Mining human antibody repertoires

Human monoclonal antibodies (mAbs) have become drugs of choice for the management of an increasing number of human diseases. Human antibody repertoires provide a rich source for human mAbs. Here we review the characteristics of natural and non-natural human antibody repertoires and their mining with non-combinatorial and combinatorial strategies. In particular, we discuss the selection of human mAbs from naive, immune, transgenic and synthetic human antibody repertoires using methods based on hybridoma technology, clonal expansion of peripheral B cells, single-cell PCR, phage display, yeast display and mammalian cell display. Our reliance on different strategies is shifting as we gain experience and refine methods to the efficient generation of human mAbs with superior pharmacokinetic and pharmacodynamic properties.

Introduction

Human antibody repertoires are collections of human immunoglobulin (Ig) genes that encode human heavy and light chains. These include \( V_H \), \( D \), \( J_H \) and \( C_H \) gene segments of the heavy chain locus on chromosome 14; \( V_L \), \( J_L \) and \( C_L \) gene segments of the kappa light chain locus on chromosome 2; and \( V_L \), \( J_L \) and \( C_A \) gene segments of the lambda light chain locus on chromosome 22 in the human genome. In addition, transgenic animals with complete or partial human antibody repertoires have been generated. Human antibody repertoires can be mined in vivo and in vitro for human monoclonal antibodies (mAbs).

Chimeric, humanized and human mAbs, which collectively are the prevailing formats of therapeutic mAbs, share human constant domains but are discerned by the origin of their variable domains. Human mAbs (also referred to as fully human mAbs) are defined as having variable domains that are entirely derived from human antibody repertoires, whereas chimeric and humanized mAbs feature variable domains that originate from non-human antibody repertoires. A key feature of human mAbs is their ability to fully blend in with the human immune system. Being indistinguishable from endogenous human antibodies, human mAbs are generally anticipated to have superior pharmacokinetic and pharmacodynamic properties compared to mAbs from nonhuman antibody repertoires. Ideally, human mAbs are not recognized as foreign by the human immune system, i.e., are not immunogenic. Human and humanized mAbs have been found to be less immunogenic than nonhuman and chimeric mAbs, the latter of which trigger human anti-mouse antibody (HAMA), human anti-rat antibody (HARA) and human anti-chimeric antibody (HAHA) responses. It is expected, but remains to be substantiated, that human mAbs are generally less immunogenic than humanized mAbs. Human anti-human antibody (HAHA) responses are mainly due to idiotypic, allootypic and glycosylation differences between human mAbs and endogenous human antibodies. Although potentially beneficial for certain therapeutic applications, it is likely that fine tuning of human mAb engineering and manufacturing will reduce the remaining immunogenicity of human mAbs.

Hybridoma technology has been extremely successful in the generation of mouse and rat mAbs; however, the initial failure to robustly apply this method to the generation of human mAbs paired with the prospect of lucrative intellectual property accelerated the development of a variety of alternative methods over the past three decades. The medical and commercial success of mAbs in the management of human disease drove this growth and diversification. Although only six out of 28 mAbs that were approved by the US Food and Drug Administration (FDA) and/or the European Medicines Agency (EMA) are human mAbs (Table 1), four of these antibodies were approved as recently as 2009, and their proportion is likely to increase, owing to an unremitting pipeline of human mAbs in Phase 3 clinical trials.
Here we review the generation of human mAbs from naïve, immune, transgenic and synthetic human antibody repertoires. Strictly speaking, only human mAbs from naïve and immune repertoires, which we summarize as natural repertoires in the next section, as well as human mAbs from transgenic repertoires, which nonetheless are products of artificial immune systems, meet the above stated criterion of having variable domains that are entirely derived from human antibody repertoires and are therefore indistinguishable from endogenous human antibodies. By contrast, human mAbs from synthetic repertoires generally contain artificial sequences that are not encoded in the human genome and cannot be generated by natural processes such as VD JH, VDJk and VKJk rearrangements and somatic hypermutation. Transgenic and synthetic repertoires are summarized as non-natural repertoires in a subsequent section. Furthermore, we distinguish between non-combinatorial and combinatorial strategies for mining human antibody repertoires. Non-combinatorial strategies retrieve human mAbs from single B-cells with the original heavy and light chain pairs (Fig. 1). Combinatorial strategies involve human antibody libraries with randomly combined heavy and light chains (Fig. 2). Hybridoma technology and phage display technology exemplify non-combinatorial and combinatorial mining tools, respectively. Both approaches have led to FDA- and EMA-approved human mAbs (Table 1).

### Natural Repertoires

Each B cell encodes a single antibody with defined specificity. The clonal diversity of the natural human antibody repertoire is defined by the number of B cells that encode antibodies with unique specificities, i.e., the number of independent clones. In an individual, this number is estimated to be at least 10^8. B cells from primary and secondary lymphoid tissues along with B cells in circulation, i.e., in peripheral blood, constitute the natural human antibody repertoire. The natural repertoire can be divided into naïve and immune repertoires depending on whether or not it has been shaped by antigen encounter. The immune repertoire has been extensively mined for human mAbs, first with non-combinatorial and later with combinatorial strategies. By contrast, combinatorial strategies have dominated access to human mAbs from the naïve repertoire (Table 2).

The bone marrow is a primary lymphoid tissue of interest for mining natural human antibody repertoires. The principle site of differentiation of hematopoietic stem cells into immature B cells, the bone marrow generates pro-B cells and pre-B cells with partially rearranged heavy and light chain genes. While these partially developed human antibody repertoires are generally not suitable for mining human mAbs, surrobody libraries that pair rearranged heavy chains with surrogate light chains of pre-B cells have been described recently. The ultimate products of B-cell development in the bone marrow are immature B cells that express cell surface IgM. Upon exiting the bone marrow and entering the circulation, immature B cells mature to naïve B cells, the latter of which express cell surface IgD in addition to IgM. Immature and naïve B cells collectively provide a fully developed human antibody repertoire that has not been shaped, at least not extensively, by antigen encounter and is therefore termed primary repertoire or naïve repertoire.

