UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela De Ciencias Biológicas E Ingeniería

TITULO: Phage Display Technology: Capabilities and Applications

Trabajo de integración curricular presentado como requisito para la obtención del título de Biólogo

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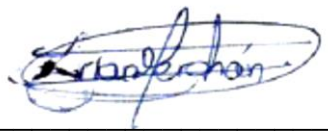

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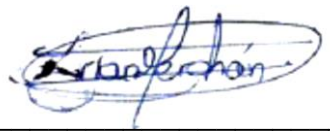

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Dedicatoria

Con mucho cariño,

Este logro se lo dedico a mi familia, quienes con trabajo y paciencia me han apoyado cada etapa de mi vida.

Brian David Merchán Muñoz

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A la Universidad Yachay Tech y a mis profesores, por brindarme la oportunidad, el ejemplo y la pasión.

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A mis amigos y compañeros, por ser una inspiración para soñar en grande.

Brian David Merchán Muñoz

Resumen

Hoy en día es posible aislar anticuerpos teóricamente para cualquier tipo de objetivo usando un virus de bacteria conocido como bacteriófagos. Con este virus se crean librerías con millones de copias de anticuerpos que posteriormente, a través de phage display, se identifica un anticuerpo específico para dicho target. La técnica involucra la recolección de genes de anticuerpos de donadores humanos, la clonación y expresión de dichos genes sobre la superficie del fago, y la identificación del fago cuya secuencia exprese un anticuerpo con las propiedades necesarias para la correcta interacción con su respectivo antígeno. Una vez identificado el anticuerpo con las propiedades deseadas, se recupera su secuencia genética y se producen múltiples copias del mismo dentro de células de cultivo. La relevancia de esta técnica radica en que los anticuerpos obtenidos al final de este proceso pueden ser usados para distintos tratamientos contra cáncer, enfermedades autoinmunes e infecciones virales, obviamente sin dejar de lado las respectivas evaluaciones de su eficiencia para aprobación y uso. El objetivo de este trabajo es explicar la biología detrás de los pasos involucrados en el aislamiento de anticuerpos mediante phage display, comprender los alcances de esta tecnología y finalmente revisar el efecto y las consecuencias de los anticuerpos que ya han sido aprobados o se encuentran en ensayos clínicos hasta la fecha.

Palabras Clave: Anticuerpo, virus, bacteriófago, phage display, sistema inmune.

Abstract

Today it is theoretically possible to isolate antibodies to any target using a bacterial virus known as bacteriophage. With this virus, libraries with millions of copies of antibodies are created, through phage display, a specific antibody for the said target is identified. The technique involves the pick up of antibody genes from human donors, the cloning and expression of these said genes on the surface of the phage, and identifying the phage whose sequence expresses an antibody with the necessary properties for the correct interaction with its respective antigen. Once the antibody with the desired properties is identified, its genetic sequence is recovered and multiple copies of it are produced within cultured cells. The relevance of this technique lies in the fact that the antibodies obtained at the end of this process can be used for different treatments against cancer, autoimmune diseases, and viral infections, obviously without neglecting the respective evaluations of their efficiency for approval and use. This work aims to explain the biology behind the steps involved in the antibody isolation by phage display, to understand the scope of this technology, and to review the effect and consequences of antibodies that have already been approved or are in clinical trials to date.

Keywords: Antibody, virus, bacteriophage, phage display, immune system.

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Note: All figures were made by the author.

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1. Introduction

To understand the relevance of phage display it is essential to start by talking about monoclonal antibodies and how their development, after the introduction of hybridoma technology by Kohler and Milstein in 1975[1], in particular, revolutionized biomedicine. Monoclonal antibodies increased the versatility of disease diagnostic tests[1]; gave a new way to the treatment of diseases, from the development of the first monoclonal antibody obtained from the mouse to the creation of the first human monoclonal antibody; allowing the treatment of diseases such as cancer, asthma, infectious and autoimmune diseases[1]. Today, the sales reflect how they became the most relevant therapeutic agents of the market. In 2002 they were valued in the market for 4 billion US dollars[1], in 2013, they reached revenues of 75 billion US dollars[2], and in 2017 their value exceeded 120 billion US dollars[3].

Later, it becomes clear how during the development of antibodies without side effects for patients, obstacles as immunogenicity, made scientists develop new technologies to overcome this problem on antibodies obtained through hybridoma technology, enabling new technologies such as phage display to be developed. In that sense, no one imagined that solving this problem would emerge a technology that would change the way drugs are developed today.

The authors of phage display technology won the Nobel prize in chemistry in 2018, which is curious because this work is a research done to obtain a degree in biology. For this reason, phage display is the perfect example of the relevance of multidisciplinarity today. During the development of this work, it is observable that even inside the biology itself that this technique involves, different branches such as immunology, microbiology, and genetics are interconnected. Despite the effort and time involved in immersing in complex issues like this, after the impact that a small virus caused around the world, there is no better time to stop and listen to what science (in this case biology) has to tell. In the same way, there is no better time to stop to understand and disseminate science affordably and understandably for ordinary people. Furthermore, a general review is provided to collect the necessary knowledge to understand and have a notion of what phage display technology is about, what it involves, and what its current and future capabilities are, followed by a list of phage display derived antibodies that evidence the impact and importance of therapeutic agents as monoclonal antibodies nowadays.

2. Problem Statement

The development of new drugs was a complex process that used to involve a large amount of analysis of possible candidates using high precision equipment, which looks for the indicated molecule with which to work and later subject it to optimization and clinical trials that verify its effectiveness, tolerance, and toxicity. This laborious process raised the cost and time of producing a drug[4]. However, phage display became so popular because it represents a form of reverse pharmacology[4] that starts from the objective to obtain the final product, and above all because it is a technique that involves low costs[4]. In addition, antibodies, being part of a defense mechanism against diseases with years of evolution, have an outstanding capacity in terms of versatility, tolerability, and bioavailability when defending the body against different diseases[5].

2.1 Objective

To write a review paper collecting the knowledge necessary to understand what the phage display technique is about, what it involves, and what its current and future capabilities are.

2.2 Specific Objectives

i. To analyze generally the steps involved in antibody isolation through phage display.
ii. To highlight antibodies' importance in the biomedical field.
iii. To illustrate the biological pathway by which a specific antibody acts.
iv. To enumerate the variety of phage display derived antibodies currently used and under clinical trials.

3. Monoclonal antibodies

Antibodies are molecules assembled by the immune system as part of the response against different infectious agents that cause diseases. To be more specific, they are part of the humoral immune system that, through B lymphocytes, is responsible for recognizing the type of antigen and developing specific antibodies against it. For this role, antibodies have two essential characteristics: specificity and the ability to confer continuous resistance for a particular antigen[6]. Therefore, taking advantage of these characteristics, scientists began to study the potential of antibodies to combat human diseases; consequently, the development of monoclonal antibodies occurred.

Human antibodies have a "Y" shape and are made up of two identical pairs of heavy chains (HC) and light chains (LC)[7], which are linked through disulfide bonds[8] (figure 1A). Each chain is divided into smaller units (of approximately 110 amino acids) called Ig domains, and it is essential to mention that the domains at the N-terminal are known to be highly variable in terms of their amino acid sequence compared to the others[9]. So, they are differentiated by taking the name of variable and constant domains (figure 1B). In the case of mammals light chains (LCs,) they can be of two classes κ or λ, both have a variable domain (VL) at the N-terminus and a constant domain (CL) at the C-terminus. On the other hand, human HCs can be of five classes: μ, δ, γ, α, and ε, which generate antibodies IgA, IgD, IgE, IgG, and IgM, each one with a different function as part of the immune system. In this case, IgAs, IgDs, and IgGs have one variable and three constant domains. While IgEs and IgMs have one variable and four constant domains. Understanding that each isotype is monomeric and defining as monomer the union of a pair of HC-LCs, it is essential to mention that the IgA and IgM classes have an extra chain called the J chain, which serves so that these isotypes can form dimers and pentamers, respectively[8]. In the case of IgG isotypes in the penultimate constant domains of heavy chains, a conserved glycosylation site is found with an essential role for effector functions that are in charge of the interactions of the antibody with other cells of the immune system[9].

