University of Setif 1
Faculty of Science and Nature
Department of Biotechnology

HANDOUT OF THE COURSE:

Immunotechnologies and health

Intended for second-year masters students Biological sciences Biotechnology and health speciality

Dr. Krache I.

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Preface

This booklet is the written support for the module "Immunotechnologies and Health" intended for students of the Master's degree in Biotechnology and Health. Bioproduction in the service of biotechnology refers to the production of biological molecules such as proteins, antibodies, membranes, or other glycolipids, including polyclonal and monoclonal antibodies.

Techniques based on immunotechnology are widely used in medical and pharmaceutical biotechnology, aimed at drug discovery and the development of diagnostic kits. These techniques are based on the use of antigens, antibodies, or immune cells in single or multiple experimental formats. The techniques using these tools will be described theoretically in this manuscript but also practically in the form of article analyses (in tutorials), oral presentations, and field trips to pharmaceutical industries. This booklet is designed following the official framework prescribed by the Ministry of Higher Education and Scientific Research. These courses are organized into two main chapters:

The first chapter explains the production techniques of polyclonal antibodies:

- Different types of antibodies ;
- Different methods of production and characterization of therapeutic antibodies.

The second chapter details the biotechnology of monoclonal antibodies:

- Different types of antibodies ;
- Different methods of production and characterization of therapeutic antibodies.

1. Techniques for the Production of polyclonal antibodies

1.1 General concepts in immunology

Immunology is a scientific discipline that focuses on the functioning of the immune system. This system consists of a complex set of individual organs and tissues through which immunocompetent cells of innate and adaptive immunity constantly circulate.

The immune system plays an essential role in protecting the body against external agents. Therefore, immunology is the science that studies the discrimination between self and non-self. To achieve this, the body must have biological mechanisms that allow it to recognize and tolerate what belongs to it (self) and to recognize and reject what is foreign to it.

Indeed, foreign substances or infectious agents constitute exogenous antigens. Meanwhile, tumor cells, for example, represent endogenous antigens that the body must eliminate. The overall result of this recognition leads to the immune response. A disturbance in any of these systems can lead to severe pathological disorders, such as immune deficiencies, autoimmune diseases, or hypersensitivity states.

1.2 Lymphoid organs

The lymphoid organs and tissues correspond to the residence of lymphocytes and other cells of the immune system, such as macrophages and dendritic cells. They are divided into two groups: central lymphoid organs and peripheral lymphoid organs. The functioning of the immune system involves a set of organs, tissues, cells, and molecules.

1.2.1 Organs

The immune system is composed of two categories of lymphoid organs (**Figure 1**), connected by two circulatory systems: the blood and lymphatic systems.

1.2.1.1 Primary lymphoid organs

These are represented by the bone marrow and the thymus. They are the sites of development and maturation of lymphocytes.

1.2.1.2 Secondary lymphoid organs

The secondary or peripheral organs include lymph nodes, the spleen, and mucosa-associated tissues (tonsils, Peyer's patches, and appendix). These organs are the sites where the immune response takes place (**Figure 1**).

