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Review Article

Hybridoma technology: is it still useful?

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ABSTRACT

The isolation of single monoclonal antibodies (mAbs) against a given antigen was only possible with the introduction of the hybridoma technology, which is based on the fusion of specific B lymphocytes with myeloma cells. Since then, several mAbs were described for therapeutic, diagnostic, and research purposes. Despite being an old technique with low complexity, hybridoma-based strategies have limitations that include the low efficiency on B lymphocyte-myeloma cell fusion step, and the need to use experimental animals. In face of that, several methods have been developed to improve mAb generation, ranging from changes in hybridoma technique to the advent of completely new technologies, such as the antibody phage display and the single B cell antibody ones. In this review, we discuss the hybridoma technology along with emerging mAb isolation approaches, taking into account their advantages and limitations. Finally, we explore the usefulness of the hybridoma technology nowadays.

1. Introduction

Monoclonal antibodies (mAbs) are universal highly specific binding proteins that were envisioned for a long time as "magic bullets" in the fight against diseases, and also important tools for other biological uses, including diagnosis and research (Parray et al., 2020). These applications were only possible with the advent of methodologies that allow the isolation of individual antibodies. The hybridoma technology was the pioneer on that. Indeed, this technique revolutionized the therapeutic and research scenario, which was further recognized by the 1984 Nobel Prize in physiology or medicine (Leavy, 2016). Other strategies have been developed for the same purpose. In this review, we explore the relevance of the hybridoma technology nowadays, how it has evolved with time, and its advantages and limitations compared with other methods that further come out.

2. Hybridoma technology

The hybridoma technology, described by Georges Köhler and Cesar Milstein in 1975 (Fig. 1), is based on the immunization of animals with the desired antigen, followed by the fusion of specific B lymphocytes with "immortal" myeloma cells. The generated hybrid cells, called hybridomas, are then cloned to obtain stable monoclonal cell lines (Köhler, and Milstein, 1975). After selecting the antibody-secreting clones of interest, the cells are transferred to large-scale culture setups to produce the antibody in the desired amounts (Holzlöhner and Hanack, 2017). B lymphocyte-myeloma cell fusion is often obtained by using the chemical compound polyethylene glycol (PEG). However, this agent can be cytotoxic at some level, and non-specific membrane fusion may occur (Tomita and Tsumoto, 2011; Smith and Crowe, 2015). Fusogenic viruses, such as the Sendai and the vesicular stomatitis viruses, are alternatives that bypass the cytotoxic effects of PEG (Smith and Crowe, 2015). Another possibility is the pearly chain method, through which the fusion occurs with the aid of an electric field and laser radiation. In this case, the contact cell surface is irradiated with pulsed laser beams to make a small perforation in the cell membrane, which enhances the chance to promote cell fusion (Ohkohchi et al., 2000; Tomita and Tsumoto, 2011). Although the pearly chain method has advantages over the PEG-mediated strategy,

Abbreviations: ASC, antibody-secreting cell; BCT, B cell targeting; cDNA, complementary DNA; CDR, complementarity determining region; Fab, antigen-binding fragment; mAb, monoclonal antibody; PEF, pulsed electric field; PEG, polyethylene glycol; scFv, single-chain variable fragment; SST, stereospecific targeting; VH, heavy chain variable domain; VL, light chain variable domain.