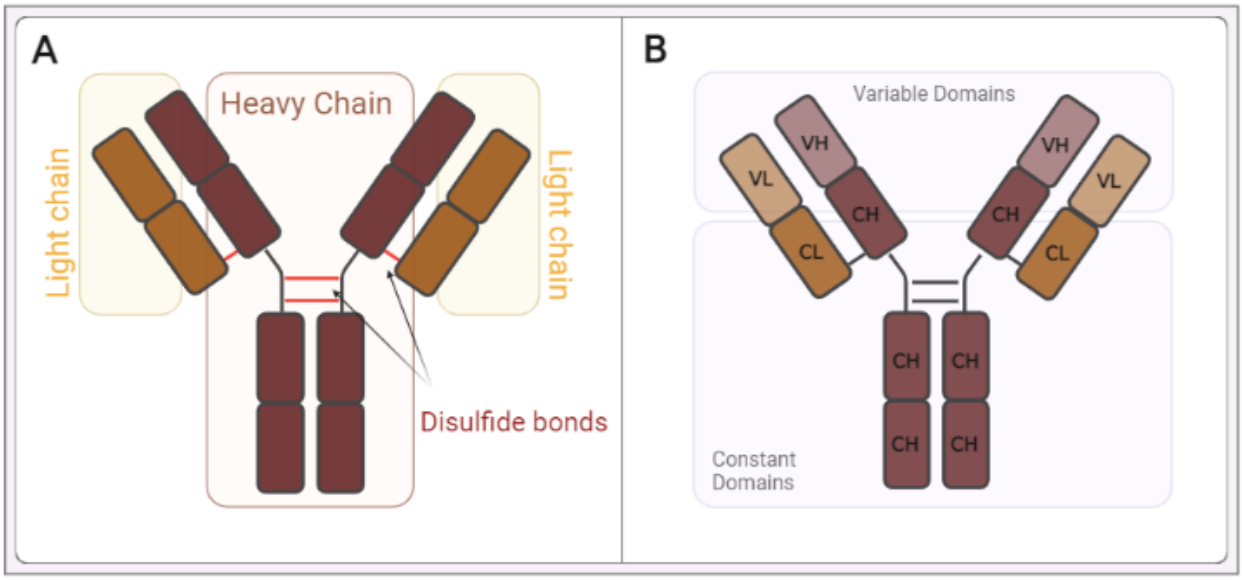

Figure 1. Classic IgG (A) shows light chains, heavy chains, and disulfide bonds, (B) shows variable and constant domains.

The second aspect, an antibody, has three functional parts: two antigen-binding sites (Fab) and the crystallizable fragment (Fc)[8] (figure 2A) Fabs and Fc are linked by a hinge region that allows Fabs to have greater flexibility than Fc. Both Fabs have the same antigen-binding sites and have a region called Fv that refers to the light chain variable domain and the heavy chain variable domain (VL and VH)[8] (figure 2B). According to the comparative analysis of several antibody sequences, it has been found that only a few amino acids in particular regions within these variable domains are responsible for said variability; these are known as hypervariable regions or complementarity-determining regions (CDRs)[9] (figure 2C). The role of CDRs is to determine the specificity of an antibody for an antigen; these are found within the so-called framework regions (FR) (figure 2C), which, unlike CDRs, FRs are conserved or similar in terms of their sequence among the different human antibodies[9]. It is important to remember that the variability of the CDRs is the result of genetic recombination between the V, D, and J genes that encode the VL of the light chain and the V and J genes that encode the VH of the heavy chain, plus the somatic hypermutation that occurs later in mature B cells[8]. Finally, each variable domain has three complementarity-determining regions CDR-L1, CDR-L2, and CDR-L3 in the case of the light chain and CDR-H1, CDR-H2, and CDR-H3 for the variable region of the heavy chain[8]. These characteristics of antibodies are mentioned below to understand the evolution of human antibodies for their use in therapy.

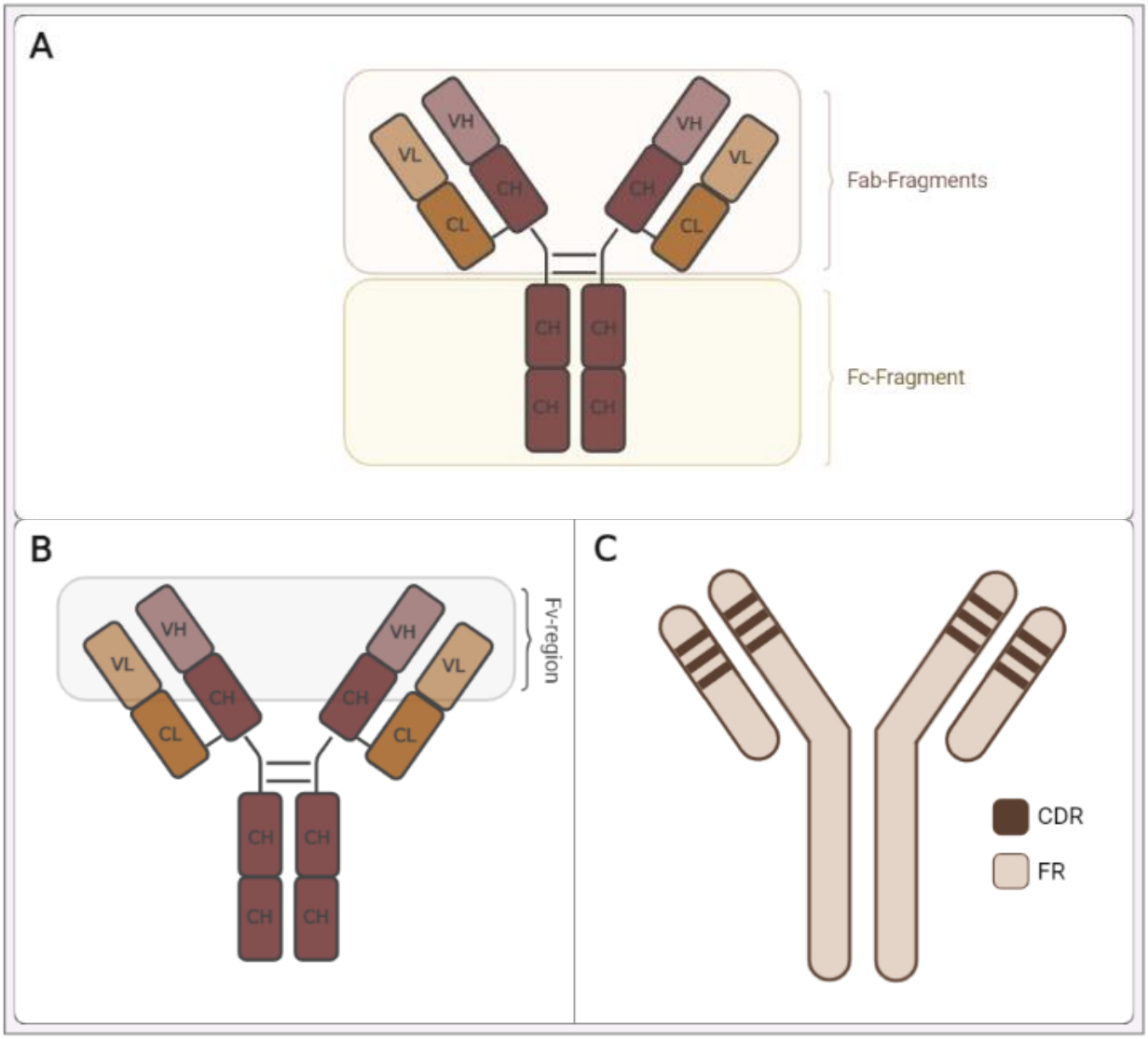

Figure 2. Classic IgG (A) shows Fab and Fc fragments, (B) shows Fv region, (C) shows complementary determining regions (CDR) and framework region (FR).

3.1 Developing of monoclonal antibodies as therapeutic agents

The term monoclonal antibody (mAb) refers to the same immunoglobulin species produced by a single B lymphocyte clone[10], and it was until 1975 that Köhler and Milstein introduced hybridoma technology and made possible the production of monoclonal antibodies in large quantities, thus promoting the study of their operation and their potential use in clinical treatments[11]. In the search for the mechanism by which such a diversity of antibodies originates, these scientists found it

necessary to have an immortal B cell by which investigate in-depth the mutations in the immunoglobulin genes[12]. Then, they took B cells extracted from the lymph or spleen glands of a mouse previously immunized with the antigen of interest and fused them with a line of myeloma tumor cells. These fused cells were grown together with a fusing agent (polyethylene glycol) in a specific medium culture (HAT) made so that only the hybrid cells could grow, the unfused cells died and in the end, the specificity of each antibody is analyzed to remain only the antibodies of interest and cloned them[12]. In this way, with hybridoma technology, it was possible to have many antibodies with known specificity to work with, and it rapidly became the conventional method to isolate mAbs.

An advantage of this technique is that when the B cells are extracted from the mice, the antibodies had already undergone the natural process of somatic hypermutation and affinity maturation within the host; therefore, they already had some potential to establish high-affinity bonds[13]. Thus, in 1986 the FDA approved the first monoclonal antibody for use in humans obtained through hybridoma technology: Muromonab CD3 (OKT3)[14,15], designed to prevent transplant rejection kidney[16].

However, due to its murine origin, some of the patients treated with Muromonab developed human anti-murine antibodies (HAMA)[14]. This immune response elicited by antibodies is known as immunogenicity[17], and it becomes the first obstacle in developing antibodies for clinical treatments. Since if a monoclonal antibody is recognized as foreign by the immune system, human anti-murine antibodies or (HAMA) anti-drug antibodies (ADA) are produced[11]. The problem with these HAMA or ADA is that they can reduce the effectiveness of treatment and even cause side effects ranging from headaches, mild gastrointestinal symptoms, transient rashes[14] to a systemic inflammatory response syndrome[11].

Therefore, in 1980, scientists decided to take the variable regions from the murine myeloma line and fuse them with the constant domains of an Ig of human origin using recombinant DNA technology to solve immunogenicity[11,18]. The result of this fusion is known as a chimeric antibody[17] (figure 3B). Although the risk of ADA response was reduced, efforts to further decrease the immunogenic response caused by regions of murine origin in chimeric antibodies continued.