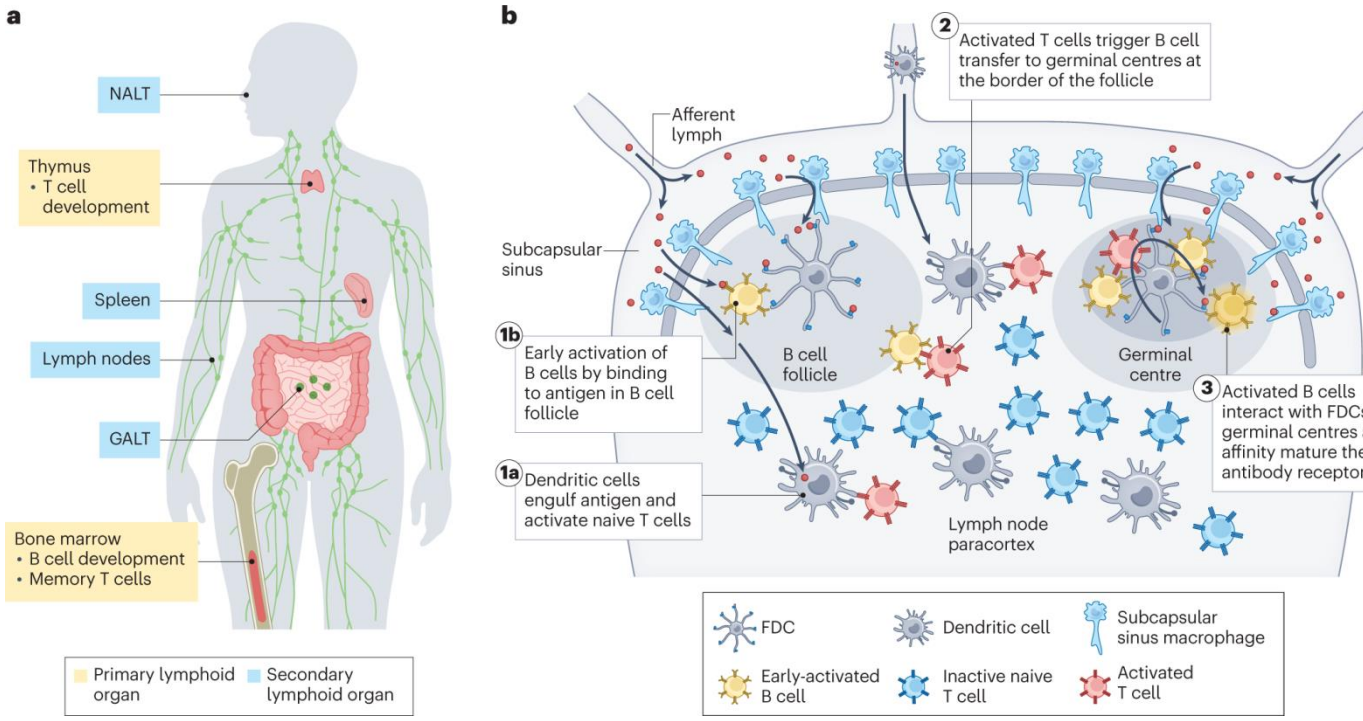


Figure 1. Primary and secondary lymphoid organs

a, Anatomical distribution of primary and secondary lymphoid organs. **b**, General organization of secondary lymphoid organs and sites of key interactions leading to adaptive immunity, using the lymph node as an example. Shown are orchestrated steps in the early activation of adaptive immune response in response to an antigen (orange). (**1a**) Dendritic cells (grey) acquire antigens trafficked into the lymph node or migrate to the lymph node from peripheral tissues carrying antigens, which they then present to naive T cells (blue) to drive T cell activation and proliferation. (**1b**) B cells (yellow) bind to antigens arriving in follicles, triggering initial B cell activation and proliferation. (**2**) Early-activated B cells receive help signals from activated CD4+ T cells at the T zone–follicle border, providing signals to drive entry into germinal centres. (**3**) Activated B cells enter germinal centres where they undergo proliferation and somatic hypermutation to affinity mature their antibody receptor through interactions with follicular helper T cells and the antigens captured on the dendrites of follicular dendritic cells (FDCs). GALT, gut-associated lymphoid tissue; NALT, nasal-associated lymphoid tissue.

1.2.2 Cellular effectors

The immune system relies on different cells for its functioning, such as lymphocytes, specialized cells that capture and present antigens, and effector cells that eliminate microbes (**Table 1**). These immune cells are divided into two major lineages: myeloid cells, which are the first to respond to a pathogen invasion, and lymphoid cells, which regulate the acquired immune response.

Table 1: Immune cellular effectors

Types	Sous types	Functions
Granulocytes or Polynuclear Cells	Neutrophils	First line of defense of the body against harmful bacteria Phagocytosis of pathogens
	Eosinophils	Destruction of parasites. They play a role in allergic reactions
	Basophils	Secretion of histamine and substances that attract eosinophils and participate in allergic reactions
Antigen-Presenting Cells (APCs)	Dendritic Cells	Presentation of antigens to T and B lymphocytes. They absorb antigens, break them down, and then prompt T lymphocytes to destroy them
	Phagocytes : Monocytes and Macrophages	Phagocytosis
Natural killer cells	Natural Killer (NK)	Destruction of cancerous or microorganism-infected cells (viruses or bacteria). Once they attach, they produce cytokines that damage and destroy cancer cells
Lymphocytes	B Lymphocyte	Synthesis of antibodies that specifically recognize antigens and bind to them, marking them so that other immune cells can find and destroy them
	T Lymphocytes	Directly attack invaders and cancer cells, direct and control the immune response through cytokine secretion
	Helper T Lymphocytes	Encourage B lymphocytes to produce antibodies and help killer T lymphocytes become more effective
	Killer T Lymphocytes (Cytotoxic T cells)	Destruction of cancer cells and virus-infected cells.