When naïve B cells encounter new antigens that bind to their surface Ig they become activated B cells, proliferate and go through differentiation processes that typically involve somatic hypermutation and class switch recombination from the earliest expressed IgM isotype to IgG, IgE and IgA isotypes. These processes are T-cell-dependent and take place in germinal centers (GC) of secondary lymphoid tissues such as lymph nodes, spleen and tonsils. Post-GC B cells with affinity and isotype matured antibodies differentiate further into antibody secreting cells and memory B cells. Antibody secreting cells can be divided into proliferating plasma blasts and fully differentiated plasma cells that no longer proliferate. Plasma cells exit secondary lymphoid tissues, enter the circulation, and migrate to the bone marrow where they may survive for weeks, months, or even years in specialized niches. Antibody titers in circulation are maintained by plasma cells residing in the bone marrow. In contrast to antibody secreting cells, memory B cells do not secrete antibodies but express cell surface Ig. Memory B cells are found in secondary

### Table 1. Human mAbs approved in the United States and the European Union

| Generic name (trade name; company) | Format (origin) | Antigen | Indications | Approval |
|------------------------------------|----------------|---------|-------------|----------|
| Adalimumab (Humira®; Abbott)       | IgG1x (phage display) | TNFα | Rheumatoid arthritis; juvenile idiopathic arthritis; psoriatic arthritis; plaque psoriasis; ankylosing spondylitis; Crohn’s disease | 2002 (FDA), 2003 (EMA) |
| Panitumumab (Vectibix®; Amgen)     | IgG2x (transgenic mouse) | EGFR | Metastatic colorectal cancer | 2006 (FDA), 2007 (EMA) |
| Golimumab (Simponi®; Johnson & Johnson) | IgG1x (transgenic mouse) | TNFα | Rheumatoid arthritis; psoriatic arthritis; ankylosing spondylitis | 2009 (FDA), 2009 (EMA) |
| Canakinumab (Ilaris®; Novartis)    | IgG1x (transgenic mouse) | IL-1β | Cryopyrin-associated periodic syndrome (CAPS) | 2009 (FDA), 2009 (EMA) |
| Ustekinumab (Stelara®, Johnson & Johnson) | IgG1x (transgenic mouse) | IL-12 and IL-23 (common p40 subunit) | Plaque psoriasis | 2009 (FDA), 2009 (EMA) |
| Ofatumumab (Arzerra®; Genmab)      | IgG1x (transgenic mouse) | CD20 | Chronic lymphocytic leukemia | 2009 (FDA), 2010 (EMA) |
Human immune repertoires are highly attractive because they contain antibodies of high affinity with minimal immunogenicity and off-target reactivity. Unlike nonhuman immune repertoires that have been mined extensively for mAbs, the human immune repertoire lacks both availability and accessibility. First of all, humans cannot be exposed to antigens at will, limiting available immune repertoires to those induced by infections, vaccinations, autoimmunity and alloimmunity. Furthermore, secondary lymphoid tissues such as the spleen, which is a preferred source of mouse, rat and rabbit immune repertoires for mAb mining, are difficult to access in humans. Access to the human immune repertoire is therefore generally limited to peripheral blood and, to a lesser extent, bone marrow. As discussed above, these compartments also contain the naïve repertoire. Combinatorial strategies allow the retrieval of human mAbs from the naïve repertoire. Typically, these mAbs need to be improved by affinity maturation in vitro to compensate for their lack of efficient selection by antigens in vivo. The next

lymphoid tissues, in circulation and in the bone marrow. Upon recall antigen encounters, memory B cells rapidly differentiate into antibody secreting cells. Both memory B cells and plasma cells can be long-lived, maintaining immunological memory for a lifetime.16

The secondary repertoire, or immune repertoire, is largely defined by the pool of post-GC B cells, plasma blasts, plasma cells and memory B cells. Expression of CD27 distinguishes this B-cell pool from other B cells. Antibody-secreting cells and memory B cells can also arise from T-cell-independent processes that play a role in both adaptive and innate immunity. Adaptive immunity to T-cell-independent antigens such as carbohydrates and lipids contributes to the immune repertoire. By contrast, cells expressing natural antibodies, which play a key role in innate immunity,17 can be considered to belong to the naïve repertoire. These two types are difficult to distinguish and typically share IgM isotype, low affinity and polyreactivity. They illustrate that immune and naïve repertoires often overlap without clear anatomical localizations or functional distinctions.

Figure 1. Non-combinatorial mining of human antibody repertoires. (A) Human antibody repertoires consist of polyclonal mixtures of a variety of B cells that express functional human antibodies, including naïve and post-GC B cells that represent naïve and immune repertoires, respectively. Post-GC B cells, which differentiate into antibody secreting cells and memory B cells, provide a human antibody repertoire that has been extensively mined with non-combinatorial strategies. (B) Hybridoma technology (top), EBV immortalization (center), and single-cell sorting (bottom) allow the isolation and expansion of monoclonal B cells which are then screened with an antigen of interest. Cell surface Ig can be utilized to first enrich B cells that express human antibodies to the antigen of interest. (C) Heavy and light chain cDNAs are amplified from mRNA of monoclonal B cells and B-cell lines by RT-PCR. (D) After cloning heavy and light chain cDNAs into ectopic expression vectors, human mAbs are purified from the supernatant of transfected mammalian cells (typically Chinese hamster ovary CHO or mouse myeloma NS0 cells) in high yields.
two sections discuss various features of naïve and immune repertoires that are exploited in non-combinatorial and combinatorial strategies for mining human mAbs.