Given this, an essential step in the evolution of monoclonal antibodies was developing humanized antibodies through the complementary-determining region (CDR) grafting technique[11]. This

technique does not take the entire variable region but only the CDRs of non-human antibodies and grafts them within a framework region of human origin[18] (figure 3C). Consequently, in 1997 the FDA approved the first humanized antibody: Daclizumab, also made to prevent transplant rejection[11].

Although humanized antibodies did not eliminate the probability of an ADA response occurring, when moving from murine to chimeric and from chimeric to humanized antibodies, it was possible to reduce immunogenicity and also increase the half-life of the antibodies: mouse (1.5 days), chimeric (10 days), humanized (12–20 days)[13]. Since the problem of immunogenicity caused by parts of the antibodies that were not of human origin could not be solved, the culminating solution for the scientists was to find a way to develop fully human antibodies in such a way that there was no foreign part in them that could trigger an immune response. However, since humans could not be immunized to produce antibodies against a particular antigen as mice could, the only way to obtain human antibodies could be by isolating B cells from donors whose immune response (the diversity of antibodies that posses) was the product of natural immunization[19].

Faced with this situation, two different methods to obtain completely human antibodies (figure 3D), and that today are the most used, was developed. In the first place, the technology that caused a breakthrough in developing antibodies was phage display. Developed in 1985, this technique allowed the expression of peptides or antibody genes on the surface of bacteriophages, which subsequently are separated according to their affinity and specificity characteristics[11]. Currently, this technology is the most used for developing antibodies, and later it is explained in more detail. To date, the FDA has approved 35 human mAbs obtained through this technology[6].

On the other hand, in 1994, transgenic mice began to be used[11]. These mice have a repertoire of human antibodies[19] so that when they are immunized, they use the human germline to produce their antibodies[13]. Thus, in 2006 the FDA approved the first human antibody produced by transgenic mice as an anti-epidermal growth factor receptor (EGFR): Panitumumab[11]. Although, immunization of mice does not always result in a successful response to antigens such as transmembrane proteins, with allosteric modifications, or with conserved or toxic antigens[19]. Depending on the immunization protocol, high-affinity antibodies can be obtained, and to date, the number of antibodies created from this technology is increasing, with 19 currently approved antibodies[11].

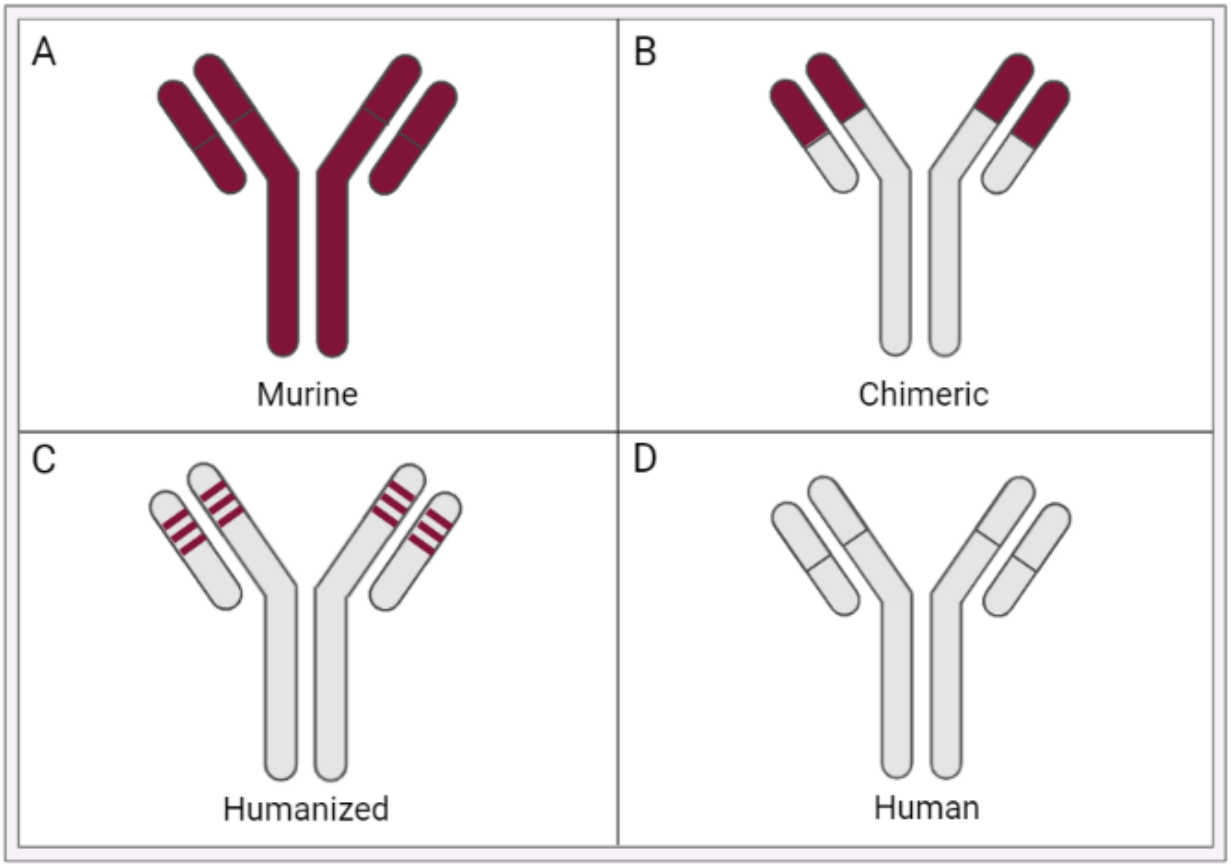

Figure 3. Classic IgG (A) shows a murine antibody, (B) shows a chimeric antibody made of variable regions from murine and constant regions from human, (C) shows humanized antibody made of CDR from murine and FR from human, (D) shows fully human antibody.

Finally, it is essential to note that to evaluate the risk of immunogenicity of an antibody, in vivo tests are carried out; these tests also help to optimize the design and development of antibodies and prevent the production of ADA. However, there are no in vitro or silicon tools that allow researchers to analyze the immunogenicity of an antibody yet. Immunogenicity is influenced by many factors such as dose, administration route, impurities contamination, aggregates resulting from Ab-Ag interaction, or even age, race, or genetic background of the patients[11]. When fully human antibodies were the option to be free of this problem, even though CDRs and frameworks come from a repertoire of human immunoglobulin genes, several fully human antibodies have reported an immune response in patients. Such is the case of Golimumab, a fully human antibody against TNFα developed for rheumatoid arthritis treatment. But it showed ADA production in 16% of patients[11]. So far, the most accurate explanation proposes that because the sequence of

the Fv region of these fully human antibodies is different from the germline, an ADA response is generated[11].

4. Introduction to Phage Display

Now is the time to understand why phage displays became so popular. Phage display is an in vitro technology able to select peptides and proteins from the surface of filamentous phage[20]. The technique performed occurred for the first time in 1985 when George Smith inserted an external DNA fragment inside a filamentous phage, specifically inside gene III that codes for the protein pIII, one responsible for encoding the phage coat. Thus, the result was a peptide displayed on the surface of the phage in the same way coat proteins are displayed around the bacteriophage genetic information (figure 4). When realizing that the display of the external peptide did not affect the virus's infectivity and that it was presented in an accessible way to be recognized by an antibody, a new way of looking at the genotype-phenotype relationship within filamentous phages was established[21]. This relationship is what makes the in vitro selection of antibodies through phage display possible, allowing the selection of those antibodies that have greater specificity, affinity, and stability to their antigen[19]. But to understand the process carried out using phage display to isolate antibodies, it is necessary to review the following concepts in the context of phage display.