1.2.3 Soluble effectors

Many circulating mediators from immune cells and surrounding tissue cells participate in several immune processes. The main mediators are:

- **Complement system**: A set of plasma and membrane proteins playing an essential role in the destruction of infectious agents and the elimination of immune complexes.

- **Cytokines**: protein molecules serving as non-antigen-specific intracellular chemical messengers. They enable intercellular communications involving the innate immune system (inflammatory response), adaptive immune response, and the hematopoietic system.
- **Antibodies (Ab)**: complex glycoproteins produced by the immune system (by plasma cells) in response to stimulation by a usually foreign component, also called an antigen. Antibodies can specifically bind to antigens to neutralize them and recruit immune system effectors, such as macrophages, NK cells, or the complement system, to eliminate microorganisms or cells expressing these antigens.

1.3 Structure and biological functions of antibodies

1.3.1 Structure

Antibodies are glycoproteins belonging to the immunoglobulin (Ig) superfamily. All proteins in this family contain at least one structural motif called an "immunoglobulin domain." This domain, composed of 70 to 130 amino acids (aa), has a characteristic secondary structure formed by two antiparallel beta sheets stacked like a sandwich.

This structure is stabilized by interactions between hydrophobic amino acids and disulfide bridges formed by highly conserved cysteine residues. There are different types of Ig domains: constant domains (IgC) and variable domains (IgV). Antibodies are made up of a combination of IgC and IgV domains, themselves located within four polypeptide chains: two heavy chains (denoted H for Heavy) and two light chains (denoted L for Light), which are identical to each other and connected by disulfide bridges. Each heavy chain includes 3 or 4 constant domains (denoted CH1, CH2, CH3, and CH4) and one variable domain VH, for a total mass of approximately 50 kDa; each light chain includes 1 constant domain CL and 1 variable domain VL, resulting in a mass of 25 kDa. Thus, a complete monomeric antibody, composed of its four chains, has a mass of about 150 kDa. This particular protein organization gives rise to the basic (monomeric) Y-shaped structure of antibodies (**Figure 2**).

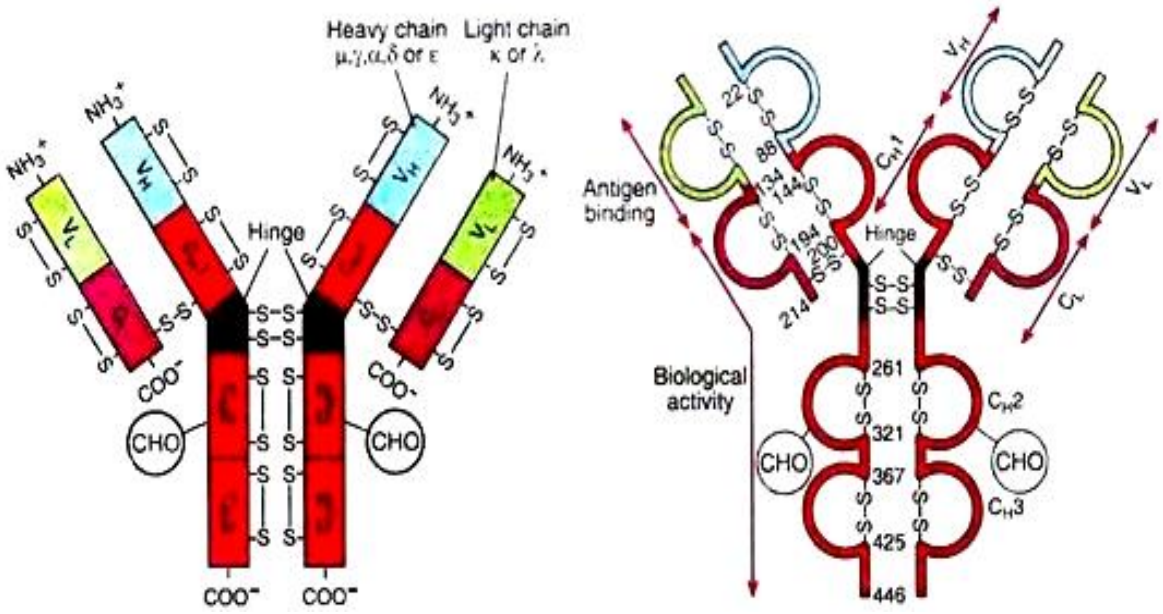


Figure 2. Schematic structure of an immunoglobulin.