**Non-Combinatorial Mining**

Hybridoma technology. The advent of hybridoma technology had a tremendous impact on biomedical research, yielding mAbs that have become an indispensable part of everyday laboratory work. Today rodent mAbs against a plethora of antigens are widely available and can be produced in almost unlimited quantities due to this robust underlying technology. Unfortunately, the therapeutic use of rodent mAbs in humans is limited by their propensity to induce strong HAMA and HARA responses that quickly neutralize the rodent mAb. Given this, the mining of human antibody repertoires using hybridoma technology was investigated extensively. Despite significant efforts, the adaptation of hybridoma technology to the generation of human mAbs has been a slow and difficult process due to the limited number of B cells stimulated with an antigen of interest and a lack of suitable drug-resistant

Figure 2. Combinatorial mining of human antibody repertoires. Polyclonal or oligoclonal mixtures of human B cells (A) are used in bulk to isolate mRNA and amplify cDNA of heavy and light chains (B). (C) Heavy and light chain cDNAs are cloned into display vectors that afford antibody libraries with a physical linkage of genotype (cDNA) and phenotype (protein). Shown as examples are filamentous phage that encode and display Fab (top) and virus-infected mammalian cells that express and display scFv (bottom). Note that heavy and light chains are randomly combined. (D) Antibody libraries are then selected by several rounds of panning on immobilized antigen (top) or screened by a single round of FACS using fluorescently labeled antigen (bottom). (E) Human mAbs from antibody libraries are manufactured the same way as human mAbs from monoclonal B cells and B-cell lines.

Table 2. Principal mining tools for human antibody repertoires

| Repertoire     | Mining tools                  |
|----------------|-------------------------------|
| Natural        |                               |
| Naïve          | Non-combinatorial             |
|                | Hybridoma technology; clonal expansion; single-cell PCR |
| Immune         | Combinatorial                 |
|                | Phage display; yeast display |
| Non-natural    |                               |
| Transgenic     | Non-combinatorial             |
| Synthetic      | Combinatorial                 |
|                | Phage display; yeast display; ribosome display |

Table 2. Principal mining tools for human antibody repertoires
myeloma cell fusion partners for the immortalization of human B cells. Thus, mouse myeloma cells were initially used for the generation of mouse-human heterohybridomas. Such hybrid cells were shown to have a strong tendency to lose human chromosomes, including chromosome 2 carrying the kappa light chain locus. While this precluded an extensive use of mouse myeloma cells for human mAb production, protocols have been established that may ameliorate the undesirable behavior of heterohybridomas and allow long-term production of human mAbs.

Given the genetic instability of heterohybridomas, the use of alternative fusion partners, particularly human lymphoblastoid cell lines and human myeloma cells, has been explored extensively. While in principle successful, the yield of viable hybridomas is typically too poor for practical applications. In addition, such hybridomas often secrete permuted Igs derived from both fusion partners. The use of certain mouse-human hybrid cells including heteromyelomas and heterohybridomas as fusion partner appears to resolve these problems, while at the same time generating stable antibody producing hybridomas. In addition, a promising human myeloma cell line allowing efficient generation of stable and highly productive human hybridomas was described, even though a more widespread use of this cell line awaits the production of a subline lacking expression of an endogenous A chain. Finally, the use of genetically engineered mouse myeloma cells allows for highly efficient formation of stable heterohybridomas with a propensity to produce affinity-matured IgG antibodies when appropriately pre-treated CD27-positive B cells are used for fusion.

As mentioned above, human hybridoma technology not only suffered from the lack of suitable fusion partners, but also from a shortage of antigen-stimulated B cells. Because ethical considerations typically prevent antigen exposure at will in humans, alternative methods to improve access to the human immune repertoire were developed. Human peripheral blood lymphocytes transplanted into immune-compromised mice were shown to give rise to a secondary humoral immune response upon antigen stimulation, and provide access to the human memory compartment through hybridoma technology.

In summary, each of the methods described is either labor-intensive or suffers from drawbacks when compared to the classical hybridoma technology. Thus, hybridoma technology has not become the method of choice for mining human antibody repertoires, leading to tremendous efforts to develop the alternative methods discussed in the following sections.

Clonal expansion of peripheral B cells. One of the most popular methods for harnessing the human immune response involves immortalization of peripheral B cells with Epstein-Barr virus (EBV). The first demonstration of EBV immortalization of human B cells into lymphoblastoid cell lines in vitro, was described almost 40 years ago. There have been numerous reports since then, although initial success was limited due to low efficiency of B-cell immortalization and cloning and the small quantities of antibody secreted. Better cloning efficiency, stability and productivity can be achieved when EBV-immortalized antibody secreting cells are fused with mouse myeloma or mouse-human heteromyeloma cells. Thus, multi-step procedures involving EBV-immortalization of peripheral B cells followed by fusion with myeloma cells and cloning of hybridomas were established. This method has yielded several human mAbs with therapeutic potential, including neutralizing antibodies against the hemagglutinin protein derived from the 1918 H1N1 pandemic influenza virus.

As an alternative to the formation of hybridomas, EBV-transformed cells have been infected with a retrovirus encoding the ras oncogene, yielding cells with similar cloning efficiencies and levels of antibody secretion. The most significant improvement of EBV immortalization came from the observation that addition of the TLR9 ligand CpG to human B cells dramatically increases the efficiency of EBV immortalization and facilitates the establishment of clonal lines stably secreting specific antibody. In this manner, mAbs potently neutralizing the SARS corona virus or the H5N1 Influenza A virus were isolated.

Strategies allowing the long-term growth and cloning of human B cells, without immortalization by transformation with a virus or an oncogene, have also been explored. In initial experiments, short-term B-cell clones were formed when cultured in the presence of EL-4B5 thymoma cells in conjunction with human T-cell plus macrophage supernatant. Improved proliferation and antibody production were found under more defined conditions, when B cells were activated by IL-4 treatment and CD40 triggering. A combination of both methods together with further refinements proved extremely efficient, allowing direct establishment of viable B-cell cultures from single, antigen-specific B cells. Similar to EBV immortalization, these B-cell clones secrete sufficient amounts of antibody into the supernatant, and production of specific antibody can be validated prior to cloning of the variable domains. Further, high throughput screening based on short term clonal expansion of activated peripheral B cells are possible and have led to the identification of HIV-1 neutralizing antibodies.

