of the constant region together form the Fab fragment, which is the most relevant in a therapeutic context. Being smaller in size (molecular weight of a Fab is about 45 kDa) they can penetrate tissues better, and absence of Fc component makes them get cleared from the body much faster. This makes the Fab fragment or its further derivatives useful therapeutic alternative in cases where only binding to an antigen is sufficient to achieve the desired therapeutic effect[7].

3 The Early Years in the Development of Antibody Technology

Quest for therapeutic antibodies with better efficacy and lesser side effect, that started at the beginning of last century, continues till date with the advent of various forms of engineered antibodies (Fig. 3).

3.1 The Early Years

Use of antibody as a therapeutic option goes all the way back to the late-nineteenth century when the German scientist Emil von Behring developed anti-toxins (earlier name of antibody) against diphtheria and tetanus toxins. Von Behring immunized animals with tiny amount of diphtheria/tetanus-causing bacteria and used serum of immunized animals to cure another set of animals that were infected with more virulent form of the disease[9, 10]. This opened up possibility of using such antiserum therapy on patients—the first instance of immunotherapy.

Paul Ehrlich standardized the antisera production in large animals (horse) and von Behring and Ehrlich together along with a commercial partner (Hoechst and Co, which was a chemical company at that time) started using the antiserum to treat patients. With this treatment, the mortality in diphtheria patients was reduced from ~ 50% to less than 25%[11]. Success in treatment of tetanus patients was even more spectacular. In a population of > 1000 soldiers injured

during World War I, the tetanus toxin antiserum treatment reduced mortality to almost zero, whereas earlier the mortality was nearly 100% for soldiers who had developed tetanus[11]. However, the initial success of antiserum therapy did not last long, and trials of the therapy in tuberculosis failed in clinic[11]. Among many of reasons for failure, including absence of the development of neutralizing antibodies, one of the major challenges was identified to be the production and purification of antibodies of defined specificity and consistent quality. For example, antiserum was identified to be a repertoire of antibodies of different specificity and affinity. Origin of this variation was ascribed to somatic mutations first by Leaderberg[12] and subsequently by Brenner and Milstein[13]. Parallel to these publications and trials of antiserum technology, research in the area of ex vivo production of antibodies, helped bring out the next revolution in developing antibodies for clinical use. Laboratory and industrial-scale production of purified and well-characterized antibodies required the development of recombinant cell and DNA technology.

3.2 Recombinant Cell Technology

In the 1930s, McMaster and Hudack isolated antibody from lymph nodes[14], and Harris and Harris identified lymphocytes as the source of antibody production[15]. In 1942, Bjørneboe and Gormsen correlated plasma cell proliferation with antibody production, concluding that plasma cells were the primary source of antibody production[16]. Many studies of the 1940s–1960s focused on physical descriptions of Bence Jones proteins (immunoglobulin light chain found in the urine, with a molecular weight of 22–24 kDa that were suggested to be monoclonal), and showed that their origin was from a single plasma cell clone (reviewed by Kaunitz[17]).

Simultaneously, mouse Myeloma cell lines were being developed by Horibata and Harris[18] in 1970, with the neoplastic mouse plasma cells made by Potter and Boyce[19] a decade earlier. Availability of the myeloma cells helped Milstein and his team at MRC Laboratory of Molecular Biology, Cambridge, in developing stable antibody-producing cells. The first breakthrough was in 1973 when Cotton and Milstein[20] were able to fuse Horibata's myeloma cells with a rat immunoglobulin producing tumor cell, S210. This 'hybrid cell' eventually paved way to Kohler and Milstein's development of hybrid myeloma or 'hybridoma' in which they were able to fuse 8-azaguanine-resistant clone (X63-Ag8) of MOPC 2.1 mouse myeloma cells with spleen cells of immunized mice that were secreting antibodies against sheep red blood cells[21]. To avoid presence of MOPC 21 heavy chain in the specific hybrids, another myeloma cell line (NSI/I-Ag4-1) was used. This was a non-secreting variant of MOPC 21 myeloma which did not express heavy chains[22]. Creation of the recombinant cell or the hybridoma won Kohler and Milstein the Physiology or Medicine Nobel Prize in 1984. Thus, hybridoma, the recombinant cell, the hybrid of lymph nodes and the cancerous myeloma cells, came to be the production house of monoclonal antibodies.

3.3 Recombinant DNA Technology

Alongside the development of cell engineering technologies, recombinant DNA technology was undergoing significant progress. Combining genetic engineering tools with ex vivo cell culture techniques resulted in the large-scale and facile production of antibodies. One of the major advances in this area was the introduction of Chinese Hamster Ovary (CHO) cells, which was first used in 1986 to produce the bio-therapeutics, tissue plasminogen activator. Since then CHO-based mAb production processes have matured considerably, reaching product titers $\sim$ 1 g/L in batch and 1–10 g/L in fed-batch processes[23] enabling their large-scale commercial production.