Antibodies can eliminate antigens in different ways (**Figure 3**). Small antigens (toxins, xenobiotics, allergens) are neutralized and then destroyed by the phagocytes. Larger antigens (pathogens) are recognized by the antibodies and then eliminated through cellular effectors (cytotoxic cells, phagocytes) or molecular (complement system).

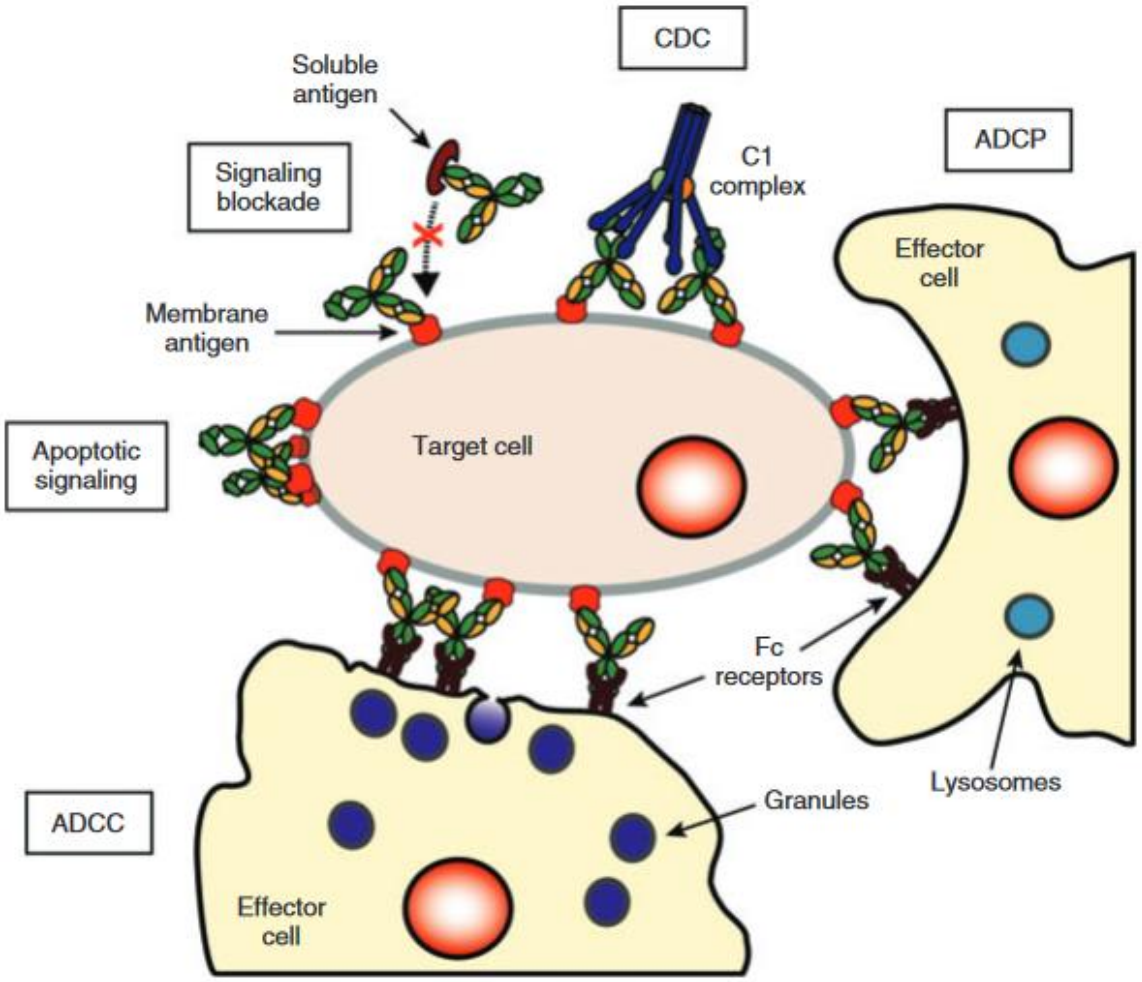


Figure 3. Potential modes of action of antibodies.
Antibodies can elicit their protective activity by blocking the interaction of soluble factors (ligands) to surface receptors by either targeting the free soluble factor or the receptor on the target cells, which may be a cancer cell. Targeting antigens on the surface of the cells may alter signaling and induce an antiproliferative and/or proapoptotic activity that can be favored by crosslinking. In addition, antibodies can activate immune effector functions such as ADCC, ADCP, and CDC. ADCC: Antibody Dependent Cell mediated Cytotoxicity ; CDC : Complement Dependent; Cytotoxicity ; CAM : Complexe d'Attaque Membranaire.