Recently, a promising method involving genetic reprogramming of human memory B cells was described. Retrovirus-mediated expression of Bcl-6 and Bcl-xL in peripheral memory B cells, combined with culturing on CD40L-expressing L cells in the presence of IL-21, converts them to highly proliferating, cell surface Ig-positive, antibody secreting B cells that can be cloned efficiently. The proteins Bcl-6 and Bcl-xL are typically expressed in GC B cells, and the resulting cells have features of GC B cells, including expression of activation-induced deaminase (AID). It is of interest whether this method will find broader application in the generation of human mAbs.

Single-cell PCR. The most straightforward, though technically challenging, non-combinatorial strategy for mining human antibody repertoires is single-cell polymerase chain reaction (PCR). If both heavy and light chain variable domains can be amplified in an efficient and reliable manner from single cells, then antibodies with original heavy and light chain pairings can be cloned on a cell-by-cell basis without the need to grow individual B-cell clones. In principle, either memory B cells or plasma cells can serve as a source of rearranged antigen-binding sites. As discussed, peripheral memory B cells are known to persist for many years after antigen exposure, thereby
offering a diverse and attractive repertoire of antigen specificities readily available in the blood. As memory B cells contain only relatively small quantities of specific RNA, the successful cloning of both heavy and light chain variable domains from a single B cell poses a significant technical challenge. In spite of this, antibodies have successfully been cloned from single peripheral B cells, including mAbs against self-antigens,\textsuperscript{61,62} tetanus toxoid\textsuperscript{65} and HIV-1 gp140.\textsuperscript{65} The efficiency of cloning relevant antibodies has been improved, e.g., by enriching specific B cells using antigen-coated magnetic beads,\textsuperscript{57} or by directly isolating antigen-specific B cells by fluorescence-activated cell sorting (FACS).\textsuperscript{63}

Compared to memory B cells, plasma cells have the disadvantage that they are present in the blood only for short periods of time after antigen exposure, after which they are only accessible in the bone marrow. Nonetheless, a known antigen exposure or challenge, e.g., a booster immunization, induces circulating plasma cells, of which a majority is specific for the antigen after approximately one week.\textsuperscript{64} Although no highly plasma cell-specific marker is known to date, they are readily isolated by FACS, e.g., based on their high expression of CD19 and CD38, and intermediate expression of CD45.\textsuperscript{65} These cells represent dedicated antibody factories and contain huge amounts of specific heavy and light chain mRNAs, thereby facilitating antibody cloning by reverse transcriptase (RT)-PCR. Numerous human mAbs have been cloned from plasma cells to date, including mAbs against tetanus toxoid and influenza virus.\textsuperscript{64-66} Recently, using a method related to microengraving,\textsuperscript{67} microwell array chips were successfully used for high-throughput screening of human antibodies secreted by single CD138\textsuperscript{+} plasma cells followed by RT-PCR cloning.\textsuperscript{68}

### Combinatorial Mining

Combinatorial mining of human antibody repertoires is intertwined with the concept of antibody libraries.\textsuperscript{8,69} Antibody libraries randomly combine heavy and light chain cDNAs obtained through RT-PCR from mRNA of B-cell pools into expression vectors that afford a compartmental or physical linkage of phenotype (protein) and genotype (cDNA). In contrast to non-combinatorial mining, the source of human antibody repertoires subjected to combinatorial mining is B-cell mRNA rather than B cells. Thus, antibody secreting cells, due to their higher heavy and light chain gene transcript levels compared to other B cells, can have much greater representation in antibody libraries. This bias can be highly desirable for the combinatorial mining of immune, but not naïve, repertoires. To increase the diversity of independent clones in antibody libraries from naïve repertoires, RT-PCR of the heavy chain mRNA can be restricted to the IgM isotype. However, combinatorial strategies have inherent biases due to the RT-PCR cloning of heavy and light chain cDNAs and due to the expression and folding of non-natural antibody fragments in non-B-cell environments. Nonetheless, high-throughput DNA sequencing recently confirmed that large antibody libraries can accurately mimic natural human antibody repertoires in terms of molecular diversity.\textsuperscript{70,71}

Like other cDNA libraries, the first antibody libraries were based on lambda phage vectors that carried heavy and light chain cDNAs and facilitated their expression upon infection of Escherichia coli bacteria.\textsuperscript{72} Lysis plaques of lambda phage on bacterial lawns were then screened with antigens of interest. This method was used to clone a diverse panel of human mAbs in Fab format to tetanus toxoid after generating an antibody library from peripheral blood lymphocytes isolated from human donors who had received a booster vaccination.\textsuperscript{73,74} A major drawback of this method is its limitation to screening as opposed to selecting antibody libraries. The number of independent clones, i.e., lysis plaques, that can be screened, even if the antigen of interest is in unlimited supply, is practically confined to 10\textsuperscript{6}.

Subsequent combinatorial strategies, e.g., phage, yeast and mammalian cell display, replaced screening with selection, allowing the mining of much larger antibody libraries approaching 10\textsuperscript{11} independent clones. Selection is facilitated by display technologies that physically link a displayed antibody fragment to its cDNA in a defined particle such as a filamentous phage, a ribosome, or a cell. Millions to billions to, theoretically, trillions of independent particles constitute an antibody library that can be mined with antigens of interest. Particles that display antibody fragments with high affinity to the antigen are isolated. Because of the simultaneous isolation of their cDNA, the displayed antibody fragments can be copied, enabling multiple rounds of selection. In other words, the physical linkage of phenotype and genotype effectively links recognition and replication. In addition, DNA sequencing may readily identify phenotype and genotype.