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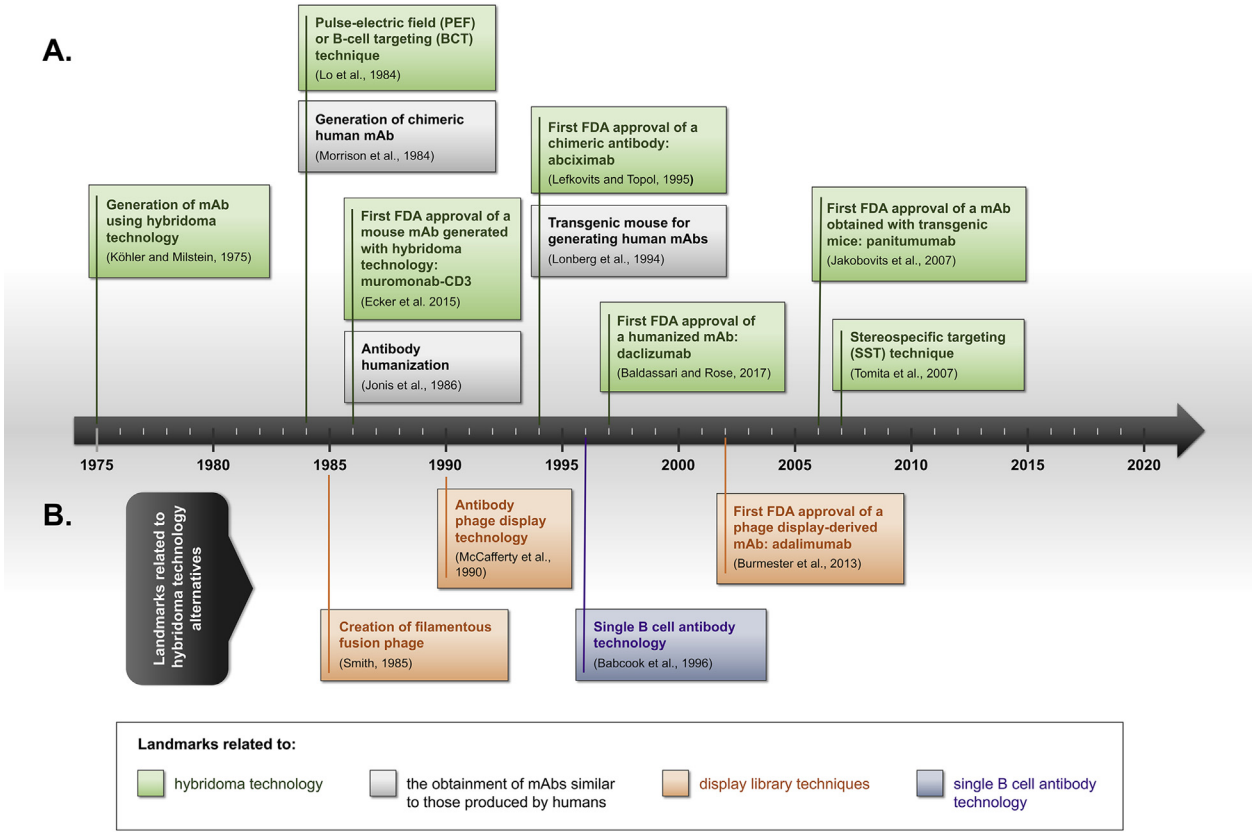

Fig. 1. Timeline of important events in the generation of monoclonal antibodies. (A) Milestones related to hybridoma technology (boxes in green) and the obtainment of mAbs similar to those produced by humans (boxes in gray). **(B)** Landmarks related to hybridoma technology alternatives: display library techniques (boxes in orange) and single B cell antibody technology (box in purple).

it still cannot selectively control the fusion of a specific B lymphocyte with myeloma cell (Tomita and Tsumoto, 2011).

Since the introduction of the hybridoma technology, mAbs have had a profound impact on medicine, providing an almost limitless source of therapeutic, diagnostic, and research reagents (Nissim and Chernajovsky, 2008; Ribatti, 2014). Given the universality and usefulness of mAbs, many discoveries came as a result of hybridoma technology, allowing the generation of antibodies directed against an antigen or even different antibodies against the same antigen (Parray et al., 2020). Among the advantages of this technique, we can list the highly reproducible mAb obtainment, once the hybridoma clones are established, the preservation of the native pairing of the combination of genes of the antibody variable and constant regions, and the *in vivo* antibody affinity maturation (Zaroff and Tan, 2019) (Table 1).