1.3.2 Isotype

There are different types of antibodies or isotypes (IgG, IgA, IgM, IgE, and IgD) that differ in terms of sequence and structure but share the common "Y" structure described above (**Table 2**). Each isotype can contain a light chain κ or λ (kappa and lambda). The diagrams show the different configurations of the secreted forms of these antibodies. It should be noted that the IgA isotype is composed of two subclasses, called IgA1 and IgA2, while IgM, IgD, and IgE have no subclasses. IgG includes four subclasses, called IgG1, IgG2, IgG3, and IgG4. For historical reasons, the IgG subclasses have different names in other species; in mice, they are

named IgG1, IgG2a, IgG2b, and IgG3. The serum concentrations indicated correspond to the average values in normal individuals.

Table 2 : Immunoglobulin's isotypes

Isotype	**Subtype**	**Heavy chain**	**MW (KDa)**	**Serum half-life (days)**	**Secreted form**	**Functions**
IgA	IgA 1, 2	**α (1 et2)**	160	6	Monomer, dimer, trimer	Mucosal immunity
IgD	None	**δ**	184	3	None	Antigen receptor on naïve B cells
IgE	None	**ε**	188	2	Monomer	Mast cell activation (immediate hypersensitivity), defense against helminthic parasites
IgG	IgG 1-4	γ (1, 2, 3 et 4)	146-165	23	Monomer	Opsonization, complement activation, antibody-dependent cell-mediated cytotoxicity, neonatal immunity, feedback inhibition of B cells
IgM	None	μ	970	5	Pentamer	Antigen receptor on naïve B cells, complement activation

1.4 **Diversity of immune System**

1.4.1 Chromosomal Recombination

In mammals, an adaptive immunity is added, which can bind a vast diversity of antigens with specificity and affinity through two highly diversified repertoires of cells (T and B lymphocytes) and receptors or soluble molecules: T cell receptors (TCR) and B cell receptors (BCR), or soluble immunoglobulins (Ig).

The gene economy coding for these receptors allows generating their diversity combinatorially, by reassociating gene fragments like a patchwork to create a combination coding for a specific Ig or TCR in a cell. Chromosomal rearrangements thus generate an

almost infinite variety of receptors from just a few dozen genes or basic building blocks (**Figure 4A**). While these mechanisms seemed well understood in detail, a much unexpected type of rearrangement has recently been described as allowing the generation of broadly reactive antibodies.

1.4.2 Affinity maturation and class switching of immunoglobulins

To adapt the immune response after encountering an antigen, the B lineage can extend the rearrangement of Ig genes thanks to the enzyme AID (activation-induced cytidine deaminase). This enzyme initiates two processes: somatic hypermutation (SHM) and class switch recombination (CSR); (**Figure 4B**).

A previously assembled VDJ segment can then be modified, and its antigen-binding site improved by SHM. A process of cellular selection favors cells presenting the BCR with the highest affinity for the antigen. Several "classes" of BCR and Ig (IgM, then IgG, IgA, or IgE, with different constant domains) are also successively produced by a B cell clone.

This "isotype switching" or "class switch" results from CSR ("switch" regions of Ig genes) between DNA sequences targeted by AID, which creates double-strand breaks. A final AID-dependent type of recombination is LSR (locus suicide recombination), a variant of the switch that deletes the entirety of IgH constant genes (responsible for the heavy chain of Igs) and thus leads to the loss of BCR and apoptosis. Furthermore, once this sequence is inserted within the VDJ gene, it undergoes hypermutation like a classic VDJ, increasing its affinity for the antigen (**Figure 4C**).