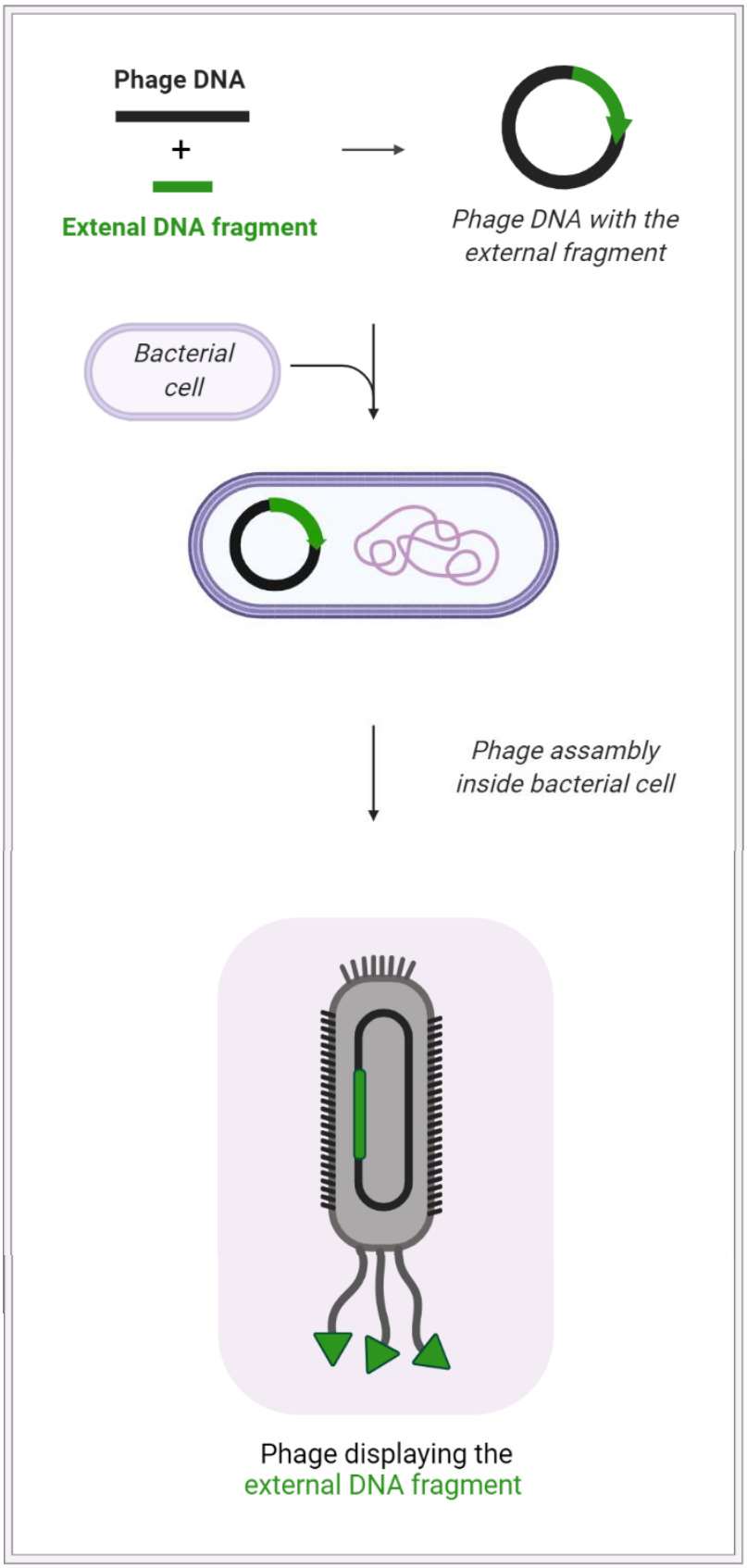

Figure 4. Process made by George Smith to present an external DNA fragment on a bacteriophage surface. George Smith inserted an external DNA fragment inside filamentous phage coat genes After bacteriophage packaging the external DNA fragment is displayed on the surface of the bacteriophage.

4.1 Filamentous bacteriophage

The word phage refers to a filamentous bacteriophage, and this group of viruses is the most used for phage display[22]. This type of virus is classified within the *Inoviridae* family of the *Inovirus* genus[22], has a single-strand circular DNA[23], and can infect gram-negative bacteria such as *E. coli*. The advantage of these filamentous phages is that they are not lytic; therefore, infected bacteria can release new phage particles without causing cell lysis[22]. The most commonly used bacteriophages in phage display are the filamentous phages M13, f1, and fd[22], being M13 the most popular. Inside the genome of the M13 phage, there are 11 genes responsible for encoding structural proteins (genes III, VI, VII, VIII, IX) and the proteins necessary for replication and assembly (genes I, II, IV, V, X, XI)[23]. Fusing an exogenous peptide or protein in any of the structural genes could interfere with the assembly of the phage, which is why the proteins most used to display a peptide or an exogenous protein are the pIII proteins (406 residues) (here I can put the rakinjack) and pVIII (50 residues). The size of the peptide or protein determines the use of one or another coat protein (pIII or pVIII) and depending on the established coat protein, one or another type of phage is used for the display[22].

Another approach in phage display uses phagemids. A phagemid is a plasmid that contains its origin of replication (ori), a phage origin of replication that allows it to replicate and pack itself from a single chain[22], multiple cloning sites, the proteins for the coat (gIII or gVIII), resistance genes and a lacZ promoter[23]. Phagemid is considered cloning vectors and requires a helper phage that provides the necessary proteins for infection and packaging process into virion particles[22]. The advantage of using phagemid is that it is easier to clone them, which allows generating larger libraries[23].

4.2 Antibody phage libraries

Antibody phage libraries consist of a considerable collection ($>10^{10}$) of protein (or peptide) sequences with unknown properties, displayed on the surfaces of millions of phages[19]. The proteins expressed on the phage can also be peptides or antibody fragments (scFv or Fab)[23]. A single-chain fragment variable (scFv) is composed only of the part corresponding to the heavy and light chains

variable regions (the Fv region), chains have a flexible peptide rich in glycine of between 10-25 amino acids that connect them[22] (figure 5A). On the other hand, the Fab corresponds to the entire antigen-binding site (figure 5B) (more about them is discussed next). This collection of millions of phages carrying different proteins is the primary source for phage display antibody discovery. According to the source, libraries are classified into four types: immune, universal, synthetic, and semi-synthetic. The most used are the Immune libraries and the Universal libraries.

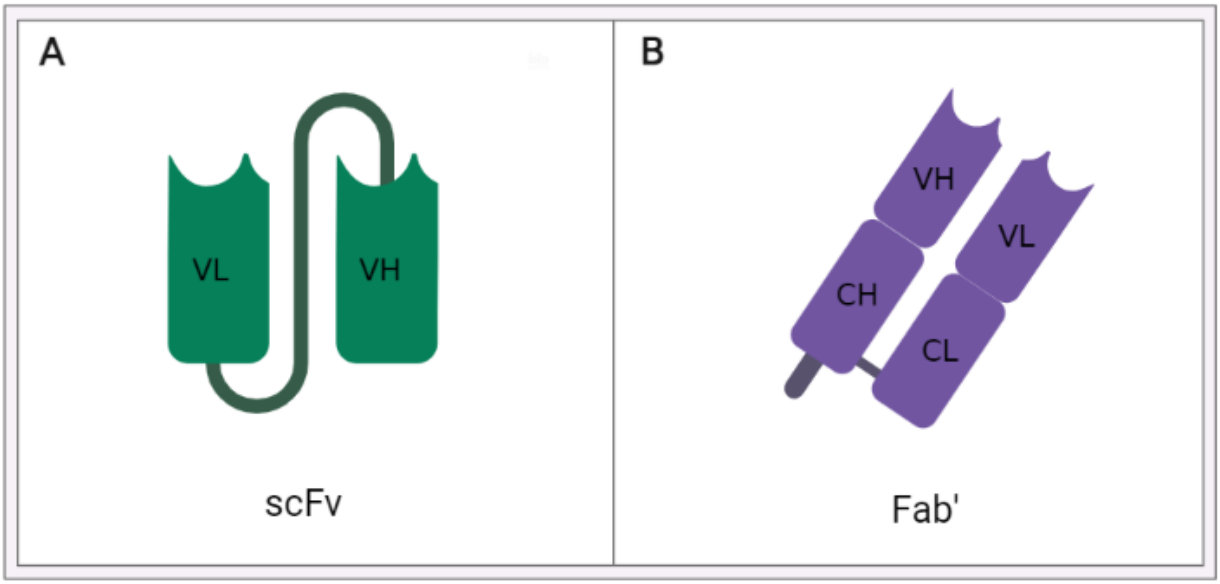

Figure 5. (A) shows a single-chain Fv (scFv), (B) shows a Fab antibody fragment.

Immune libraries originate from blood cell samples from immunized donors from which the IgG mRNA genes are extracted. The cells can be obtained from infected individuals, patients who suffered an infection or disease, or by extracting B cells from lymph nodes, spleen, bone marrow, or tonsils from vaccinated patients[13]. An advantage of generating immune libraries from this type of individual is that the antigen has already activated their B cells; that is, they have already undergone an affinity maturation process that predisposes the antibody clones to recognize antigens similar to the antigen of interest[23]. Therefore, antibodies with higher affinities can be isolated and can be used directly for therapy. A single immune library would be enough to obtain antibodies capable of recognizing different epitopes or targets of the same pathogen, or even different pathogens, depending on individuals' immunization history of individuals[13]. In the

medical field, immune libraries are more used to discover antibodies that fight infectious pathogens such as the human immunodeficiency virus (HIV), West Nile virus, or cancer patients[19].

As already mentioned above, due to the ethical problems involved in immunizing humans, an alternative developed to overcome this was transgenic animals capable of expressing a repertoire of human antibodies[13]. In the case of immune libraries, there are cases in which immunized transgenic animals, taking advantage of their ability to produce human-like antibodies, are used to build an immune library from their response. In addition, since it is an immunization generated from the antigen of interest, the isolated antibody will have high affinity and high stability[13]. That is the most significant advantage of using immune libraries; once the creation of an antibody library starts from the response generated by the immune system after presenting an antigen of interest, it is much easier to obtain an antibody with the desired affinity. However, the problem is that some antigen immune responses could not be generated correctly or could be unpredictable[13]. Another drawback is that each antigen requires its immunization, which involves more laboratory processes and costs, without mentioning the ethical issues of using animals for immunizations[24].

On the other hand, universal libraries originate from IgM mRNA obtained from peripheral blood, spleen, and bone marrow lymphocyte B cells from non-immunized and healthy donors[13]. The principal characteristic of this repertoire of human antibody genes is that they are the closest to the germline of human antibodies; thus there is a low risk of immunogenicity[19]. This type of library does not depend on the immunization history of an individual, antibodies against non-immunogenic antigens, hydrophobic targets, or even toxic antigens can be isolated[19]. They are also called "single-pot" libraries because they contain such a broad repertoire for antigen specificity that they can theoretically coat any possible antigen[19]. However, since these antibodies are isolated from B cells that did not undergo an affinity maturation process, the antibodies obtained as a result tend to have a relatively low affinity (between 4 nM to 220 pM)[13]. In this sense, it is essential to mention, the affinity of the antibody obtained is related to the size of the library used. Using a small library between 10^7 and 10^8 clones, antibodies with micromolar to nanomolar ranges take place. Starting with a library between 10^{10} and 10^{11} clones, antibodies with nanomolar and peak molar ranges resulted. The diversity and size of the libraries can be increased by isolating rearranged V genes from multiple donors and by combining B cells from peripheral blood

lymphocytes, tonsils, and bone marrow[13]. In any case, the antibodies isolated from universal libraries can go through a stability and affinity optimization process if necessary.