**Phage display.** The generation and selection of peptide libraries displayed on filamentous phage marked the advent of display technologies.\textsuperscript{75} The use of phage display for the generation and selection of antibody libraries, displayed in either scFv\textsuperscript{76-78} or Fab\textsuperscript{79,80} format, revolutionized the field of mAbs and antibody engineering in the early 1990s, arguably accelerated by an intense competition between researchers at the Medical Research Council (Cambridge, UK) and The Scripps Research Institute (La Jolla, California, USA). Over the past 20 years, phage display has proven to be the most robust and versatile mining tool for human antibody repertoires, yielding human mAbs to virtually any antigen of interest.\textsuperscript{8} The process of multiple rounds of selection on a purified antigen or on antigen-expressing cells, referred to as panning, can be adapted to positively or negatively select a range of desired antibody properties, such as affinity, specificity, manufacturability and catalytic activity. While scFv (~25 kDa) and Fab (~50 kDa) still dominate the phage display format, human antibody repertoires have also been mined through the display and selection of single variable domains (~12.5 kDa) of heavy and light chain.\textsuperscript{81,82}

Typically, recombinant fusion to the N-terminus of phage protein pIII or a fragment of pIII provides the physical linkage of antibody fragment and phage, facilitating monovalent display in phagemid systems and multivalent display in phage systems.\textsuperscript{83} Other multivalent display systems use phage proteins pVIII and pIX as fusion partners.\textsuperscript{79,84} For the generation of human mAbs with high affinity, monovalent Fab display through phage protein pIII is generally preferred.\textsuperscript{85} Phage display relies on the expression...
and folding of antibody fragments in the periplasm of gram-negative bacteria, typically *Escherichia coli*. Although this compartment provides an oxidizing milieu suitable for disulfide bridge formation and mimics the endoplasmic reticulum of eukaryotic cells surprisingly well, prokaryotic factors that skew the selectable diversity of antibody libraries are inevitable. For example, the lack of chaperones in bacteria can lead to inadequate folding of scFv and Fab and result in toxicity.

Human antibody libraries displayed on phage were generated from immune and naïve repertoires at about the same time. The first human immune repertoire mined by phage display were based on peripheral B cells and bone marrow of vaccinated or infected human donors and yielded human mAbs to tetanus toxoid, HIV-1 gp120, and other viral antigens.95-98 Notably, antibody libraries derived from bone marrow of HIV-1-positive human donors with serum antibodies to DNA and other autoantigens have also been accessed by phage display.99-101 Tumor-infiltrating B cells provide a unique immune repertoire in cancer patients that has been mined with phage display.102 The bone marrow was conceptualized as the central bank for antigen-experienced B cells that carried a record of antibody responses to past viral exposures. Autoimmune repertoires from human donors who have serum antibodies to DNA and other autoantigens have also been accessed by phage display.103-105 Tumor-infiltrating B cells provide a unique immune repertoire in cancer patients that has been mined with phage display for potentially therapeutic human mAbs to tumor antigens.106-108 Alternatively, peripheral B cells from cancer patients vaccinated with autologous tumor cells have been used to generate and select antibody libraries by phage display.109 Peripheral B cells and phage display were also employed to mine an all-human repertoire of a cancer patient who had been treated with allogeneic hematopoietic stem cell transplantation, revealing serum antibodies to tumor antigens that might have potential as therapeutic human mAbs.110

The first human naïve repertoire mined by phage display was based on peripheral B cells and yielded human mAbs to a variety of different antigens that had not been encountered in vivo.111 Importantly, this method allowed the generation of human mAbs to human antigens.112 These studies demonstrated that the random combination of heavy and light chain can yield human mAbs with new specificities. Due to the overlap of naïve and immune repertoires, as well as the bias of mRNA from antibody secreting cells, antibody libraries from naïve repertoires typically contain a mixture of heavy and light chain cDNAs with and without somatic hypermutations. Human mAbs derived from naïve repertoires generally have lower affinities compared to human mAbs from immune repertoires, but this shortcoming can be overcome by increasing the number of independent clones, thereby generating a larger antibody library.113-115 However, it is now common to mature the affinity of human mAbs derived from naïve repertoires with subsequent antibody engineering methods that are also based on display technologies. Methods that involve the randomization of certain sequences within heavy or light chain are discussed in a subsequent subsection on synthetic repertoires.

A method that preserves natural V<sub>H</sub>-DJ<sub>H</sub>, V<sub>J</sub> and V<sub>L</sub> sequences and yields human mAbs indistinguishable from endogenous human antibodies involves the shuffling of heavy and light chains.116 This method, known as chain shuffling, can yield excellent human mAbs from moderately diverse antibody libraries with 10<sup>4</sup> independent clones.117 Chain shuffling can also be used to convert mouse mAbs to human mAbs via sequential replacement of mouse heavy and light chains with human heavy and light chain libraries, respectively, followed by selection through phage display.118 Adalimumab, the first human mAb approved by FDA and EMA (Table 1), was derived from a mouse mAb to human TNFα via this method.119 A similar method, also based on phage display, preserves only the complementarity determining region (CDR) 3 sequences of mouse heavy and light chains, i.e., HCDR3 and LCDR3, while converting everything else to human sequences.120

A general concern about the de novo generation of human mAbs from naïve and synthetic repertoires in vitro is their lack of exposure to negative selection processes in vivo which are integral to immune tolerance mechanisms and efficiently remove undesirable off-target reactivities. Addressing this concern, phage display has also been used to mine non-combinatorial antibody libraries, i.e., antibody libraries that retain original heavy and light chain pairings of human antibody repertoires through in-cell RT-PCR.121 This method is of particular interest for the identification of human antibodies in autoimmune repertoires.