Next major technological breakthrough in development of monoclonal antibodies was the phage display library. It was originally proposed by Smith[24] in 1985 when he validated the display of exogenous proteins on filamentous phage by fusing the peptide of interest into the minor coat protein pIII of the nonlytic filamentous phage fd or M13. This technology relied on use of filamentous phage to display a large number (10^8–10^{10}) of proteins on their surface from which the desired protein, even an antibody fragment could be screened out[25]. Phage library has popularly been used in displaying various antibody fragments like Fab or scFv. As shown in Fig. 6, Fab is the antibody binding arm of an antibody whereas scFv is not actually a natural part of the antibody. It is a fusion protein formed by recombining the variable regions of the heavy and light chain (V_H and V_L) through a 7–20-amino acid-long linker.

Synthetic DNA fragments encoding either scFv or Fab fragments are made by incorporating sequences into phagemid vectors like pBluescript KS(–). The phagemid is packaged into phage particles with the assistance of a helper phage to produce the antibody display phage. Next, the 3′ portion of gene III (from a phage) is amplified

and combined with an antibody sequence using overlap extension PCR. The phagemid library is electroporated into *Escherichia coli* cells. The desired clone is selected out from the library by repeated screening (a method called panning) with the target antigen, sequenced and subcloned into an appropriate expression vector[26]. In current practice, diversity of therapeutic antibodies and antibody fragments depending on the end usage is brought about by the vectors used.

With the advent of recombinant technology and expression systems, antibody-yielding hybridoma has almost been entirely replaced by various forms and formats of recombinant antibodies and their derivatives. Such immuno-engineering efforts yielded newer therapeutic options and hold an even larger promise of delivering improved therapeutics that we shall try to assess in remaining parts of this review.

4 Present and Near Future of Engineered Antibodies

4.1 Recombinant Antibodies

Monoclonal antibodies developed using the hybridoma technique entered clinical practice in 1985 when the first monoclonal antibody, Muromonab (Orthoclone OKT3), was approved by US FDA to prevent kidney and other organ transplant rejections[27]. Usage of this antibody was rather restricted since it gave rise to serious side effects called Human Anti-Mouse Antibody (HAMA) response because of its mouse origin[28]. To overcome this challenge, mouse–human chimeric antibodies were envisaged. Chimeric antibodies, with varying degrees of mouse and human components in the structure have been developed (Fig. 4), and are discussed in this section.

In 1984, Morrison et al. paved the way to recombinant antibody by creating "mouse–human antibody molecules of defined antigen-binding specificity by taking the variable region genes of a mouse antibody-producing myeloma cell line with known antigen-binding specificity and joining them to human immunoglobulin constant region genes using recombinant DNA techniques" in their own language[29]. Rituximab (Rituxan) and Infliximab (Remicade) were the first chimeric antibodies (suffix -ximab) that targeted the CD20 and TNF-α antigens, respectively. Rituximab was approved for cancer indications like non-Hodgkin's lymphoma, chronic lymphocytic leukemia and autoimmune disorders like rheumatoid arthritis, whereas Infliximab was for various autoimmune disorders like Crohn's disease, psoriasis, ulcerative colitis and ankylosing spondylitis.

Amount of mouse component was further reduced from 65% in chimeric antibody to 95% by 'humanizing' the antibody using a technique called "CDR grafting" invented by Greg Winter and colleagues[30, 31]. CDRs from the heavy chain variable region of mouse antibody B1-8, which binds the hapten NP-cap (4-hydroxy-3 nitrophenacetyl caproic acid) was joined to the corresponding CDR of a human myeloma protein[31]. The hapten affinity of the 'new humanized' antibody was reported to be 1.9 μM, which was comparable to that of the original mouse antibody at 1.2 μM. The first such humanized antibody (suffix -zumab), Trastuzumab (Herceptin), which binds to the extracellular region of HER2/neu cell surface protein was approved by FDA in 1998 for treatment of HER2/neu+ metastatic breast cancer. Six years later, in 2004, FDA approved another humanized antibody, Bevacizumab (Avastin)—the first anti-angiogenic drug that neutralizes various isoforms of VEGF-A protein. Bevacizumab was initially approved for treatment of metastatic colorectal cancer and later on the approval was extended to other indications like metastatic non-small cell lung cancer, metastatic breast cancer, glioblastoma and clear-cell renal carcinoma. Approval for breast cancer was withdrawn by FDA in 2012 as some of clinical trial results indicated lack of improvement in progression-free

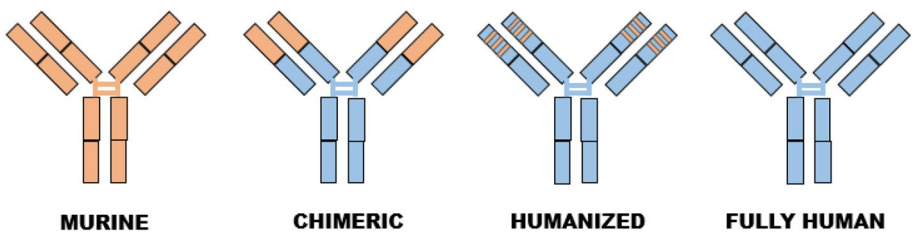

Figure 4: Schematic representation of murine (mouse), chimeric, humanized and human antibodies showing relative composition of mouse and human components.

survival[32]. However, Bevacizumab continues to be used in the clinic for many off-label indications, most notable being its usage to treat age-related macular degeneration (AMD)[33].