Finally, there are also universal synthetic or semi-synthetic libraries. The origin of these libraries is synthetic sequences, or a mixture of natural and synthetic sequences, respectively[19]. Synthetic libraries are completely random CDR regions inserted into fully synthetic antibody sequences. While in semi-synthetic libraries, CDR regions with a limited number of variations are used[13]. To create a synthetic library, scientists rely on bioinformatic analysis of different data as antibodies epitopes, antigen-antibody interactions, structure prediction, variable fragment recombination, and affinity maturation design [23]. Because these sequences are produced randomly, they do not have natural influence or redundancy exerted by evolution[23]. For that, this type of library is the ideal option to isolate antibodies against self-antigens[13]. These synthetic sequences are not eliminated, as happens with B cells in autoimmune diseases in which their immune system attacks and eliminates them. In the same way, this type of library is an acceptable option to obtain antibodies against lipids, carbohydrates, and proteins with post-translationally modifications[13].

4.3 Antibody fragments

Peptide sequences developed for therapeutic purposes are known as antibody fragments. As mentioned above, complete proteins and antibodies are displayed on phages and antibody fragment sequences. Within the sequence of an antibody fragment, the antigen-binding site is kept, and some or all of its constant regions are extracted, this does not affect its binding properties and an antibody fragment may even present better physical properties than a complete antibody version, depending on the requirements[25]. For that, the antibody fragments found in clinical trials have increased as their advantages become evident[26].

The most popular format of antibody fragments is the single-chain fragment variable (scFv) (figure 5A); as mentioned above, these fragments are composed only of the variable domains of the heavy and light chains attached by a flexible peptide between 10 - 25 amino acids rich in glycine[22]. Due to their small size, scFv are easier to clone and well-tolerated by bacteria, which means an efficient display and high expression levels[26]. ScFv can also cross tumors much faster than Fabs or complete IgGs, which become viable to study intracellular signaling pathways[13]. In addition, they can bind

to sterically restricted epitopes that Fabs and IgGs cannot access, have a short retention time in tissues not involved in therapy, and are rapidly cleared from the bloodstream[13]. The unique drawback scFv has, they are less stable than Fabs and can form oligomers and multimers[13].

On the other hand, Fabs are the whole antigen-binding site. That is a complete light chain with the variable domain and the first constant domain of a heavy chain[26] (figure 5B). The interaction of its VH / VL and CH1 / CL chains makes this antibody format more stable than scFv[27]. However, Fab fragments that are too long have the risk of causing toxicity in *E. coli*; therefore, fragments production is affected. In addition, lacking Fc effector functions in antibody fragments remove their ability to eliminate infected cells or recycle antibody molecules[13]. Moreover, scFv and Fab are great in neutralizing pathogens.

It is essential to note that due to its size, it is more convenient to make improvements in an antibody fragment; even when you want to improve a complete antibody, making improvements to the fragment version of the antibody and then converting that improved fragment back into a full antibody format is advantageous to researchers [25].

4.4 Biopanning

The following concept deals with how the selection of antibodies from libraries is carried out and is known as biopanning. Due to the diversity of clones found within a library, the appropriate antibody is extremely rare inside it[19]. In the case of immune libraries, as they contain a relatively small repertoire, they present a higher percentage of a specific antibody for the antigen of interest to be found. To put this in context, in an immune library, an antigen that fits between 10^3-10^6 that does not fit can be found, while in a universal library the difference can be an antigen that fits between 10^7-10^9 that does not[19]. For that reason, when working with universal libraries, the selection process must be meticulous in isolating a high-quality antibody.

The first step in biopanning is the phage antibody library incubation with the antigen of interest for a specific time to allow the binding[28]. That, by immobilizing the target antigen on a solid surface (figure 6), usually using magnetic beads, column matrices, or plastic surfaces with high protein binding capacity[19]. The selection is also an option to be carried out with adherent cultured cells or even with cells in suspension whose receptors on the surface are the target[22]. Once the

phages with a particular affinity are bound, the next step is to remove phages whose characteristics do not present specificity or affinity through several washes; usually, by stringent washing[19] (figure 6).

Subsequently, the bound phages are eluted by incubating them in a low pH buffer or competitive elution[28] (figure 6). Finally, the infection stage uses the phages previously separated to amplify them infecting *E. coli* bacteria to produce a new antibody library. That new library undergoes another round of biopanning[19] until two or three rounds to enrich the antibodies significantly [22].

It is essential to mention, to determine the strategy to employ, parameters such as the antigen of interest, the quality of the library, the binding and washing conditions; must be contemplated. The strict control of these conditions is vital to pre-design the properties of an antibody, such as epitope specificity, conformational specificity, and whether or not it presents interspecies cross-reactivity[19].

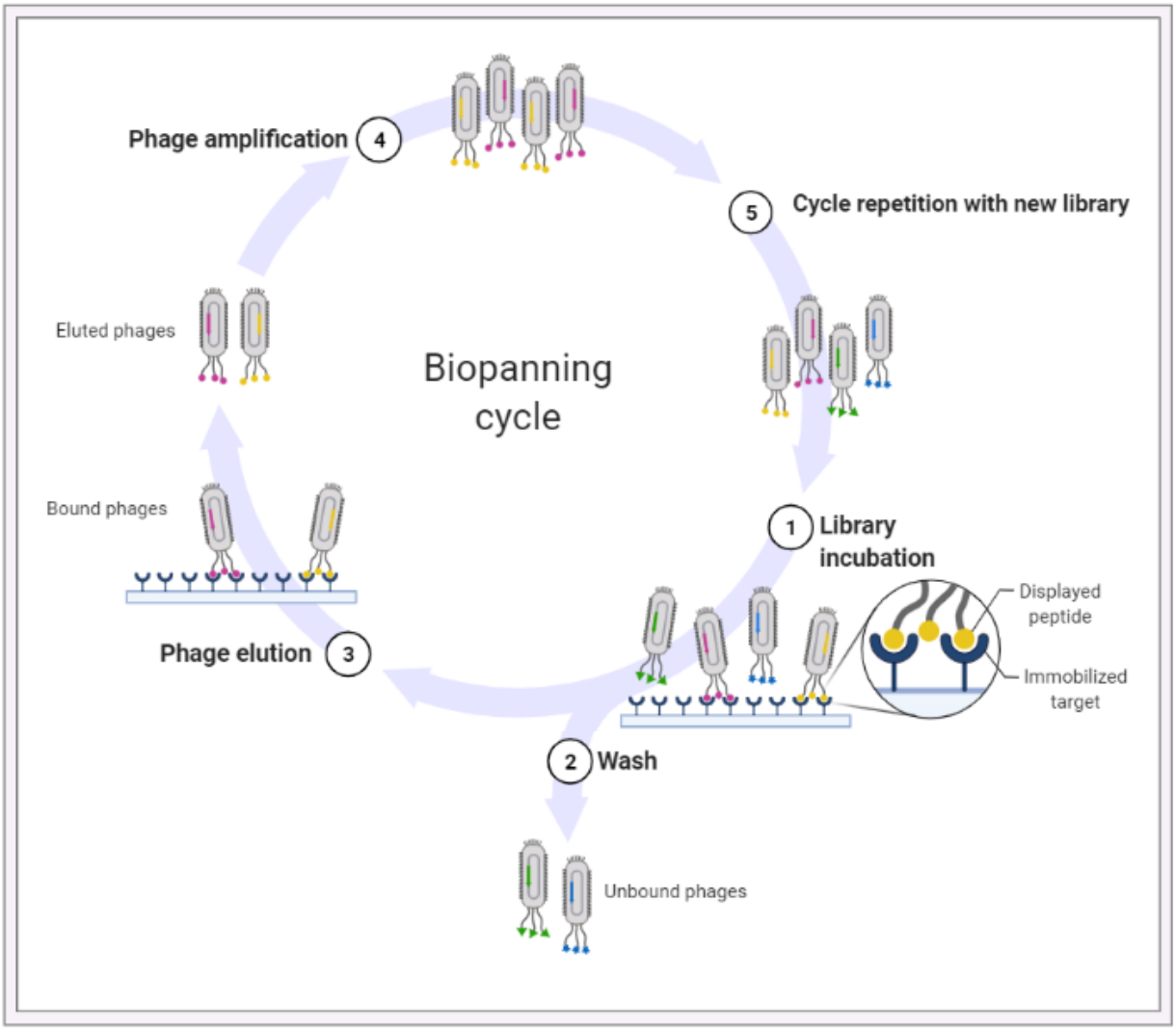

Figure 6. Biopanning cycle: (1) First step in biopanning involves immobilizing the target antigen on a solid surface. (2) Washing phages with undesired properties. (3) Elution of phages that have affinity for the target. (4) Amplification of possible candidates. (5) Repetition of the cycle with new candidates' library.