**Yeast display.** The expression, folding and display of antibody fragments on the surface of yeast cells from the yeast *Saccharomyces cerevisiae* involve eukaryotic processes that are similar to those encountered in the natural B-cell environment. Antibody fragments are channeled through endoplasmic reticulum and Golgi apparatus, ensuring proper disulfide bridge formation and N-linked glycosylation.111,112 Thus, yeast display may address some of the above mentioned biases prokaryotic systems impose on antibody libraries. In fact, in a direct comparison based on the same human antibody repertoire, yeast display yielded a more diverse set of human mAbs than phage display.113 The physical linkage of antibody fragment and cell is provided through recombinant fusion to the C-terminus of the secreted protein Aga2, which covalently associates with the cell surface protein Aga1.114 Formats of antibody fragments that have been used for yeast display include scFv,115 Fab115 and scFab.116

Initially limited by the number of independent clones that could be transformed, yeast display has become a powerful mining tool for large antibody libraries from human naïve repertoires.117-119 In addition, yeast display has been successfully employed for the mining of human immune repertoires.113,120 Interestingly, the haploid/diploid lifecycle of yeast can be exploited in recombinatorial strategies that generate large antibody libraries in diploid cells by mating a haploid cell pool harboring a heavy chain library with a haploid cell pool harboring a light chain library.118,120 Such recombinatorial strategies based on yeast display have also been used for chain shuffling.121

In contrast to phage display, antibody libraries displayed on yeast cells are simultaneously screened and selected with an antigen of interest. Screening is carried out by flow cytometry and selection by FACS. The ability to detect and sort individual binding events during the selection process has made yeast display...
an exceptional method for the affinity maturation of mAbs, including human mAbs derived from naive repertoires by phage display. Mammalian cell display. A screening/selection system based entirely on expression of human antibodies in their natural environment, i.e., the mammalian cell, may be best suited for the mining of human antibody repertoires. Within this system, all necessary components for human antibody synthesis and processing are available at physiological levels. Compared to yeast cells, antibody repertoires expressed in mammalian cells are less biased by properties other than antigen binding. Several laboratories have therefore developed strategies for human mAb mining by mammalian cell display. The most straightforward approach is to transfect an antibody library with a vector directing antibody expression to the cell surface by means of a C-terminal transmembrane region, thereby allowing for selection of cells expressing specific antibody based on antigen binding, e.g., by FACS.

In a proof-of-concept study involving the expression of a mixture of two different CD22-specific scFv on the surface of human embryonic kidney 293T cells, it was estimated that an up to 240-fold enrichment can be achieved with only 2-fold affinity differences. It remains to be shown whether this approach will allow for isolation of antibodies from more complex mixtures, such as a library. A general limitation of screening by plasmid transfection is the potential to deliver multiple plasmids per cell, leading to the expression of an ill-defined number of different antibodies on each cell. It would then require multiple rounds of selection in order to enrich for specific antibodies, even if the library diversity is small. This problem can be circumvented with viral expression vectors. As infection rates can be dosed precisely, conditions can be used where one cell expresses exactly one antibody species.

One viral system suitable for screening of human antibody libraries on the surface of mammalian cells is based on vaccinia virus. Using separate heavy and light chain libraries, African green monkey kidney BSC-1 cells are co-infected, human IgG antibodies expressed on the cell surface and cells encoding specific antibody isolated by FACS. Because heavy and light chains are expressed from separate vectors, infection rates are kept high to ascertain that a substantial fraction of cells expresses functional antibody. As a consequence, some of the cells isolated will express more than one heavy and light chain pair, and several rounds of selection are beneficial. After screening, specific recombinants can be recovered from small numbers of selected cells, perhaps even single cells.

A two-step method for the isolation of human antibodies by mammalian cell display has been described recently. Using virus like particles with highly repetitive arrays of antigen, pools of B cells specific for an antigen of interest are first isolated from peripheral blood lymphocytes of immunized or naturally immune human donors by FACS. Recombinant, antigen-focused scFv libraries are then generated from these B cells and screened by mammalian cell surface display using a Sindbis virus expression system. Infection of baby hamster kidney cells at a low multiplicity of infection ensures that one cell expresses only one antibody species, thus allowing identification of antigen-specific antibodies in a single round of FACS. Due to the highly replication-competent nature of the Sindbis vector, viruses encoding a specific antibody can readily be amplified from single-sorted cells. Thus, the binding properties of antibodies can be characterized prior to cloning, sequencing and subsequent production as whole IgG. Towards a clinical application of the technology, therapeutic mAbs against nicotine and influenza A M2 protein have been isolated, characterized and validated preclinically to date. A drawback of mammalian cell display is that the number of cells that can be handled at the same time is limited. This is particularly true if the screening is done under conditions where one (or close to one) antibody species is expressed per cell, limiting the diversity of libraries that can be conveniently handled to about $10^7$ independent clones. This is suboptimal for combinatorial libraries and pre-selection of B cells for antigen specificity is advisable. It is therefore no coincidence that the human mAbs isolated by mammalian cell display to date are derived from pools of B cells enriched for antigen specificity. Because these enriched combinatorial libraries are of low diversity, there is a high likelihood that the resulting human mAbs contain natural pairs of heavy and light chains, i.e., are derived from clonally related B cells. An advantage common to all screening systems based entirely on mammalian cell expression is that they have a built-in selection for efficient expression in mammalian cells. As antibody good manufacturing practice (GMP) processes typically involve expression in mammalian cells, this is expected to have a significant impact on the cost of goods.

Non-Natural Repertoires

Transgenic repertoires. More than 20 years ago, a proof of concept study first demonstrated that human Ig gene segments introduced into a mouse can be accessed by the mouse immune system and used to express functional antibodies. In this approach, a human heavy chain mini locus containing $V_H$ D and $J_H$ gene segments linked to a $C_{\mu}$ gene was introduced into an embryonic stem cell that was used to develop a transgenic mouse. In this mouse, a large fraction of lymphoid cells expressed human antibody genes and this led to measurable levels of hybrid IgM with human heavy chains and mouse light chains in the serum. Further, it was shown that conventional hybridoma technology could be used to recover recombinant monoclonal IgM, thus laying the ground for the production of human antibodies in so-called humanized mice.