Muromonab-CD3, also called orthoclone (OKT3), was the first mAb approved by the Food and Drug Administration (FDA), in 1986, for therapeutic use in humans (Fig. 1) (Ecker et al., 2015). That is a murine hybridoma-derived mAb targeting CD3 on mature peripheral T cells to avoid organ allograft rejection (Colvin and Preffer, 1991). However, the occurrence of a human anti-mouse immune response has limited the clinical applicability of murine mAbs in humans (Gonzales et al., 2005). The most appropriate strategy for obtaining therapeutic mAbs would come with the use of human hybridomas, but attempts to obtain these hybrid cells failed, mostly due to their genetic instability (Smith and Crowe, 2015). On the other hand, technological advances allowed the structural modification of these molecules, and the first achievements on that made feasible the removal of antibody murine markers, giving rise to chimeric mAbs containing fragments of variable regions of the murine antibody light and heavy chains linked to human immunoglobulin constant regions. The chimeric mAbs are originated from mouse myeloma cells transfected with chimeric genes, producing antibodies with human features and the same antigen specificity of the antibody originally generated in mice (Morrison et al., 1984). Abciximab (c7E3 Fab) was the first chimeric antibody approved by the FDA, in 1994, to inhibit platelet aggregation in high-risk angioplasty cases (Fig. 1) (Lefkovits and Topol, 1995). Following studies led to a process known as antibody humanization, which grafts non-human antibodies complementarity determining regions (CDR) into human antibody scaffolds. That is obtained using non-human antibody framework regions as CDR graft acceptors (Jones et al., 1986; Safdari et al., 2013). In 1997, the FDA approved the first humanized antibody, called daclizumab (Fig. 1), which is indicated for prophylaxis of acute organ rejection in patients who received a kidney transplant and, subsequently, it was also allowed for the treatment of adults with recurrent forms of multiple sclerosis (Kim and Baker, 2016; Baldassari and Rose, 2017). In the next decade, a great advance happened with the obtainment of appropriate transgenic animals for generating fully human mAbs (Lonberg et al., 1994). This achievement was possible due to several methodological advances that allowed the integration of the human immunoglobulin gene loci into the mouse genome in a stable way, along with the inactivation of the endogenous murine immunoglobulin genes (Osborn et al., 2013; Murphy et al., 2014). Other transgenic animals, such as cattle, rabbits, and rats, can also be exploited for the biological production of human antibodies (Flisikowska et al., 2011; Osborn et al., 2013; Matsushita et al., 2014). The genetic manipulation of the genome was made such that the transgenic animal immunization with the antigen of interest turns possible the generation of murine hybridomas secreting human mAbs. The first hybridoma-derived human mAb isolated from transgenic animals – panitumumab – was approved for therapeutic use in 2006 (Jakobovits et al., 2007) (Fig. 1).

The hybridoma technology has remained at the forefront of the mAb generation field (Zaroff and Tan 2019). Currently, more than 90% of the

Table 1
Advantages and disadvantages of technologies used to generate monoclonal antibodies.

Technology	Advantages	Disadvantages
Hybridoma		
Original technique	- Preserves the native pairing of variable and constant regions gene combination. - Antibody chimerization and humanization methods and transgenic animals can be used to obtain mAbs for therapeutic use in humans. - Antibodies undergo *in vivo* affinity maturation.	- Known and available antigen targets are needed. - Low efficiency on cell fusion and hybridoma isolation. - It is required a relatively long period for generating the cell line and the selection of a specific hybridoma. - Hybridoma cell lines may be genetically unstable. - Constant risk of cell culture contamination.
B Cell Targeting	- More efficient cell fusion compared to the original hybridoma technique. - Use only B lymphocyte selected by antigen. - Possibility to simultaneously generate at least 3 specific mAbs against different antigens, using a single mouse.	- Electrostatic field applications might be challenging. - High technical expertise is needed.
Stereospecific targeting	- More efficient cell fusion compared to the original hybridoma technique. - Generation of mAbs that recognize native antigen conformations, instead of linear structures. - DNA immunizations are cheaper than the original hybridoma technique and allow the generation of antibodies against complex or non-conventional antigens.	- Might be more time-consuming and expensive than the previous techniques, particularly if cell lines for immunization, cell fusion, and screening steps are not available. - As it is necessary to perform an electric fusion, it also has the disadvantages of the BCT technique.
Antibody phage display	- Animal host is not required. - The screening of a large number of clones increases the chances of generating good mAbs. - Potential to isolate mAbs against toxic and non-immunogenic antigens. - Possibility to redesign natural CDRs for generating mAbs of improved specificity and affinity. - Display libraries are commercially available.	- The diversity of the phage library depends on the bacterial transformation efficiency. - Antibody formats are limited to scFv and Fab. - Building a phage display library is expensive.
Single B cell	- High efficiency in obtaining specific mAbs, compared to hybridoma technology. - Possibility to isolate mAbs from vaccinated or naturally immunized human subjects. - Isolation of native mAbs with the preservation of natural cognate VH and VL pairing. - No need to culture B cells. - Potential to isolate functional mAbs against conformation determinants that are difficult to emulate *in vitro*. - It is possible to distinguish B cells at different stages of development and differentiation. - In experimental studies, B cells can be isolated from multiple samples without the need to euthanize animals. - Antibodies undergo *in vivo* affinity maturation.	- Single-cell sorting devices are expensive. - RT-PCR procedures might be challenging. - Antibodies targeting B cell markers are not available for all species.

antibodies approved for therapeutic use were generated by this technology, most of them in chimeric or humanized versions (Parray et al., 2020). However, the dominance of this method is accompanied by its low efficiency. Hybridoma-based mAb generation is marked by long screening processes, suboptimal selection of specific mAb-secreting cells, a mAb validation that is rarely possible at an early stage, not to mention that the availability of the purified antigen target is needed (Harlow and Lane, 1988). To optimize antibody generation, several variants of this technology have been developed over the years. Examples are the B Cell Targeting and the Stereospecific Targeting techniques, which are described below.