4.5 Antibody isolation through phage display resume

Now, integrating the concepts mentioned above, the antibody isolation process can be described as follows:

First, it is essential to have or to create a phage antibody library (figure 7). That requires antibody gene fragments production. This step involves immunizing an animal with an antigen of interest to extract the mRNA from its B lymphocytes, to perform a cDNA synthesis from them[24] (this is in the case of using an immune library. If it were a universal library, it would start from mRNA extraction from blood cells). The genetic information of cDNAs is not specific for a single antibody but can express different types of antibodies for various antigens [24]. Then, using restriction enzymes, the genes associated with the different types of antibodies are cut and cloned into phagemid vectors. In such a way, after phage packaging, library creation is achieved with a diversity of functional antibody sequences displayed on the surface of thousand of phages[24].

The next step is selection. Selecting candidates within the library diversity is the process mentioned above as biopanning (figure 8). To find the specific clone for the antigen of interest, researchers rely purely on the property of the antibody to bind to its respective antigen[24]. As mentioned above, to end up with high-affinity antibodies, it is essential to biopanning 3 to 5 times[24].

Antibodies obtained after this step are already feasible as therapeutics, however, they have to go to clinical trials for further modifications and optimizations. Once the evaluations required for approval determine that their use is beneficial to health, they are used in treatment.

5. Monoclonal antibodies developed through Phage Display on the treatment of diseases

5.1 The first phage display derived antibody

The first result of this technology is Adalimumab, a fully human IgG1-κ antibody developed through phage display and approved by the FDA for rheumatoid arthritis treatment[19]. This antibody acts on the tumor necrosis factor TNF-α in circulation or on the surface of cells, blocking the interaction of TNF-α with its receptors p55 and p75, thus preventing the activity of cytokines[29] and consequences such as inflammation, fever, sepsis, acute phase responses, survival and cell proliferation[19]

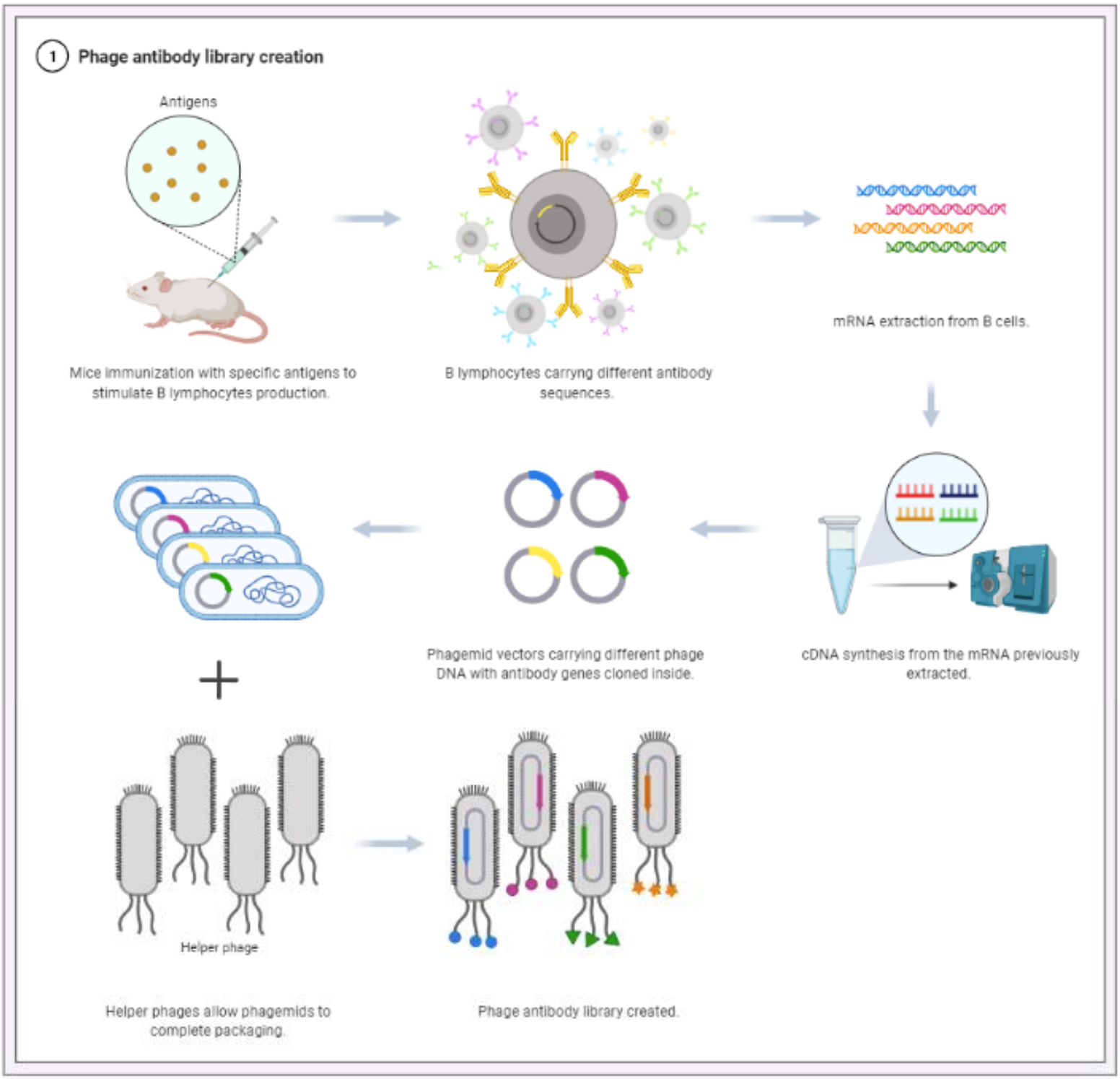

Figure 7. Illustrative resume of a library creation process.

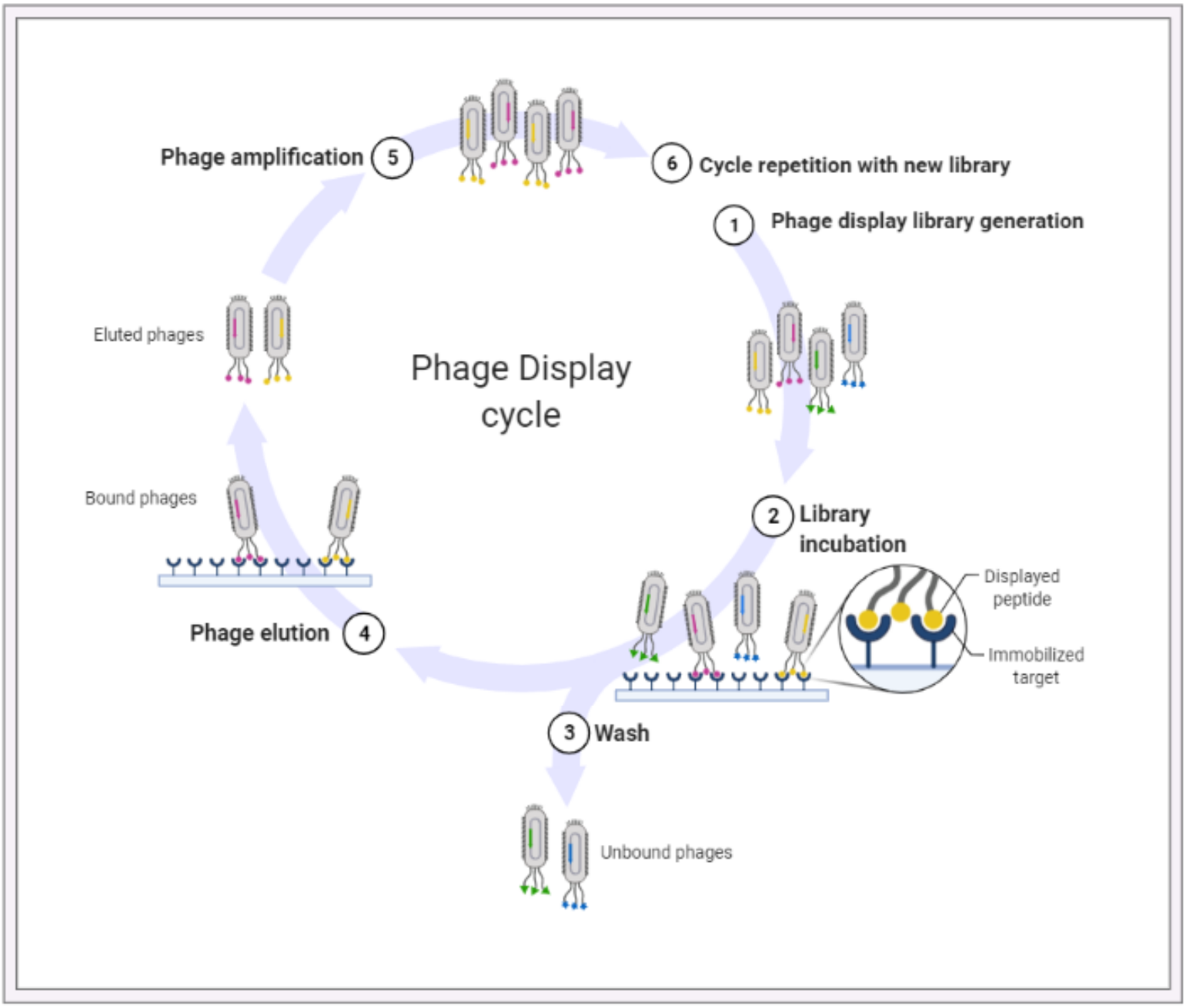

Figure 8. Complete phage display cycle: (1) Library creation is resumed in previous figure. (2) The library incubation (the first step of biopanning) involves immobilizing the target antigen on a solid surface. (3) Washing phages with undesired properties. (4) Elution of phages that have affinity for the target. (5) Amplification of possible candidates. (6) Repetition of the cycle with new candidates' library.