Producing completely human monoclonal antibodies was achieved through the phage display library technology. These antibodies (suffix -umab) did not elicit any HAMA response[34]. The biggest commercial success of this technology is the anti-TNF-α drug Adalimumab (Humira) that was developed jointly by BASF Pharma and Cambridge Antibody Technology, using the phage display library platform[35]. Adalimumab was approved by FDA in 2002 for certain autoimmune indications (rheumatoid and plaque psoriasis), and has been used widely in the clinic leading to it being named the second in the list of all time best selling prescription drugs in history[36].

Due to their advantages, over the last decade majority of approved antibodies were either humanized or fully human (Fig. 5).

4.2 Antibody Fragments

Modular nature of the antibodies with distinct functions of the Fab and Fc fragments leads to the belief that individual fragments, in particular the Fab fragment which contains antigen-binding characteristics of the antibody, can also find their way to clinic. Being smaller in size, antibody fragments can penetrate tissues that are inaccessible to full-size mAbs[37]. Also, absence of glycosylation on the Fab fragment makes its production possible in bacterial expression systems, thereby reducing the complexity and cost of production[38].

The Fabs Abciximab (Reopro), Fab fragment of a chimeric antibody against platelet glycoprotein IIb/IIIa, was the first antibody fragment approved by FDA to prevent the risk of thrombosis due to coronary intervention[39]. Another commonly used Fab fragment, Ranibizumab (Lucentis), was approved by FDA in 2006 for treatment of AMD[40] as an anti-angiogenic agent. Ranibizumab was originally derived from a murine monoclonal antibody[41]. cDNAs encoding the V_L and V_H domains were isolated using reverse transcriptase-polymerase chain reaction (RT-PCR) from the RNA of hybridoma cells. These cDNAs were cloned and fused to human constant light (C_L) and heavy chain (C_{H1}) domains. Several framework residue substitutions near the CDRs were made to improve binding to the VEGF antigen. The heavy and light chains were moved into a phage display vector. Site-directed mutagenesis of the CDRs was used to improve antibody/antigen binding. To generate a Ranibizumab-producing bacteria, E. coli 60E4 cells were transformed with the final construct[41]. Subsequently in 2008, Certolizumab pegol (Cimzia), Fab fragment of a humanized anti-TNF-α antibody conjugated with polyethylene glycol to increase its half-life in blood was approved for clinical use[42].

A number of other Fab fragments have been approved as antidote of specific toxins and drug overdose such as Digifab (for digoxin overdose), croFab (anti-venom) and the more recent Idarucizumab (Praxbind) for reversal of Dabigatran anticoagulant activity. However, despite the initial excitement, none of the Fab fragment drugs have been as clinically successful as their full IgG counterparts. One of the reasons could be the unchanged efficacy of the fragments when compared to the full antibodies. For example, Certolizumab pegol did not show any marked improvement in efficacy compared to its full-length counterpart (Adalimumab) in rheumatoid arthritis[43]. Similarly, comparison of efficacy between Ranibizumab and Bevacizumab (full-length version) was explored in multiple studies, most of them failing to show any specific advantage of Ranibizumab over the other[44, 45]. Many other antibody fragments that were in development stage[46, 47] could not cross the regulatory hurdle.

scFv The Fab fragment still has some constant regions of the antibody that can be further eliminated to create even smaller structure with only the variable regions[48]. By combining the V_H and V_L domains with a peptide linker, one can create a single-chain (scFv) antibody, or the V_H and V_L may be linked with a disulfide bond to form dsFv (Fig. 6). Additionally, diabodies are homodimers of scFv fragments bound with peptide linkers.

A whole IgG is bivalent—it can bind to two molecules of the antigen using both its arms. Reduction of the whole IgG to smaller fragments reduces its valency and hence antigen-binding activity. For this reason, Fab, scFv or dsFv typically have lesser binding strength than the parent IgG. However, such limitations are overcome by making specific mutations in the CDR region to improve its affinity. Additionally, the smaller scFv or dsFv are easier to manufacture and could potentially be modified more easily. Nevertheless, scFvs have not demonstrated high efficacy in the clinic. For example, Efungumab, an scFv fragment to neutralize candida infection failed efficacy tests[49], and Pexelizumab, another scFv fragment that was targeting C5 protein of human complement system to reduce aftereffects of heart

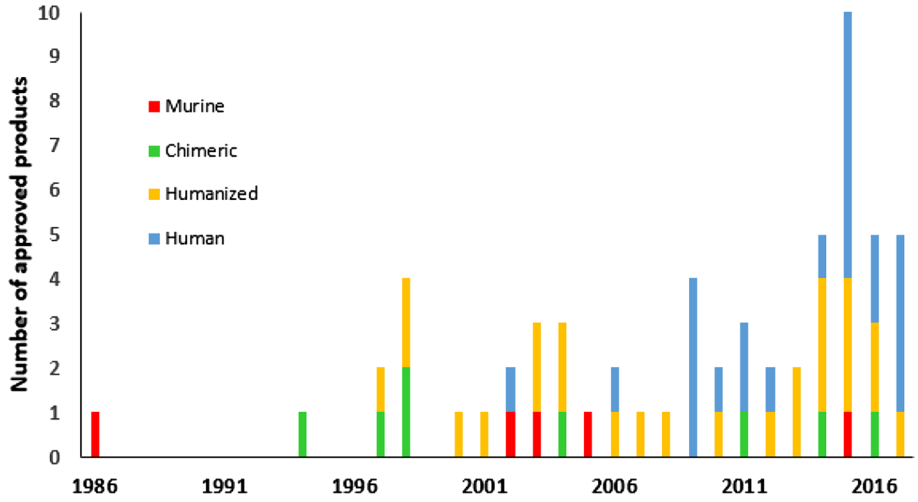

Figure 5: Categories of new therapeutic antibodies approved by FDA since 1986. Humanized and human antibodies (gold and blue bars) dominate last 10 year's approval.