2.1. B Cell Targeting (BCT)

The B Cell Targeting (BCT) method, also known as Pulsed Electric Field (PEF), was described by Lo et al., in 1984 (Fig. 1) (Lo et al., 1984). It is based on two central points: the preselection of B lymphocytes recognizing the antigen of interest, and the further B lymphocyte fusion with myeloma cells by using direct current electrical pulses (Tomita and Tsumoto, 2011). Briefly, specific biotin-labeled antigen binds to the corresponding B lymphocytes, which are subsequently recovered by using streptavidin, giving rise to a B lymphocyte-antigen-biotin-streptavidin complex (Tomita and Tsumoto, 2011; Greenfield, 2019). Then, such B lymphocyte complexes are co-cultured with biotin-labeled myeloma cells and the resulting mixture is exposed to PEF to promote cell fusion (Lo et al., 1984).

This last step, the most critical one, is characterized by the cell membrane destabilization after electrostatic field exposure, which eases the occurrence of fusion between cell membranes (Greenfield, 2019). For that, a strong electric field is formed vertically between electrodes arranged in parallel and guides the alignment of the B lymphocyte-myeloma cell complexes along with it, favoring the fusion of the membranes close to each other. No electrical fusion occurs in complexes arranged in any other direction (Tomita and Tsumoto, 2011). Different research groups have explored the application of electrostatic pulses for generating hybridomas (Wojchowski and Sytkowski, 1986; Werkmeister et al., 1991; Hewish and Werkmeister, 1989). In general, the cell fusion mediated by electric field was found more efficient than the achieved with PEG, a cytotoxic agent (Tomita and Tsong, 1990; Awsiuk et al., 2019), with improvements not only in the number of fused cells but also in the hybridoma growth rate. The BCT technique demonstrated five-to-ten times greater efficiency in the formation of hybridoma cells secreting the antibodies of interest, in comparison with the PEG-mediated method. However, based on the reported data, such fusion efficiency does not seem to go far beyond 20% (Tomita et al., 2006), and the BCT protocol is more complex than the original hybridoma one. Another point to note is that the electrofusion yields are low when the fusion partner cells have different sizes, although this is a limitation that can be overcome with the use of nanosecond pulse electroporation (Rems et al., 2013).

The BCT method can also be used for the simultaneous generation of at least three to five mAbs against different antigens, using a single mouse (Awsiuk et al., 2019), which reduces not only the laboratory work but also the number of animals needed for isolating mAbs. This procedure, known as multitargeting, is based on mouse immunization with multiple antigens, followed by the immunoglobulin B-cell receptor-guided selection of B lymphocytes sensitized by each of the desired antigens. As a disadvantage, immunosuppression caused by immunization with several antigens may occur (Table 1) (Tomita and Tsumoto, 2011; Awsiuk et al., 2019).

2.2. Stereospecific targeting (SST)

Early descriptions of conformation-specific mAbs were published in the 1960s (Janeway and Sela, 1967), highlighting the characteristic of these antibodies in specifically recognizing only one type of stereoisomer of a given chemical compound. It is known that stereospecific mAbs have high specificity for their ligands, which is helpful for diagnostic and therapeutic approaches. However, the generation of these mAbs is technically challenging, particularly in the case of highly structured and well-preserved targets. Examples are extracellular loops or domains of multi-transmembrane proteins, such as membrane-bound receptors (Hazen et al., 2014). The Stereospecific Targeting (SST) method was proposed to address this problem (Tomita et al., 2007) (Fig. 1) and consists of four phases.