Production of human antibodies in genetically modified mice was immediately recognized as a highly attractive approach for the generation of human mAbs against human antigens, as mice can readily be immunized with any antigen of interest and are not tolerant to most human proteins. While the mice described by Brüggemann et al. only expressed a human heavy chain mini locus in the presence of the endogenous mouse heavy and light chain loci, the production of fully human antibodies became feasible with the development of mice with additional genetic modifications. In two different laboratories, mice were generated that combined disruptions of the endogenous mouse heavy and
The endogenous mouse lambda light chain locus was left unmodified by both groups because it only contributes to 5–15% of the antibody repertoire. In the report by Lonberg et al., the human heavy chain locus comprised 3 Vπ, 16 D, all 6 Jπ, and the Cπ and Cμ constant region gene segments, whereas the light chain locus comprised 4 Vπ, all 5 Jπ, and the Cκ gene segments. The mice described by Green et al. carried a repertoire of similar diversity, with the heavy chain locus including 5 Vπ, all 25 D, all 6 Jπ, and the Cπ and Cμ constant region gene segments, and the light chain locus including 2 Vπ, all 5 Jπ, and the Cκ gene segments. The human transgenes in such mice had undergone Vπ-D-Jκ and Vπ-Jκ rearrangements with N region diversification, i.e., additions or deletions of nucleotides at the Vπ-D-Jκ and Vπ-Jκ junctions, class switch recombination and somatic hypermutation. Despite the limited repertoire present in both transgenic mouse strains, immunization led to the generation of specific human antibodies against several antigens that were accessible by conventional hybridoma technology. In this respect, transgenic mice have led to a revival of hybridoma technology for the mining of human antibody repertoires.

The finding that transgenic mice containing human heavy and light chain mini loci are capable of mounting a specific humoral immune response comprising human antibodies is surprising in at least two respects. First, it demonstrates that hybrid B-cell receptor complexes consisting of human surface Ig and mouse Igα/Igβ (CD79a/CD79b) chains are functional in vivo and permit largely normal development and function of B cells. Second, only a fraction of the natural heavy and light chain V gene repertoire appears to be sufficient for the generation of antigen-specific antibodies, putting the relative importance of combinatorial diversity into perspective. For the production of antibodies against protein antigens using hyperimmunization protocols, the two non-germline encoded sources of clonal diversification, i.e., additions or deletions of nucleotides at the Vπ-D-Jκ and Vπ-Jκ junctions, class switch recombination and somatic hypermutation, appear to be sufficient. Indeed, it was shown that the HCDR3 repertoire is in large part created by junctional diversity, and sufficient for most antigen specificities.

Despite the initial success of the transgenic mice carrying mini loci, human mAbs against few antigens were isolated from these mice. The immune responses in these mice were inferior to those of wild type mice, with reduced numbers of mature B cells and lower levels of circulating IgGs; however, incorporation of a significantly larger repertoire of Vπ gene segments led to increased levels of pre-B and B cells. Mice that incorporated the majority of the human heavy chain and kappa light chain loci were found to recapitulate the human antibody response even better by restoring B-cell compartments to near normal levels. Compared to the transgenic mice carrying mini loci, the animals with near complete loci displayed higher levels of human serum Ig, better class switch recombination efficiency and increased clonal diversity and magnitude of the human antibody response. Accordingly, a panel of human mAbs with high affinity against three human antigens was isolated. In contrast to mice, approximately 40% of serum IgGs in humans contains a lambda light chain. To further enhance the diversity of humanized B-cell responses, transgenic mice were generated that carried, for the first time, large portions of all three human Ig loci, including the human lambda light chain locus, in a background in which the endogenous mouse heavy and kappa light chain loci had been inactivated. Upon immunization, these mice generated antibodies containing both human kappa and lambda light chains. To generate more complete humanized mice, the to date largest fraction of the human germline repertoire was introduced by microcell-mediated chromosome transfer. In this manner, transchromosomal mice were generated that express the complete heavy chain and kappa light chain repertoire in absence of mouse heavy and kappa light chains. However, instability of the chromosome fragment containing the kappa light chain locus led to a substantial reduction in the generation of hybridomas, a problem that was solved by crossing IgH transchromosomal mice with Igκ transgenic mice. It is interesting to note that of the six human mAbs on the market today, five are derived from humanized mice (Table 1). Of these, panitumumab is derived from Abgenix’ XenoMouse, whereas the other four are derived from Medarex’ UltiMab platform, which is based on the HuMab mouse, the Kirin TC mouse and a cross of the two, the KM mouse.

Natural antibody responses are polyclonal. There are cases in which immunotherapy using polyclonal antibodies (pAbs) instead of mAbs is expected to be more efficient. This includes, for example, the treatment or prevention of diseases caused by different viral or bacterial strains or combating viruses prone to formation of escape mutants. In principle, human pAbs could be produced in humanized mice. While mice are well suited for the generation of mAbs, their small body size makes them unsuitable for the production of useful amounts of therapeutic or preventive pAbs. One method of accessing the potential of human pAbs is to use recombinant pAbs, i.e., defined cocktails of mAbs. In addition, due to advances into the understanding of humoral immune systems of large farm animals combined with significant technological advances, it may soon be possible to generate human pAbs by hyperimmunization of transchromosomal cattle.
Antibody libraries that are based on human naïve repertoires but introduce additional sequence diversification through randomizing single or multiple CDR sequences represent, by definition, human synthetic repertoires; however, not all synthetic repertoires are based on randomized CDR sequences. Using master framework sequences derived from a single human VH and VL gene segment, Söderlind et al. introduced and recombined a complete set of CDR sequences derived by RT-PCR from human naïve repertoires. Yet another approach of sequence diversification in vitro is based on dispersing mutations throughout heavy and light chain variable domain sequences, as opposed to focusing mutations on defined segments such as CDR sequences. This can be achieved through error-prone PCR or mutagenic Escherichia coli strains. Like focused mutagenesis, dispersed mutagenesis has been utilized for the affinity (or catalytic activity) maturation of human mAbs, usually by phage display and, more recently, by yeast display.