surgery also failed to show any efficacy in clinical trials[50]. The small size of scFv resulted in its fast clearance through the kidney and diminished its therapeutic effect.

While not being considered as individual molecules for clinical therapeutics, modified forms of the scFv format have started to gain traction in the clinic for cancer immunotherapy. Some examples of the formats that are being developed currently include bispecific antibody, CAR T cells and intrabodies, which will be delved into in subsequent sections of this review.

5 Bispecific Antibodies

Antibodies are typically monovalent, that is, they are specific to only one antigen. The only exception is IgG4 which shows hetero-bivalency in vivo through a process called Fab-arm exchange[51]. Unlike IgG4, bispecific antibodies (bsAb) are not naturally occurring molecules; antibodies with binding capacities for two different antigens may be engineered. Initial efforts of making bsAb focused on fusing two hybridoma's to generate 'hybrid hybridomas' that can secrete such antibody[52]. With developments in molecular biology

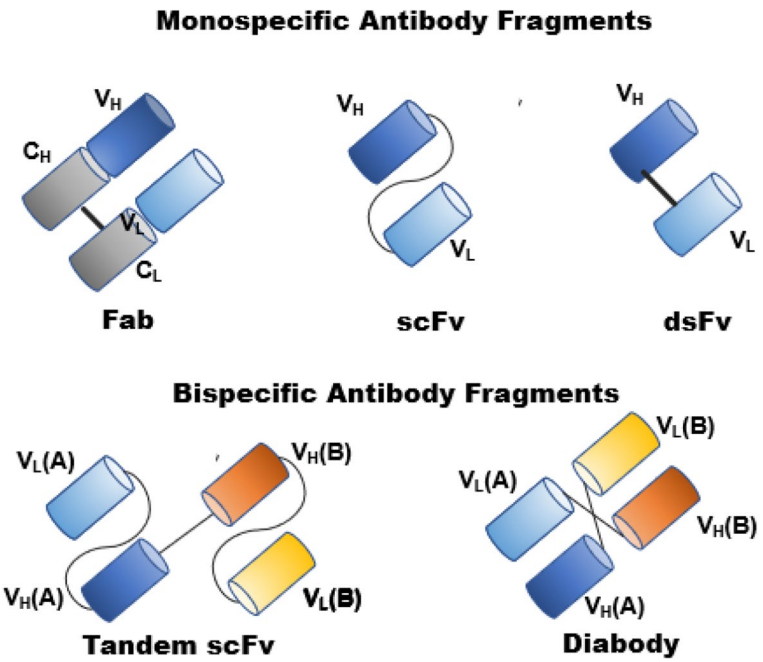

Figure 6: Schematic structure of mono-specific and bispecific antibody fragments.

techniques and introduction of phage display library, it is much easier to design and produce bsAb.

Based on their structure bsAb can be of two categories—(1) IgG-like and (2) non-IgG-like. (1) IgG-like structures have a distinct Fc region fused with V_H and V_L domains of each of the individual antibodies in different combinations resulting in variants such as quadromas, knobs-into-holes, dual-variable domains (DVD)-Ig, and $(IgG)_2$. (2) On the other hand, a non-IgG-like structure has only the V_H and V_L domains of the two antibodies joined together with peptide linkers in various orders. Tandem scFv, diabody, tandem diabody, nanobody are examples of non-IgG-like structures. Schematic illustrations of these formats are shown in Fig. 6. More details of the structure can be found in some recent reviews[53–55].

There is tremendous interest in developing bsAb due to their numerous potential applications. An example of this appeal is the fact that nearly 20% of first-in-human trials of antibodies in 2016 were with bispecific format, a number that was less than 10% a few years ago[56]. Most straightforward design of a bsAb is a dual-antigen inhibitor that tries to neutralize effects of two antigens with a single drug. Such two-antigen inhibitors in clinical development include oncology drugs against VEGF + Angiopoietin 2, DLL4 + VEGF, HER2/neu dual domain, EGFR + C-Met and a whole plethora of dual cytokine inhibitors like TNF-α + IL17, IL1a + IL1b, IL4 + IL3 for autoimmune disorders[54]. It can be argued that such double-header bsAbs are practically no different than a combination drug of two monoclonals. Nevertheless, this strategy is particularly useful for anti-viral mAb therapy. Most mAbs target glycoproteins of a virus that facilitates cell entry. Such glycoproteins show a high degree of sequence variability across species and strains and hence a bivalent therapy against multiple epitope increases effectiveness of the therapy. This is perhaps the reason that a number of anti-viral bsAbs are in development[57].