A modification in the original hybridoma technology was performed already in the first step, the animal immunization. The immunogen is administered intramuscularly in the DNA form (Tomita et al., 2007), which guides the expression of the antigen in its native form. Thereby, the chances of inducing the production of functional mAbs are greater, even against the most challenging targets (Liu et al., 2016). Compared to protein inoculation, gene immunization allows the efficient testing of different designs of immunogens, does not require purification of proteins from a pathogen, circumvents the difficulty of expressing and purifying antigens in large quantities, and can also be used to obtain antibodies against several proteins at the same time through immunization with several nucleic acid sequences that encode different proteins or different subunits of the same protein (Liu et al., 2016), which are relevant advantages for generating high-quality mAbs. Although the DNA immunizations can be considered not very immunogenic in some cases, the use of immunomodulators, if necessary, does not interfere negatively in the conformation of the antigen. Also, among the options of entry pathways for DNA immunization, the intrasplenic administration may be still more efficient, since a single dose of DNA is sufficient to generate the desired antibody responses, with reduced immunization period and technique cost, compared to the traditional protein administration (Parray et al., 2020). On the other hand, the antigen glycosylation pattern, that differs from the occurring in humans, as well as the possibility of inducing immune tolerance and generating anti-DNA antibodies may be problems when using this approach (Khan, 2013). The transduction of myeloma cells to express the antigen is a limitation that sums to those described for BCT. In a recent update, an additional intraperitoneal injection containing cells that express the target antigen has been proposed to increase the humoral response and ensure the recognition of antigenic structures. The idea is to promote a further stage in the B cell maturation. Indeed, an increase in serum antibody titers, when compared to the results of gene immunization only, could be observed (Table 1) (Yamasaki et al., 2020). The second step involves the preselection of conformational epitope-recognizing B cells. For this, isolated splenic cells are incubated for a short period with myeloma cells transduced with a vector carrying the antigen gene for the formation of B lymphocyte-myeloma cell complexes (Shabani et al., 2010). The third step is the cell fusion itself, which occurs by using electrical pulses as described for the BCT method. The screening of hybridomas secreting the desired mAbs, the fourth step, makes use of the native antigen targets expressed on a cell surface. The clone selection may include an additional step to discard the undesirable clones by using recombinant protein, which may contain partially denatured structures (Yamasaki et al., 2020). The SST method provides more than 50% positivity for B lymphocyte-myeloma cell fusion, and more than 24% of the generated clones were found to secrete the desired mAbs (Yamasaki et al., 2020).

3. Antibody phage display technology

The antibody phage display technology, initially reported in 1990 (McCafferty et al., 1990), is considered a powerful tool to generate mAbs (Fig. 1). The methodology, based on the phage display concept described by George Smith in 1985 (Smith, 1985), consists in the development of a combinatorial antibody phage library – that is, a huge collection of phages displaying antibody fragments – and the subsequent screening of the antibodies that recognize the antigen of interest.

To generate an antibody phage library, firstly it is necessary to clone

antibody gene fragments into vectors. Both filamentous M13 phage and phagemid, which combines the characteristics of plasmids and phages (Tikunova and Morozova, 2009), can be used as vectors. Comparatively, while the first one has all the ability to produce phage particles and display antibody, the phagemid needs to infect bacteria with a helper phage, that is required to package the phagemid as single-strand DNA into virion particle (Barbas et al., 1991; Lowman, 2013; Almagro et al., 2019). In both cases, vectors are used to transform *E. coli* by electroporation. After obtaining the phage display library, the antibodies displayed on the vector surface are screened through a process called biopanning (Wu et al., 2016). It should be noted that the antibodies are most often displayed in single-chain variable fragment (scFv) or antigen-binding fragment (Fab) forms.

There are four types of antibody display libraries: immune, naïve, semisynthetic, and synthetic. The immune libraries are obtained from immunized animals or humans and are mostly used to discover antibodies against infectious pathogens (Trott et al., 2014) or antigenic targets in cancer patients (Thie et al., 2011; Frenzel et al., 2016). This library contains a restricted antibody repertoire that underwent antigen-driven *in vivo* selection (Barbas et al., 1991; Orum et al., 1993; Frenzel et al., 2016), which differs from the other phage display libraries, known as "universal", that theoretically provide binders for all possible antigen structures (Frenzel et al., 2016). The naïve antibody libraries are generated from a pool of B lymphocytes of non-immunized donors, and one successful example is the scFv library licensed from Cambridge Antibody Technology (CAT; now part of MedImmune/AstraZeneca) (Javle et al., 2014; Almagro et al., 2019). While the naïve libraries are derived from natural antibody gene repertoires, the synthetic ones are entirely based on *in silico* design to obtain individual antibody amino acid sequences (Fuh, 2007), bypassing the need to isolate antibody genes. The semisynthetic libraries, on the other hand, are created using both naturally and synthetically (*in silico*) randomized CDRs. In this library type, it is possible to redesign natural CDRs to improve the chance of finding antibodies with high specificity and affinity (Orum et al., 1993; Fuh, 2007).