Owing to the fact that the discussed antibody libraries blend in vivo and in vitro diversity, they are sometimes referred to as semi-synthetic. By contrast, fully synthetic antibody libraries are entirely designed and engineered in vitro. A fully synthetic antibody library that combined a single human VH and VL gene segment with randomized HCDR3 and LCDR3 sequences was mined successfully for human mAbs to human antigens. Further restrictions that mimic natural CDR sequence diversity in the heavy chain variable domain and use a fixed light chain variable domain yielded human mAbs that closely resemble endogenous human antibodies. These were improved further by introducing natural CDR sequence diversity in the light chain variable domain. CDR sequence diversity can be further restricted to a four-amino-acid code (Ala, Asp, Ser and Tyr) or even a two-amino-acid code (Ser and Tyr), demonstrating the versatility of synthetic repertoires and providing molecular insights into specificity and affinity of antibody/antigen interactions. Restricting sequence diversity in fully synthetic and semi-synthetic repertoires has also been used for tailoring antibody libraries toward the selection of human mAbs to a particular class of antigens, such as peptides and carbohydrates. Fully synthetic antibody libraries that are more complex and based on >1010 independent clones (in scFv or Fab format) reassemble a variety of natural CDR and framework sequences and combine these with randomized CDR sequences. A fully synthetic antibody library with diversification in all six CDR sequences was reported more recently.

In addition to phage display, fully synthetic antibody libraries have been mined successfully with ribosome display. Ribosome display is an in vitro display technology that does not require transformation of prokaryotic or eukaryotic cells. Ribosome display has been employed to both accomplish and guide the affinity maturation of human mAbs. Other less common display technologies, such as retroviral display, have also been used for mining synthetic repertoires. In addition to the conventional scFv and Fab formats, synthetic repertoires have facilitated the display and selection of single human variable domains and, recently, human constant domains with diversified sequences in non-CDR loops of the Ig fold.

Another exciting advance in the area of human synthetic repertoires has been the generation and selection of antibody libraries that feature an expanded genetic code for the incorporation of unnatural amino acids into CDR sequences. Collectively, synthetic repertoires have begun to outshine all other human antibody repertoires with respect to diversity and versatility. Human mAbs from synthetic repertoires are poised to become preferred reagents for diagnostic and basic applications; however, their utility for preventive and therapeutic interventions remains to be established in clinical trials.

**Outlook**

Increased availability and improved accessibility of human antibody repertoires have made human mAbs the entity of choice for therapeutic mAbs. Today, human mAbs to virtually any antigen of interest can be generated. The clinical performance and the commercial viability of currently approved human mAbs provide a robust platform for the development of future generations of human mAbs. Not only will these broaden the scope of targeted antigens and indications, but also improve the activity of intervention through better pharmacodynamic and pharmacokinetic properties. Although an initial goal in the development of human mAbs was to render them indistinguishable from endogenous human antibodies, subtle antibody engineering and expression strategies that retain low immunogenicity, but tune affinity, effector functions and circulatory half-life, as well as lower the cost of goods, are being employed in the development of the next generation of human mAbs.

Of the currently approved human mAbs (Table 1), one was derived from a naïve repertoire and five were derived from transgenic repertoires. It will be interesting to compare human mAbs from different sources with respect to their pharmacokinetic and pharmacodynamic performance. For example, the human anti-human TNFα mAb, adalimumab, which was derived indirectly from a naïve repertoire as described above, induces a HAH response in a significant percentage of patients, adversely affecting serum concentrations and clinical responses. The recent approval of human anti-human TNFα mAb, golimumab, which was derived from a transgenic repertoire, provides a first opportunity to clinically investigate whether human mAbs derived from different human antibody repertoires against the same antigen differ in terms of immunogenicity.

The immune repertoire is a natural repertoire that has not been fully exploited for the mining of human mAbs. Sophisticated in vivo processes that shape the immune repertoire afford endogenous human antibodies with high affinity, minimal immunogenicity and minimal off-target reactivity. The immune repertoire has become increasingly accessible through new methods that facilitate the enrichment, isolation or expansion of antigen-specific post-GC B cells, plasma blasts, plasma cells or memory B cells from peripheral blood. The development of these methods has been spurred by the discovery of endogenous human antibodies of potential therapeutic utility in normal human donors, as well as in those with inflammatory diseases, infectious diseases, and cancer. Endogenous human antibodies that are triggered...
by preventive or therapeutic interventions, such as vaccination or allogeneic hematopoietic stem cell transplantation, are particularly attractive. An ideal mining strategy for this exceptional source of human mAbs is mammalian cell display as it provides an environment that closely resembles B cells. Consequently, this platform is anticipated to contribute to the development of future generations of human mAbs.

Finally, it is important to keep in mind that endogenous human antibodies are polyclonal whereas human mAbs, by definition, are monoclonal. In general, polyclonal or even oligoclonal responses are thought to be more proficient in mounting effector functions compared to monoclonal responses.179 While non-recombinant human pAbs purified from human donors have long been used in preventive and therapeutic interventions, recombinant human pAbs would afford much broader applicability.180 Thus, despite current practical and regulatory challenges in manufacturing and formulation, human pAbs likely will be a factor in future antibody therapy. Mining strategies that yield a panel of human mAbs to a variety of different epitopes of an antigen of interest are suitable for the development of human pAbs. Nonetheless, it remains possible that cocktails of human mAbs that are individual products of conventional mining strategies are still inferior to truly polyclonal human antibodies that emerged as complex mixtures in vivo. Thus, evolving mining strategies from monoclonal to polyclonal screening and selection offers a worthy challenge for antibody engineers.

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Conflict-of-interest and financial disclosure statements

R.R.B. is an employee of Cytos Biotechnology AG and holds stock options in the company.

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