Another class of bsAbs is that which clamps two antigens together to create a novel or synergistic mechanism of action. Emicizumab[58], a bsAb recently approved by FDA (November 2017) for hemophilia A is a prime example. Blood factor VIII (FVIII) is deficient in hemophilia A, and the standard treatment regimen is external administration of recombinant or plasma-purified FVIII three times a week. Besides creating a large treatment burden on patients, the external FVIII also elicits immunogenic response, reducing efficacy of treatment. Emicizumab, whose two arms bind to other blood factors FIX and FX, works on the principle that FVIII co-activates FIX and FX by spatially bringing them together. The bsAb essentially mimics the function of FVIII by acting as a clamp between the other two factors[58]. Recent clinical study of this antibody on hemophilia patients established its efficacy and absence of anti-drug antibody response[59].

Bispecific T cell engager (BiTE) Abs have also been developed, primarily for treatment of hematological malignancies. One arm of such antibodies target a T-cell surface antigen, like CD3, and the other targets a tumor-associated antigen (TAA). Due to this structure, the BiTE brings TAA expressing tumor cell close to a cytotoxic T cell forming a synapse between them. Release of proteases like perforin and granzymes from the cytotoxic T cell initiates lysis and apoptosis of tumor cells (Fig. 7). The first such BiTE developed was Catumaxomab (Removab), synthesized by fusing a mouse IgG against epithelial cell tumor antigen EpCAM, and a rat IgG against CD3[60]. This drug was approved for clinical usage in Europe but not under the FDA regime. Blinatumomab (Blincyto), a more recent BiTE, was given accelerated approval by FDA for acute lymphoblastic leukemia (ALL) in 2014 and full approval in 2017 after completion of clinical studies. It is a bispecific tandem scFv connecting anti-CD19 mouse scFv with anti-CD3 mouse scFv[61]. Blinatumomab in ALL shows response rate of 60% or more in various clinical studies[62, 63]. BiTEs are also designed for solid tumors, and few of them including EpCAM + CD3 (lung, gastric, colorectal and breast cancer), CEA + CD3 (gastrointestinal cancer) and PSMA + CD3 (prostate cancer) have entered Phase I clinical trial, and many more are in preclinical development[64]. It will be interesting to see if these antibodies can match success of their counterparts in hematological malignancies.

Another emerging area for utilization of a bsAb is its use as a 'shuttle' or a 'trojan horse' to reach inaccessible areas in tissues or body. In such designs, one arm of the antibody attaches to a 'transporter' antigen that ferries it to the desired location, and the other arm attacks the target. In a study with transgenic mice, Niewoehner et al[65] used a transferrin receptor (Tfr) + amyloid β antibody to cross blood–brain-barrier of the animals and attach to amyloid β + regions in brain. Wec et al[66] designed a bsAb that blocks receptor binding site of host endosomal protein NPC1 in

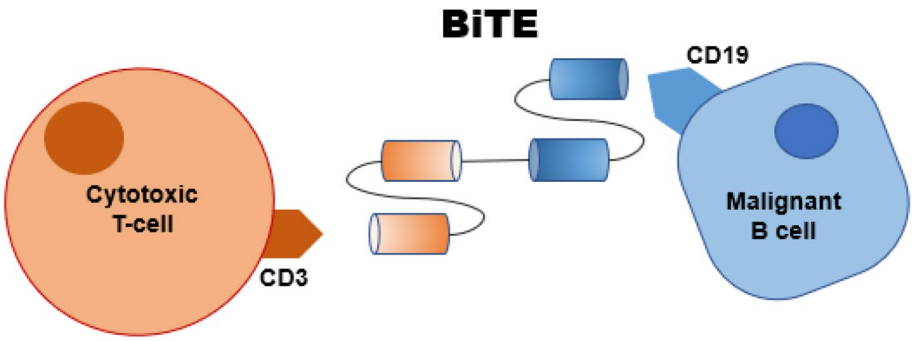

Figure 7: A BiTE-like Blinatumomab forms a cytosolic synapse between the T cell and malignant B cell.

Ebola virus. However, this epitope is exposed only when the virus is in host endosome. Access to endosome was gained by making second arm of the bsAb specific to a glycoprotein expressed on virus surface. This glycoprotein-specific arm of the bsAb hitches a ride to the endosome, where the other arm blocks the receptor binding site to stop propagation of virus to the host.

Such bi-, tri- or even higher specificity antibodies that create synergy between their multivalent actions are prime examples of immuno-engineering that is likely to rule the landscape of immunotherapy for the next few decades.

6 CARs, BiKEs and TriKEs

The first cellular therapies, involving chimeric antigen receptor (CAR) T cells, were recently approved by the US FDA for clinical use. Tisagenlecleucel (Kymriah) was the first to be approved in August 2017 for B cell precursor acute lymphoblastic leukemia (ALL)[67] and 2 months later Axicabtagene Ciloleucel (Yescarta) was approved for certain types of non-Hodgkin lymphoma including diffuse large B cell lymphoma[68]. For these two drugs the CAR construct resembles an antibody structure with an extracellular anti-CD19 scFv domain, a transmembrane spacer domain and an intracellular signaling domain[69]. It offers an autologous adoptive cell therapy in which T cells are extracted from a patient by leukapheresis, and in a GMP facility the cells are transduced with the CAR construct, expanded, and subsequently reinfused to the patient[70]. In a study with thirty ALL patients (some of whom had prior stem cell transplantation or other forms of T cell therapy), 27 (90%) showed a state of remission after 1 month of treatment with CD19 CAR-enriched T cells and 6-month remission-free survival was as high as 67%[71]. Further discussion on CAR T cells may be found in a review by A. Chandele in this issue.