Building the phage display library is the most important step of this technology. There is a directly proportional relationship between the size of the antibody library and the probability of finding a particular antibody (Burioni et al., 1997; Almagro et al., 2019). The Next-Generation Sequencing (NGS) is an important tool to analyze the variability, the sequence composition, and the size of antibody phage display libraries (Rouet et al., 2018). The construction of a phage display library is more expensive than generating hybridomas after animal immunization. However, the antibody screening step of the phage display method is faster and cheaper (Hentrich et al., 2018).

The first antibody discovered by phage display (CAT library) as well as the first human antibody approved for therapy was adalimumab (Humira®) (Fig. 1) (Burmester et al., 2013). It is an IgG1 mAb that binds tumor necrosis factor-alpha (TNF-α) and prevents the interaction of this inflammatory cytokine with the corresponding receptor. Having been discovered from an scFv phage library, gene manipulation was needed to obtain the final IgG format (Machold and Smolen, 2003). This antibody has been used for the treatment of patients with moderate to severe rheumatoid arthritis, among other autoimmune diseases.

Although the phage display library is a promising technology for the development of antibodies, it has limitations. The diversity of the phage library depends on the bacterial transformation efficiency and is limited to the 10^{10}-10^{11} variant antibody maximum repertoire of the phage display library. This restriction can be overcome by mRNA and ribosome display strategies, which are *in vitro* cell-free methods having a bigger library size and a higher displayed antibody diversity (10^{14} variants) (Hudson and Souriau, 2003; Kunamneni et al., 2020). It should be also considered that phage display-selected mAbs are generated in *E. coli* and therefore are not glycosylated; the use of eukaryotic display platforms, like yeast (Doerner et al., 2014) and mammalian expression systems (Zhu and Hatton, 2017), is a possibility to circumvent that. Other antibody phage display methodology disadvantages are the propensity to generate biased repertoires and the loss of information of antibody natural pairing (Saggy et al., 2012) (Table 1).

4. Single B cell antibody technology

Several technological platforms have been proposed to generate mAbs from hybridomas. An inherent characteristic of these methods is the need to fuse B lymphocytes with myeloma cells (Köhler, and Milstein, 1975) and this was, for a long time, a required step to isolate single antibodies of known specificity. In the last few decades, technical advances have allowed the detection and isolation of single functional B lymphocytes from heterogeneous primary cell populations, as well as the antibody gene amplification and cloning without the need to immortalize the selected antibody-secreting cell (ASC). These single B lymphocyte approaches, collectively known as "single B cell antibody technology" (Fig. 1) (Babcook et al., 1996), revealed attractive and useful to generate neutralizing mAbs in a rapid way for several applications (Tiller et al., 2008), including the management of emerging pathologies. Indeed, an increasing number of mAbs against infections caused by viral agents, such as HIV (Scheid et al., 2009a), Dengue (Durham et al., 2019), MERS-CoV (Wang et al., 2018), and SARS-Cov-2 (Cao et al., 2020), were obtained with such technology. The following items briefly describe the basic concepts and benefits of the single B cell antibody technology.

4.1. Identification and isolation of single B cells

The screening and isolation of ASC can occur in a random or antigen-specific manner, from peripheral blood or lymphoid tissue samples. For random selection, B cells can be recovered by flow cytometry (Smith et al., 2009) or can be picked from tissues by micromanipulation (Küppers et al., 1993). For antigen-specific selection, multi-parameter flow cytometry or other fluid-based approaches are generally used (Clargo et al., 2014; Meng et al., 2015; Rajan et al., 2018). Flow cytometry systems are efficient to recover single cells (Battye et al., 2000) and an example is their successful use to isolate IgG + memory B lymphocytes reactive to gp140 from donors with HIV (Scheid et al., 2009a, 2009b). In this case, anti-CD19 and anti-IgG antibodies, along with biotinylated gp140, were used to select the desired cell subset. Such methodology led to the generation of anti-gp140 mAbs with different antigen neutralization activities (Scheid et al., 2009b).

It should be noted that antigen-specific IgG + B cells comprise just a small percentage of circulating cells and, to identify and isolate them, reagents targeting B cell surface markers are desirable. A variety of antibodies are available to detect human B lymphocytes, which makes it even possible to distinguish cells at different stages of development and differentiation. This is an advantage of the single B cell technology over the original hybridoma technique. On the other side, the scenario is not the same when it comes to isolating non-human subsets. Indeed, we do have antibodies against mouse B lymphocyte markers (Starkie et al., 2016), such as CD45R and CD19, but the sorting of B cells from most of the other species (rabbit and guinea pig, for example), although feasible (Starkie et al., 2016; Lei et al., 2019), becomes challenging due to the low or absent repertoire of appropriate B cell-targeting antibodies. Another point that should be considered is related to cost: the use of expensive sorting devices integrates an important part of the procedures to isolate antigen-specific single B lymphocytes from a polyclonal mixture. Alternatively, other strategies can be used, including antigen-coated magnetic beads (Adler et al., 2017), cell-based microarrays (Jin et al., 2011), and soft lithographic methods for micro engraving (Love et al., 2006). The downside? These techniques are also costly or require extensive knowledge.