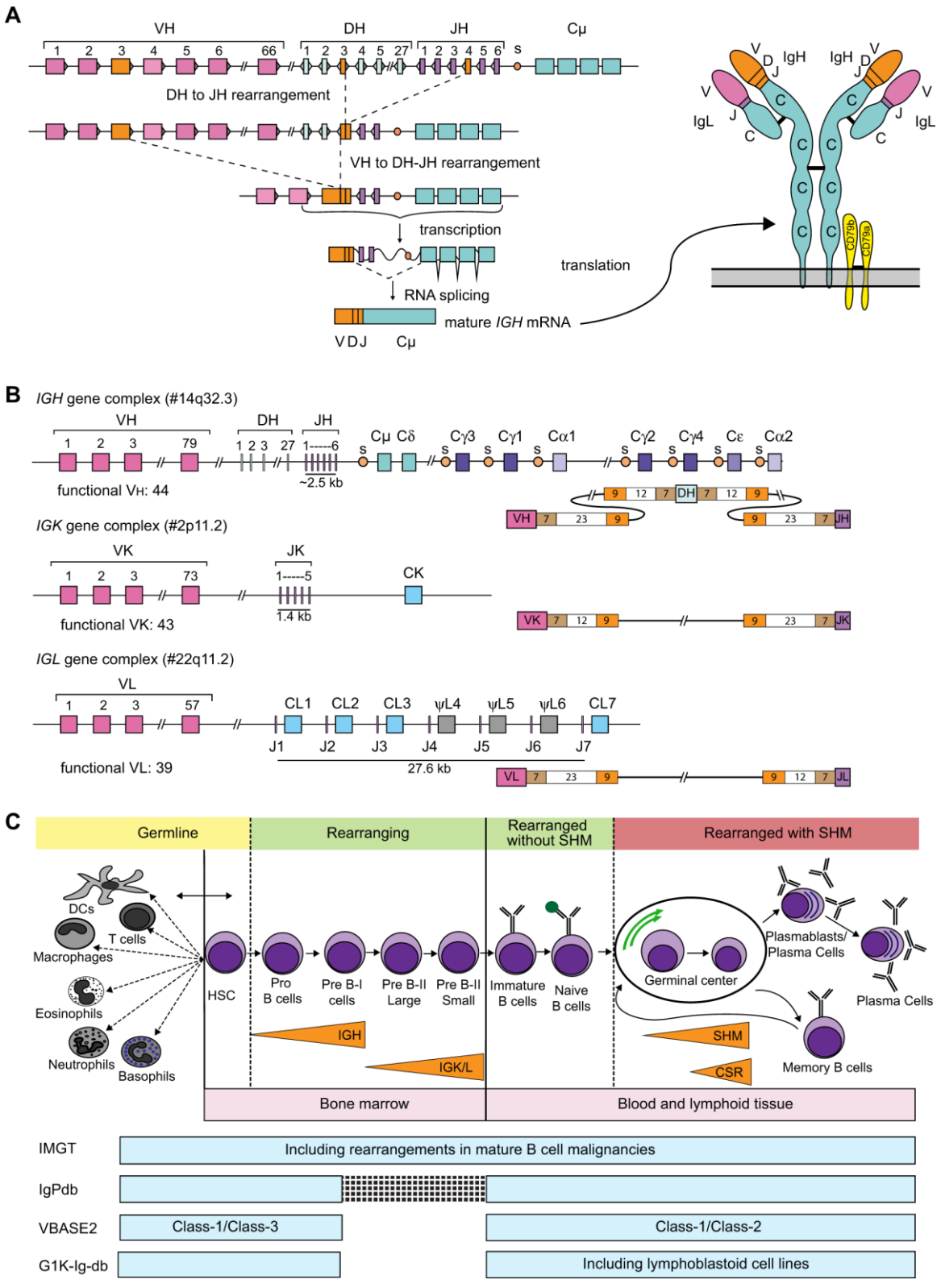


Figure 4. Generation and assessment of diversity in Immunoglobulin (IG) loci.

A. In the first step of *V(D)J* recombination in the *IGH* locus a *D* gene is coupled to a *J* gene. Subsequently, a *V* gene is coupled to the *DJ* joint. The *VDJ* exon is transcribed and spliced to the *IGHM* exons. An analogous process takes place in the Ig light chain genes. When a functional IgM protein is transported to the plasma membrane with anchoring molecules CD79a and CD79b and assembled with a functional Ig light chain, it forms a complete antibody molecule. **B** Schematic overview of the three *IG* loci: *IGH*, *IGK*, *IGL* and the structure of their corresponding Recombination Signal Sequences (RSS). Genomic