Similar to the BiTE antibody platform discussed earlier, there are ongoing efforts to develop bispecific killer cell engagers (BiKEs). Natural killer (NK) cells, a part of the innate immune system, are known for their ability to recognize aberrant cells (such as tumor cells) through a whole array of cell surface receptors. A bispecific antibody that is capable of binding to one of the NK cell activating receptors as well as an antigen expressed on a tumor cell could potentially be a potent therapeutic. One example of such a molecule is a bispecific antibody developed by Gleason et al.[72], with one arm targeting CD16 protein that activates NK cells and the other arm attaching to CD33 protein expressed on acute myeloid leukemia (AML) cells. Treatment of CD33+ myeloid-derived suppressor cells (MDSC) with the CD16 + CD33 BiKE successfully reversed immunosuppression of NK cells and induced apoptosis of MDSC. Further, the same group of researchers extended the bispecific molecule to a tri-specific engager with an additional arm binding to IL-15 which is a signal for NK cell development, proliferation and activation. Bringing circulating IL-15 near the NK cells through third arm of the TriKE activated the NK cells and yielded more effective reversal of NK cell suppression in AML patient samples[73]. While there is tremendous interest in using monoclonal antibodies and CAR T cells in cancer immunotherapy currently, the non-specific innate immunity imparted by NK cells makes BiKEs and TriKEs interesting therapeutic agents that could soon be seen as an alternative option in clinic.

7 Fc-Fusion Proteins

In addition to the CDR region of antibodies, many other proteins like the receptor can also bind an antigen. Recombinant version of the receptor can be made to act like a drug that neutralizes the target protein. However, this approach faces two problems—first, just the sequence from receptor may not make a stable protein and second, it may be too small and get cleared in renal system without staying long in blood circulation. We saw in last section how Certolizumab pegol solved this problem by conjugation with polyethylene glycol. Fc-fusion is another solution in which the receptor sequences are fused with Fc portion of human IgG. Apart from imparting stability to the structure, the Fc portion also binds to FcRn receptor that allows it to stay in circulation for a longer time[74]. Further, it allows purification of the molecule from cell-based expression systems using Protein-A column—a great help in manufacturing[75].

First such Fc-fusion protein to reach clinic was Etanercept (Enbrel, Fig. 8), a fusion of 75 kDa extracellular region of TNF Receptor II and human IgG Fc[76]. As a TNF-blocker it was approved by FDA in 1998 for various autoimmune disorders including rheumatoid arthritis, psoriatic arthritis, plaque psoriasis and ankylosing spondylitis. At the time of its approval, a competing anti-TNF drug Infliximab was already in clinic, and in a few years the eventual market leader Adalimumab made its entry. However, Etanercept was able to keep its position in market since it showed comparable efficacy to its monoclonal antibody big brothers. A meta-analysis of results from 21 clinical trials of rheumatoid arthritis patients showed that in the short-term Etanercept has more efficacy than Infliximab and similar efficacy as Adalimumab[77]. Another meta-analysis[78] in psoriatic arthritis concluded for joint diseases Etanercept has a better efficacy, whereas Adalimumab offers a better skin response. Along with such comparable efficacy results, many studies indicated Etanercept offering the lowest cost of treatment[79, 80]—perhaps the reason why it did not fade away in competition.

Another Fc-fusion protein that stood up to the monoclonal antibody challenge, and emerged as the leader is Aflibercept (Eylea, Fig. 8) or the VEGF-trap made out of VEGF receptors and human IgG Fc fusion[81]. Aflibercept was originally approved by FDA in 2011 for treatment of AMD, and later on, the approval was extended to diabetic macular edema. It was also approved for treatment of metastatic colorectal cancer in another name (Ziv-Aflibercept). VEGF receptor arms of Aflibercept were far more efficient in trapping circulating VEGF molecules as was seen in comparative clinical studies with other VEGF blockers[82]. A systematic review by Sarwar et al[83] concluded that though Aflibercept and Ranibizumab showed similar efficacy in AMD patients, the once-in-8-week dosing regimen (compared to monthly dosing for Ranibizumab) reduced the treatment requirement for Aflibercept.

Unlike Etanercept and Aflibercept, Dulaglutide (Trulicity) is an Fc-fusion protein that acts more like a ligand itself than a trap for its target GLP-1 receptor[84]. Made by fusion of human GLP-1 peptide and IgG4 Fc, it increases the half-life of natural GLP-1 from 2 min to 4–5 days supporting once-in-a-week dosing[85]. Many other Fc-fusion proteins like the IL-1 blocker Rilonacept (Arcalyst), T cell co-stimulation inhibitor Abatacept (Orencia) and Belatacept (Nulojix) and blood clotting factors like FVIII–Fc-fusion Eloctate and FIX–Fc-fusion Alprolix have been approved by FDA over the last decade. Though clinical success of many of these newer fusion proteins are not well established yet, performance of market leaders like Etanercept and Aflibercept have enthused researchers to continue the engineering efforts using the Fc-fusion platform.