4.2. Single-cell immunoglobulin gene transcript amplification, cloning, and expression

Having isolated single B cells, the next step is the immunoglobulin gene amplification. The cells are lysed, the cDNA is synthesized by reverse transcription of total mRNA, and the full-length immunoglobulin genes for the variable and constant regions of the light and heavy chains are amplified by PCR (Tiller et al., 2008). The obtained fragments are cloned into linear expression cassettes to further generate the immunoglobulin domains in cell-based expression systems (mammalian or bacterial cells). In scenarios without the cultivation of the recovered B cells, the cDNA is synthesized from single-cell material. The antibodies are typically expressed in Fab form (Clargo et al., 2014), but it is also possible to express them in other formats, including full-length IgG and single-chain variable fragment (scFv) (Meng et al., 2015; Rajan et al., 2018).

These procedures summarize a common protocol route for protein expression. However, more robust and sophisticated systems are also available. That is the case of the "single-cell RT-PCR-linked *in vitro* expression" (SICREX) platform, through which the antibodies are expressed outside a cell unit (Jiang et al., 2006; Ojima-Kato et al., 2015). In this system, the protein synthesis occurs in a mixture containing the transcription/translation machinery from *E. coli*, and therefore the gene-cloning, transformation, and cultivation procedures are not needed. As a consequence, the time to generate the antibodies is greatly reduced to just a few days. Here we also have a drawback: incorrect folding of the antibody domains sometimes occurs.

From a broad perspective, the single B cell antibody technology, just like the other methods discussed in this review, has its advantages balanced by downsides, revealing a singular panel that characterizes it. Compared with the current hybridoma technology, though, the single B cell approaches have some positive points that stand out and even exceed those exposed above. It can be included here the potential to (a) isolate mAbs reactive to conformational determinants that are difficult to emulate *in vitro*; and (b) in experimental studies, collect multiple samples after the immunization period without the need to euthanize the animals (Tiller et al., 2008; Starkie et al., 2016; Rajan et al., 2018). But the biggest advantage of single B cell approaches is the possibility to isolate neutralizing mAbs from vaccinated or naturally immunized human subjects, as well as from those with autoimmune diseases. The high-throughput screening of individual ASC repertoire based on phenotypic and genotypic features allows the analysis of the human immune response to pathogens (Shi et al., 2019), accelerates the search for neutralizing mAbs of therapeutic relevance, and also provides insights for a rational vaccine design strategy (Scheid et al., 2009b).

Overall, the recent advances in the single B cell field trace a path that was out of reach when César Milstein and Georges Köhler found on the hybridoma creation the magic solution to isolate mAbs (Köhler, and Milstein, 1975). Table 1 summarizes some of the advantages and drawbacks of the single B cell antibody technology, in comparison with the hybridoma and phage display techniques.

5. Discussion

Given the foregoing, we can consider that the choice of the method to be used for obtaining an antibody must be guided by the purpose of the demand. The first demonstration that mAbs could be isolated came with the hybridoma technology, which made feasible the use of these molecules for a variety of biological applications. The task was revealed to be not as practical as it might seem, though. Hybridoma-derived immunoglobulins are of animal origin and, to be used as therapeutic tools, need to be converted into human mAbs. Such protein structural change can be currently achieved with established antibody chimerization and humanization protocols or the use of appropriate transgenic animals, in strategies that were crucial for the obtainment of the therapeutic mAb repertoire available today but are known to be costly, time-consuming, and technically challenging (Safdari et al., 2013). The limitations are not restricted to that. The low efficiency of the B lymphocyte-myeloma cell fusion and the further hybridoma cell isolation are important bottlenecks of this technology, not to mention the constant risk of cell culture contamination and the genetic instability of the generated hybridoma cell lines (Harlow and Lane, 1988).