Four trials, ARMADA, DE011, DE019, and STAR, stand out when it comes to demonstrating the efficacy of Adalimumab for rheumatoid arthritis; these trials were carried out in a placebo-control mode and lasted between 6 and 12 months[30]. Two of them, ARMADA and DE019, were carried out by applying 40 mg of Adalimumab by subcutaneous injection together with methotrexate. In the ARMADA trial, according to the criteria of the American College of Rheumatology (ACR 20), out of a total of 271 patients, a superior clinical response was observed in 67.2% of the patients treated with Adalimumab, in contrast to 14.5% in patients treated with methotrexate alone[30]. On the other hand, in the DE011 trial, the adalimumab effect as monotherapy was studied in 554 patients under the same mode of administration (40 mg by subcutaneous injection), but they were divided into a group that received a weekly dose and another that received one dose every other week. Although in both groups improvements were observed, within the group receiving a weekly dose, a superior clinical response in 53% of patients was found, compared with 46% in the other group[30]. This study showed that Adalimumab administered as monotherapy requires shorter doses to achieve greater efficacy[30]. Similarly, analysis of ARMADA, DE019, and STAR trials, using the SF-36 questionnaire to assess the life quality of patients, showed that they not only felt an improvement concerning their physical state but also of their emotional state. The results of that analysis revealed that patients felt less pain and fatigue, which meant that they could carry out the activities of "more normal" life[30].

It is worth mentioning that the cross-talk of the different pathways related to TNF interferes at various physiological levels, which made it impossible to develop a specific anti-TNF therapeutic to prevent the entire cascade response that it produces. However, antibodies have been developed to suppress a wide range of inflammatory autoimmune diseases related to TNF. Today there are five different types of biological antagonists for TNF on the market. Adalimumab, infliximab, Golimumab, certolizumab pegol, and etanercept[19] (Frenzel et al., 2016). On the other hand, Adalimumab, by demonstrating long-term efficacy in clinical trials and, above all safety, was approved by the FDA on December 31, 2002, for moderate and severe treatment of rheumatoid arthritis in monotherapy or combination with other anti-rheumatic drugs. It was finally marketed under the brand name Humira, a Human Monoclonal antibody In Rheumatoid Arthritis[19], and as a curiosity, to date, it is one of the best-selling drugs in the world, and in 2019 it accumulated $ 19.1 billion in sales[3].

5.2 Last phage display derived antibody

On the other hand, the last of the antibodies approved and developed through phage display is Avelumab[3]. A fully human IgG1 - λ antibody interacts with programmed death-ligand 1 (PD-L1) and blocks its binding with PD-1 and B7.1 receptors present on T cells and antigen-presenting cells[31]. Normally when PD-L1 interacts with PD-1 and B7.1 receptors, it represses the activity of cytotoxic cells responsible for killing cancer cells. Therefore, blocking this interaction should activate antitumor immune responses[31]. In vitro experiments have revealed that Avelumab differs from other PD-L1 / PD-1 interaction blocking drugs in that it shows the ability to induce antibody-dependent cell-mediated cytotoxicity (ADCC). Being a total IgG that conserves its native Fc region, it is believed that this allows it to induce an ADCC and promote T-cell activation[31,32].

In that sense, the PD-L1 / PD-1 interaction involves a wide range of physiological responses. For this reason, the application of Avelumab has been evaluated in different types of cancer. Trials have been conducted to test the efficacy of Avelumab in different types of cancer such as breast cancer, pancreatic cancer, ovarian cancer, urothelial cancer, and mesothelioma[31]. In early 2017, it was approved in the United States to treat metastatic Merkel cell carcinoma (mMCC)[3]. In a study (NCT02155647) designed to evaluate the response of Avelumab according to the Response Evaluation Criteria in Solid Tumors (RECIST), 88 patients were evaluated, with an average age of 72 years with a diagnosis time between 6 and 17 months[31]. After administering an intravenous infusion of avelumab with 10 mg/kg every 2 weeks for an average of 10 months, a positive response was observed in 79% of patients with a mean progression-free survival (PFS) of 2.7 months overall survival (OS) of 11.3 months[33]. In the same year, the FDA approved Avelumab for the treatment of metastatic urothelial carcinoma based on the results of study NTC02603432[3], and in 2019 it was approved for therapy of high-level renal cell carcinoma in based on the results of the study NCT02684006[3].

Today there is a great diversity of antibodies for different diseases such as psoriasis, myeloma, glioma, asthma, Alzheimer's disease, breast cancer, renal cancer, and prostate cancer. In this sense, the variety of studies for developing new therapeutic agents makes evident the feasibility

and versatility of the process. Table 1 lists phage display-derived antibodies approved or under clinical trials below.

Table 1. List of phage display derived antibodies approved or under clinical trials.

Antibody name	Antibody format	Target antigen	Indications	Phase
Adalimumab	IgG1-κ	TNFAα	RA[34]	Approved 2002
			PSA[35]	Approved 2002
			AS[35]	Approved 2006
			CD[35]	Approved 2007
			Psoriasis[36]	Approved 2008
Adecatumumab	IgG1	EpCAM	Breast cancer[37], prostate cancer[38]	Phase II
			Myeloma[39], HL[40]	Phase I
Anetumab	IgG1-λ	MSLN	Mesothelioma, mesothelin-expressing ovarian cancer, non-small cell lung cancer and pancreatic cancer[41]	Phase II
Amatuximab	IgG1-κ	MSLN	Mesothelioma, mesothelin-expressing pancreatic[42,43]	Phase II
Atezolizumab	IgG1-κ	PD-L1	Renal cancer[44]	Phase I

			Urothelial Carcinoma[44]	Approved 2016
			Urothelial bladder cancer[44]	Approved 2017
			Breast cancer[45]	Approved 2019
Avelumab	IgG1-λ	PD-L1	mMCC, metastatic urothelial carcinoma[46]	Approved 2017
			RCC[47]	Approved 2019
Belimumab	IgG1-λ	BLyS	SLE[48]	Approved 2011
			Vasculitis[49]	Phase III
Bertilimumab	IgG4-κ	CCL11	Bullus pemphigoid[50]	Phase II
Bimagrumab	IgG1-λ	ActRII	Type 2 diabetes[51]	Phase II
Carlumab	IgG1-κ	CCL2/MCP-1	Prostate cancer[52]	Phase II
			Pulmonary fibrosis[53]	Phase II
Cixutumumab	IgG1-λ	IGF1R	NSCLC, HCC, solid tumors[54,55]	Phase II
Foravirumab	IgG1-κ	Rabies virus glycoprotein	Prophylaxis of rabies[56]	Phase III

Fresolimumab	IgG4-κ	TGFβ	Scleroderma, metastatic breast cancer, NSCLC, fibrosis, focal segmental glomerulosclerosis[57–59].	Phase II
Ixekizumab	IgG4-κ	IL17A	Psoriasis[60]	Approved 2016
			PSA[60]	Approved 2017
			AS[61]	Approved 2019
Mapatumumab	IgG1-λ	TRAIL-1	Multiple myeloma, colorectal cancer, NSCLC, NHL, cervical[62–64]	Phase II
Mavrilimumab	IgG4-λ2	GM-CSFRα	RA, GCA, COVID-19[65–67]	Phase II
Moxetumomab pasudotox	Murine IgG1	CD22	HCL[68,69]	Approved 2018
Namilumab	IgG1-κ	GM-CSF	RA, AS, psoriasis[70–72]	Phase II
Necitumumab	IgG1-κ	EGFR	NSCLC[73]	Approved 2015
Opicinumab	IgG1-κ	LINGO 1	MS, and Optic neuritis[74,75]	Phase II
Tanibirumab	IgG1-κ-λ	VEGFR2	Solid tumors[76]	Phase I
Utomilumab	IgG2-λ	4-1BB	Breast cancer, B-cell lymphoma[77,78]	Phase II
Ganitumab	IgG1-κ	IGF-1R	Metastatic colorectal cancer[79]	Phase II

Caplacizumab	Humanized VH-VH.	VWF A1 domain	aTTP[80]	Approved 2018
Ramucirumab	IgG1-κ	VEGFR2	Gastric cancer, NSCLC[81]	Approved 2014
			Colorectal cancer[82]	Approved 2015
			HCC[83]	Approved 2019
Ranibizumab	Fab-IgG1-κ	VEGFA	nAMD[84]	Approved 2006
			MEfRVO[85]	Approved 2010
			DME[86]	Approved 2012
			CNV[87]	Approved 2016
			Diabetic retinopathy[88]	Approved 2017
Darleukin	L19 scFv-IL2 fusion, diabody	EDB-FN	Metastatic melanoma[89]	Phase III
Fibromun	L19 scFv-TNFα fusion, diabody	EDB-FN	Glioma[90]	Phase II
Raxibacumab	IgG1-λ	Anthrax PA	Inhalation anthrax[91]	Approved 2012
Otilimab	IgG1-λ	GM-CSF	RA[92]	Phase III
Seribantumab	IgG2-λ	HER3	Ovarian cancer, breast cancer[93]	Phase II
Tralokinumab	IgG4-λ	IL13	Asthma[94]	Phase III