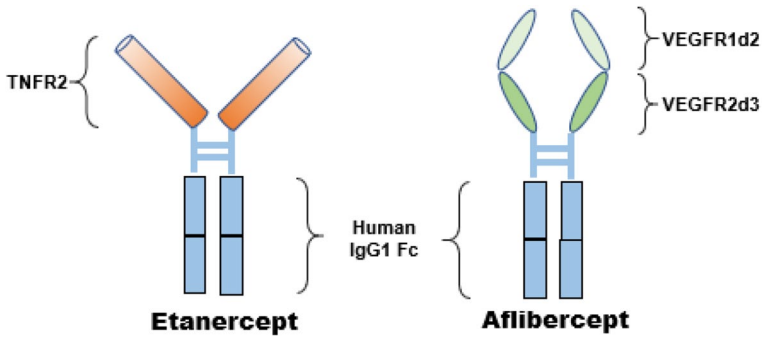

Figure 8: Design of Fc-fusion proteins—etanercept and aflibercept.

8 Intrabodies

Therapeutic antibodies discussed till now target either circulating antigens or receptors on cell surface. However, many times the target is intracellular. Microinjection of antibodies into cells was successfully tried out in 1980[86] but the method did not find popularity due to obvious limitations in its wider usage. Ten years later, heavy and light chains of an IgM antibody were separately expressed in primate kidney cells and were directed to nucleus or cytoplasm[87]. This was the first instance of developing antibodies inside a cell by inserting a plasmid from outside. This effort opened up the new expanding field of research on intracellular antibodies, or 'intrabodies'.

Intrabodies are directed to express in specific parts of the cells by inserting location-specific tags in plasmid. Most common locations of intrabodies are the cytoplasm or the endoplasmic reticulum (ER). However, intrabodies can be made to target nucleus or mitochondria as well. Cytoplasmic intrabodies cannot retain the full Ig structure since the disulfide bonds get reduced in cytoplasm, and hence single-chain antibodies are the preferred format for cytoplasmic expression. ER-targeted antibodies, on the other hand, can be expressed in a native Ig form and are mostly used to target cell surface receptors. Exhaustive reviews of both types of antibodies and recent development in each category can be found here[88–90].

Inhibition of receptors with an intracellular receptor tyrosine kinases domain has been tried out using both ER-targeted and cytoplasmic intrabodies. For example, an scFv specifically binding to EGFR was constructed from hybridoma, and was introduced to NIH3T3 cells targeting the ER[91]. This intrabody bound to the extracellular domain of EGFR protein inhibiting its surface expression. In another study, anti-EGFR scFv fragments were enriched from a phage display library and were expressed in the cytoplasms of primate kidney cells (COS-7) or human tumor cells (A-431)[92]. In both cases, localization of the antibody bound to intracellular regions of EGFR was confirmed. It would have been interesting if there was any functional data available on comparison of cell viability using both approaches.

Other than scFv, single-domain antibodies consisting of only the V_L or V_H domain are also used extensively for targeting intracellular proteins. Such antibodies were traditionally generated by immunizing animals such as camel, llama or sharks. However, using phage display it is fairly easy to develop such antibodies in vitro. Quite a number of such single-domain intrabodies mostly targeting viral proteins or tumor-specific antigen have been developed and studied[93]. One specific example is the single-domain antibody against HIV Nef1 protein. In transgenic mice, this antibody was able to rescue the CD4+ T cell maturation defects caused by Nef1[94]. To the best of our knowledge, the only clinical trial for an intrabody was reported in 2000 for an anti-HER2/neu scFv fragment encoded in adenovirus for ER targeting. This was a Phase I trial with fifteen ovarian cancer patients and focus only on safety of the use of adenovirus plasmid. There was no control/placebo group to evaluate any clinical efficacy parameters. The results did not indicate any adverse effect related to the use of adenovirus-based plasmid[95].

A few recent studies further strengthened the intrabody platform for effectively targeting intracellular proteins. By developing conformation-specific antibodies, and converting them to a scFv intrabody plasmid, Chirichella et al[96] were able to inhibit acetylation of HIV integrase protein in host cells and thereby limit viral integration activity. In another study[97], a unique method of degrading a cytosolic protein target was proposed using the anti-target antibody and TRIM21, an ubiquitin ligase. This method, termed as Trim-away, was demonstrated in a proof of principle experiment with mCherry cell line overexpressing TRIM21 and GFP, the protein of interest in this case. Microinjection of anti-GFP antibody to the cells resulted a TRIM-mediated rapid degradation of GFP with a half-life of 16 min. This method can easily be adopted using an scFv intrabody plasmid (instead of microinjection) to degrade and neutralize any cytosolic protein within a few minutes of their expression.

9 Discussions

Monoclonal antibodies have established themselves as the cornerstone of efficacious treatment in cancer, autoimmune diseases and many other therapeutic areas with minimal off-target effects.