Since the mid-1980s, several methods have been developed to work around these limitations, starting with changes in hybridoma technology. Examples are the proposed BCT and SST protocols, that brought relative improvements in the B lymphocyte-myeloma cell fusion efficiency, but instead turned the hybridoma technique more complex and hard-working, compared with the original methodology. Alterations in the other steps of this technology, such as the selection of the desired antibody-secreting cells, have been also described (Manz et al., 1995; Hanack et al., 2016; Listek et al., 2020); however, despite indeed accelerating the mAb identification process, the need to generate hybridomas remains. Based on different principles, the antibody phage display method emerged as the first alternative to the hybridoma technology. It brings important advantages, such as the potential to isolate mAbs against toxic and non-immunogenic antigens, and the possibility to generate, for the first time, antibodies without using experimental animals. On the other hand, an important limitation is the need to have an available and previously identified target antigen, which is also valid for the hybridoma technology.

Despite improvements in the hybridoma technology, and the development of antibody display (Winter et al., 1994), chimerization and humanization strategies (Winter and Milstein, 1991), a major advance came with the discovery of tools to isolate mAbs directly from single B cells. Besides not strictly depending on B cell culture and the use of experimental animals, the single B cell antibody technology allows a simple and rapid generation of mAbs with therapeutic potential without the need to previously know the target and have it available. This is a promising technique with the potential for even isolating functional mAbs against conformation determinants that are difficult to emulate *in vitro* but, currently, it still has low accessibility, particularly compared to the hybridoma methodology.

Overall, all the technologies discussed above revealed useful for obtaining therapeutic antibodies against several disorders, including infectious diseases. More than a hundred mAbs described against the Ebola virus illustrate that (Saphire et al., 2018) and, among them, some hybridoma-derived antibodies were used to develop therapeutic cocktails, such as ZMapp, composed of three chimeric mAbs (Qiu et al., 2011, 2014; Pettit et al., 2016), and REGN-EB3, comprising three human mAbs generated by using appropriate transgenic mice (Pascal et al., 2018). Other examples of antibodies generated toward the Ebola virus are the phage display-derived mAb KZ52 (Maruyama et al., 1999), and the single B cell-isolated antibody Mab114, obtained from a human survivor (Corti et al., 2016). But when considering emerging diseases, the hybridoma technology does not seem to be the most appropriate, particularly taking into account the need to obtain therapeutics in a short time. In this situation, the single B cell antibody technology seems to better respond to the urgent demand for functional mAbs, which is illustrated by the experience in the recent COVID-19 pandemic. In a period less than one year, at least 14 single B cell-derived human mAbs or mAb cocktails were obtained against SARS-CoV-2, the causative agent of this disease, and five of them entered Phase 2/3 clinical trials (Tuccori et al., 2020). Another positive point of the single B cell antibody technology is the possibility to isolate the desired mAbs without previously knowing the antigen target, which could be particularly helpful in infectious disease cases. However, all that does not exclude the potential application of other methodologies in the fight against emerging pathogens. Indeed, a panel of neutralizing mAbs elicited against SARS-CoV-2 was obtained from phage display libraries (Noy-Porat et al., 2020), and even hybridoma-based strategies have been explored for that purpose (Wang et al., 2020).

So, is the hybridoma technology still useful? The reported data so far

indicate yes. Beyond being a pioneer, this methodology is very popular. Several of the most recently generated mAbs were discovered on murine hybridomas (de Aguiar et al., 2016; Sanches et al., 2016; Parray et al., 2020), including some of the most successful FDA-approved antibodies, such as the immune checkpoint inhibitors nivolumab (anti-programmed cell death protein 1; anti-PD-1) (Robert et al., 2014) and atezolizumab (anti-programmed cell death protein ligand 1; PD-L1) (Fehrenbacher et al., 2016), used in the management of non-small cell lung carcinomas, head and neck cancers, melanomas, renal cell carcinomas, and several other tumors (Parray et al., 2020). Despite the emergence of new promising technologies for generating mAbs, it seems that none of them was able to provoke a technological shift up to now, remaining the hybridoma-based strategies in a leadership position.

Credit author statement

T.M.P. and F.B.V.C. wrote the "Hybridoma technology" section. C.B. and G.S. wrote the "B Cell Targeting (BCT)" section. B.H. and E.R.S. wrote the "Stereospecific Targeting (SST)" section. J.H.O., J.Z.M., and R.B.A. wrote the "Antibody phage display technology" section. R.B.A. wrote the "Single B cell antibody technology" section and prepared the timeline figure. All authors contributed critically to the review preparation, discussed the covered topics, and approved the final text. R.B.A. and J.Z.M wrote the discussion section, revised all the text, and answered the reviewer.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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