Ianalumab	Defucosylated IgG1-κ	BAFF-R	pSS[95]	Phase III
Teleukin	F16 scFv-IL2 fusion, diabody	A1 domain of tenascin-C	AML[96]	Phase I
Xentuzumab	IgG1-λ	IGF-I,	NSCLC, solid tumors[97]	Phase II
Setrusumab	IgG2-λ	SOST	OI, post-menopausal women with low BMD[98]	Phase II
Briakinumab	IgG1-λ	IL12 and IL23	Psoriasis[99]	Phase III
Guselkumab	IgG1-λ	IL23	Psoriasis[100]	Approved 2017
Lanadelumab	IgG1-κ	pKal	HAE[101]	Approved 2018
Tarextumab	IgG2-κ	Notch2/3	Pancreatic Cancer[102]	Phase II
Elgemtumab	IgG1-mk	HER3	Breast cancer, gastric cancer[103]	Phase I
Gantenerumab	IgG1-κ	Amyloid-β	Alzheimer's disease[104]	Phase III
Emapalumab	IgG1-λ	Interferon-gamma	HLH[105]	Approved 2018
Orticumab	IgG1-λ	oxLDL	Atherosclerosis[106]	Phase II
Tesidolumab	IgG1-λ	Complement 5	Geographic Atrophy, AMD[107]	Phase II

TNFAα, Tumor necrosis factor-alpha; RA, Rheumatoid arthritis; PSA, Psoriatic arthritis; AS, Ankylosing spondylitis; CD, Crohn's disease; JIA, Juvenile Idiopathic Arthritis; UC, Ulcerative colitis; HS,

Hidradenitis suppurativa; EpCAM, Epithelial cell adhesion molecule; HLA-DR, Human Leukocyte Antigen–DR isotype; HL, Hodgkin's lymphoma; HLA-DR, Human Leukocyte Antigen–DR isotype; MSLN, Mesothelin; NCI, The National Cancer Institute; PD-L1, Programmed cell death-ligand 1; NSCLC, Non-small cell lung cancer; SCLC, Small cell lung cancers; RCC, Renal cell carcinoma; HCC, Hepatocellular carcinoma; mMCC, metastatic Merkel cell carcinoma; BLyS, B-lymphocyte stimulator; SLE, Systemic Lupus Erythematosus; CCL11, CC chemokine ligand 11; ActRII, Myostatin/activin type II receptor; CCL2, CC chemokine ligand 2; MCP-1, Monocyte chemoattractant protein 1; IGF1R, Insulin-like growth factor 1 receptor; TGFβ, Transforming growth factor β; IL17A, Interleukin-17A; TRAIL-1, TNF-related apoptosis- inducing ligand receptor 1; NHL, Non-Hodgkin's lymphoma; GM-CSFRα, Granulocyte macrophage-colony stimulating factor receptor alpha; GCA, Giant cell arteritis; COVID-19, novel coronavirus 2019; HCL, Hairy cell leukemia; GM-CSF, Granulocyte macrophage-colony stimulating factor receptor; EGFR, Epidermal Growth Factor Receptor; LINGO-1, Leucine-rich repeat and Ig containing Nogo receptor interacting protein-1; MS, Multiple sclerosis; VEGFR2, Vascular endothelial growth factor receptor 2; Ang-1 and−2, Angiopoietin 1 and 2; VWF, von Willebrand factor; aTTP, Acquired thrombotic thrombocytopenic purpure; VEGFR2, Vascular endothelial growth factor-receptor 2; VEGFA, Vascular endothelial growth factor A; nAMD, Neovascular age-related macular degeneration; MEfRVO, Macular edema following Retinal Vein Occlusion; DME, Diabetic macular edema; CNV, Visual impairment due to choroidal neovascularisation; MM, Multiple myeloma; EDB-FN, Extradomain-B of fibronectin; IL2, Interleukin 2; PA, Protective antigen; OS, Osteoarthritis; HER3, Human epidermal growth factor receptor 3; BAFF-R, B-cell-activating factor receptor; CLL, Chronic lymphocytic leukemia; ETH, Swiss Federal Institute of Technology Zürich; pSS, Primary Sjögren's syndrome; AML, Acute myeloid leukemia; IGF-II, Insulin-like growth factor II; SOST, Sclerostin; OI, Osteogenesis imperfecta; HPP, Hypophosphatasia; BMD, Bone mineral density; VEGFR-3, Vascular endothelial growth factor receptor-3; FGFR2, Fibroblast growth factor receptor 2; TFPI, Tissue factor pathway inhibitor; FXI, Coagulation factor XI; VTE, Venous thromboembolism; COPD, Chronic obstructive pulmonary disease; TLR-3, Toll-Like Receptor 3; DKK1, Dickkopf 1; EDA-FN, Extra-domain A of fibronectin; HER2, Human epidermal growth factor receptor 2; LYPD3, Ly6/PLAUR domain-containing protein 3; pKal, Plasma kallikrein; HAE, Hereditary Angioedema, TRAIL-R2, TRAIL receptor 2; DR5, death receptor 5; SCLC, Small cell lung cancer; HER3, Human epidermal growth factor receptor 3; HNSCC, Head and neck squamous cell carcinoma; Aβ, Amyloid-β; FZD, Frizzled receptor; PNH, Paroxysmal nocturnal haemoglobinuria; AMD, Age-related macular degeneration; HLH, Hemophagocytic lymphohistiocytosis.

Up to the date this table was made, there are 14 approved monoclonal antibodies derived from phage display and more than 7 are under medical studies[3]. To get an idea of the leading companies behind the development of these antibodies we have MophoSys's HuCAL with the highest number of monoclonal antibodies 19 understudies and 1 approved; AstraZeneca (CAT libraries) with 10 understudies and 5 approved monoclonal antibodies; and finally, Dyax with 9 understudies and 4 approved [3].

In the same way, despite the versatility of the technology allows isolating antibodies for different medical conditions. The trend shows that CAT libraries have obtained a greater number of antibodies indicated for non-cancer treatments[3]. On the other hand, with Dyax libraries, greater success is observed with monoclonal antibodies for treatments in oncology[3]. While in the case of

MorphoSys's HuCAL libraries, the development of antibodies is observed in equal amounts for both cancer and non-cancer treatments[3].

Finally, the most common format used by the aforementioned libraries is that of an Immunoglobulin G (IgG)[3].

6. Conclusions and Future perspectives

As a challenge for future (curious) researchers, understanding the biology behind this technique, getting to this point, has left me with more questions than answers. One is: after the impact of covid-19, what are the capabilities that this technique can provide in the development of a vaccine against the covid-19 virus?

In this regard, during the development of this work phage display has continued to evolve, a remarkable step it has taken concerning a possible treatment for the covid-19 virus involves the development of an antibody in "Nanobody" format, this antibody interacts with the receptor of the virus in charge of triggering the infection, thus helping to avoid contagion or stopping the infection[108]. The nanobody named "Nanosata-1C-Fc" reported by Ye et al. (2020) is within a new category of antibodies called nanobodies that correspond only to a variable domain of a heavy chain, within which there are four framework regions as a base structure and three complementarity-determining regions for interaction with the antigen. This nanobody can bind to the receptor binding domain of the spike protein of the virus, thus blocking its interaction with the human angiotensin-converting enzyme 2 (ACE2) [108], a receptor with which the virus interacts to trigger the infection. Nanosata-1C-Fc is the first nanobody to demonstrate efficacy in an animal model, in this case the hamster[108], and the results of the experiments show that it has an affinity for the spike protein that is 3000 times greater than that of the ACE2 receptors[108]. Although transferring the results observed in the animal model is complex, the potential that this new series of nanobodies presents to combat the covid-19 virus will undoubtedly continue to give good results.

Finally, the key potential of phage display lies in the freedom it offers researchers to manipulate conditions in vitro, giving them greater control over the properties expressed by the resulting

antibody. It is possible to work in conditions of high temperatures, pH, denaturants, UV exposure, with non-aqueous solutions, proteolytic enzymes. Either find new antigen epitopes or specific conformations, combining libraries to test a different response, perhaps less immunogenicity or more stability to obtain a slow blood clearance. On the other hand, the increase in disease treatment antibodies since the development of phage display clarifies two things; the impact of this technology as a great scientific and research complement, and the impact that antibodies have on human health and the economy.

The more science advances, the more they interact and the more they interconnect. Phage display is the result of this interaction. In addition, the digital tools (which are reflected in and without which the development of this work would not have been possible) for the creation and spread not only of information but of knowledge that we have today, without a doubt, make that the interaction between the different branches of science is increasingly inevitable. With the pandemic, likewise, it was unavoidable the fall of the veil that would reveal the lack of attention and relevance that science had; things seem to take different directions now. For this reason, the interconnection potentiality in multidisciplinarity challenges the scientists' curiosity to explore depths and complexities similar to knowledge such as phage display. It is quite an intrigue to know what comes next.

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