Though the idea of using antibodies for therapy originated with the early work of von Behring and Ehrlich, it was the invention of hybridoma technology by Kohler and Milstein that made laboratory production of monoclonal antibody possible. Initial shortcomings of murine antibodies in clinic were overcome using recombinant DNA technology with migration from murine to chimeric to humanized antibodies. Finally, the development of phage display technique by Smith created an in vitro platform to identify and select

antibody fragments at much lesser cost and time than the hybridoma development. Antibody fragments generated by phage display library along with the capability of engineering the fragments using recombinant DNA technology has propelled development of many newer immunotherapy formats that have been successful in the clinic. The estimation of the global antibody therapeutic market of USD 140 bn by 2024[98] perhaps is an underestimation of the future contribution of antibody to therapeutic world since immuno-engineered therapeutic products like single-chain-variable fragments (scFv) or CAR T cells are typically not considered as monoclonal antibodies in such market research. Perhaps, the next big breakthrough in immunotherapy would be intrabodies, which would attack a target within the cell and shut off its expression. As the regulators are working towards a data-driven approach to assess the safety of gene therapy[99], it will not be too long before intrabodies enter the clinic.

Ongoing research on intrabodies may soon be translated to clinic for targeting intracellular proteins. The fertile landscape of immuno-engineering is likely to come up with newer forms of therapy more rapidly than ever.

Undoubtedly, the approval of BiTE and CAR T cell therapy has brought increased focus on immuno-engineering. Clinical efficacy of CAR T cells appears to be much superior to many engineering antibodies such as the BiTE, Blinatumomab. However, it is to be kept in mind that CAR T cell therapy requires much more infrastructure support than an off-the-shelf drug, and hence patient cohorts for CAR T cell therapy are much smaller. On smaller exploratory studies, which is a fairer comparison platform between the two, Blinatumomab shows similar response rates as CAR T cell therapy[100]. Further, the high cost of CAR T cell therapy[101] and requirement of high-end infrastructure is going to limit its widespread usage in clinic. In that context, development of off-the-shelf drugs like BiTEs and BiKEs will surely make more effective inroads to clinic and expand its applicability from hematological malignancies to solid tumors as well.

Away from the spotlight of immunotherapy in cancer, new immunotherapy options are emerging to counter viral infections as well. Palivizumab (Synagis), the first and till date the only FDA-approved anti-viral mAb (against RS virus) was introduced way back in 1998, and since then no other anti-viral mAb appeared in clinic. However, the outbreak of many deadly viruses in the last decade or so has rejuvenated immuno-engineering efforts to develop effective anti-viral mAbs. Earlier we discussed the usage of bsAbs to target multiple epitopes of a viral protein or to act as a trojan horse to enter host endosomes and neutralize viral proteins. However, there is increased evidence that apart from the direct action, the anti-viral mAbs also create indirect 'vaccine-like' effect by engaging with the host immune system[102]. Many such anti-viral mAbs are currently in advanced stages of development and we are likely to see them entering the clinic within a few years. Additionally, other recent antibody engineering efforts such as antibody–drug conjugates and Fc engineering (mutations and glycoengineering for increasing efficacy and stability) have resulted in therapeutics that are already in use or being tested in clinical trials.

Antibodies in most forms, full length or fragments, have found and will continue to find usage in the clinic. However, as evident from the discussions above, fragments though useful will never be able to entirely replace the full-length antibodies. Bispecific antibodies, though they present a whole repertoire of structural marvels, will be limited in usage due to the inherent difference in the concentration of two targets. Unless and until the two target proteins are always in equal ratio, bispecific antibodies will find limited usage, except activations of killer cells as in the case of BiTE, BiKE, etc. On the other hand, it is our opinion that a majority of antibodies developed for clinical use in the future will be based on scFv expressed as fusion proteins with various partners depending on the end usage. Intrabodies, especially conjugated with TRIMM, render the large collection of intracellular targets available for therapeutic intervention. Very recent FDA approval of voretigene neparvovec-rzyl (Luxturna), the first ever in vivo gene therapy drug, in December 2017[103] opens the door wide for intrabodies to be approved for clinical usage in near future.

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Dr Sujan K. Dhar completed his PhD in theoretical physics from Indian Institute of Science, Bangalore, and subsequently moved to computational biology. He was the Chief Technology Officer of Connexios Life Sciences, a systems biology-based drug discovery company. He co-founded and served as Chief Executive Officer of Abexome Biosciences, working with researches and pharmaceutical companies to develop antibodies and antibody-based solutions. He continues his association with biopharmaceutical research and development in his current role as the Director of Beyond Antibody LLP.

Dr Manjula Das holds a PhD in Microbiology and Molecular Biology from Indian Institute of Science, Bangalore. She worked at McGill University, Montreal, on cancer biology and subsequently at MIT, Boston, on gene regulation networks during her post-doctoral studies. She was part of many biotechnology companies including Metahelix Life Sciences as senior scientist, Connexios Life Sciences as CSO and Bioneeds PLC as Head, Bioanalytical. Manjula was the co-founder and CSO of Abexome Biosciences where she led a team to develop a large repertoire of antibodies and assays for use in research and diagnostics. She is currently the Director of Beyond Antibody LLP and also Principal Investigator in Mazumdar Shaw Centre for Translational Research, Bangalore, where she leads the tumor immunology programme.