position of the loci is indicated in brackets. In *IG* loci each rectangle depicts one of the variable (*V*), diversity (*D*), joining (*J*) and constant (*C*) genes, and circles (assigned with "s") depict switch regions. The number of known functional genes, as listed in the IMGT, is indicated underneath each scheme. RSS structure schemes depict the position of heptamers (7), nonamers (9) and spacers (12/23) relative to *V*, *D* and *J* genes. **C** Hematopoietic stem cells in bone marrow, give rise to cells of both myeloid and lymphoid origin. While most of the cell types retain *IG* genes in their germline configuration, precursor B cells rearrange first Ig heavy chain and then Ig light chain genes to form a functional antibody. B cells with the functional B-cell receptor migrate to the periphery where they can recognize antigen. Upon antigen recognition and receiving help from T cells, B cells enter germinal center reaction during which they undergo intensive proliferation, improve affinity for antigen by the introduction of somatic hypermutations (SHM) in rearranged *IG* genes, and may change their effector functions in the process of class-switch recombination (CSR). This results in the formation of memory B cells and antibody-secreting plasma cells. *IG* genes can be sequenced from any B-cell type. However, in (virtually) all cells other than B cells, they will be in their germline configuration. Precursor B cells and naive mature B cells carry rearranged *IG* genes, which can be further modified by the presence of SHM in post-germinal center cells. Light blue block underneath B-cell maturation scheme depicts the sources of *IG* sequencing in the three existing *IG* databases: IMGT (ImMunoGeneTics), IgPdb, VBASE2 and the *IG* gene sequence data from the 1000 genome project .

1.5 Biotechnology of polyclonal antibodies

Yet, although they have been used in therapy for more than a century, it was only after the technological advances of recent decades that researchers could master the antibodies that pharmaceutical industries are developing on an almost exponential basis. Because of their structural complexity, therapeutic antibodies are biomedical and they are a growing part of it. If cancer, immunology and rheumatology were the precursor fields, they are now used in almost all areas of medicine: today they revolutionize the medical sector, by offering new therapeutic prospects for previously incurable diseases.

1.5.1 Definition

Polyclonal antibodies are of human or animal origin. The animals most used for production are mice, rabbits, goats... Polyclonal antibodies come from several different B lymphocyte clones, directed against several antigenic determinants, called epitopes, constituting the same antigen. They do not have the same physicochemical and biological characteristics. They are therefore heterogeneous and have a wide specificity.

They have many undesirable cross-reactions. The results obtained in the laboratory with polyclonal antibodies are never rigorously reproducible from one immune-serum batch to another, since these are dependent on the immune response of the individual or the immunized animal which is never identical. Their production in humans or animals is ethically difficult to accept. This makes procurement difficult. The production technique is

easy and fast. The cost is low for small quantities but high for large quantities requiring a high number of animals, restrictive farming conditions and expensive infrastructure.

1.5.2 Production of polyclonal antibodies

To obtain polyclonal antibodies, an animal (for example a rabbit, a goat...) is immunized several times with an antigen preparation and an adjuvant in order to increase its immune response. When the production of polyclonal antibodies (immune serum) becomes important, the animal's blood is recovered and the antibodies are purified in the laboratory, the production involves the following steps:

- Preparation of immunogens according to the typology of the immunogen (cellular antigen, protein, peptide, nucleic acid, chemical molecule, etc.);
- Immunisation of animals;
- Antibody purification for use in molecular biology experiments such as ELISA, affinity chromatography and immunotransfert tests. Non-specific proteins must be eliminated using elution solutions containing the free antigen or specific buffers to concentrate purified antibodies, often by ultrafiltration or precipitation;
- Validation and quantification consists of measuring the concentration of antibodies using a specific ELISA test then use UV spectroscopic methods to estimate the concentration. Specificity validation must be added to ensure that polyclonal antibodies are specific to the target antigen;
- Storage and preservation of antibodies for future use by lyophilization and subsequently store them in low temperature solution to maintain their activity.

The production of specific antibodies is the consequence of a complex event involving many factors, including:

1. Choice of animal species (adult, young and healthy), lineage within a species,
2. The nature and dose of the antigen, its molecular weight... etc.
3. Immunization protocol: route of administration (subcutaneous, intradermal, intramuscular, intra peritoneal and intravenous. The oral route is exceptionally used for the preparation of an immune serum, Cellular antigens are injected by venous route

or in some cases by intra peritoneal route) and dose spacing, use of adjuvant (**Figure 5**).

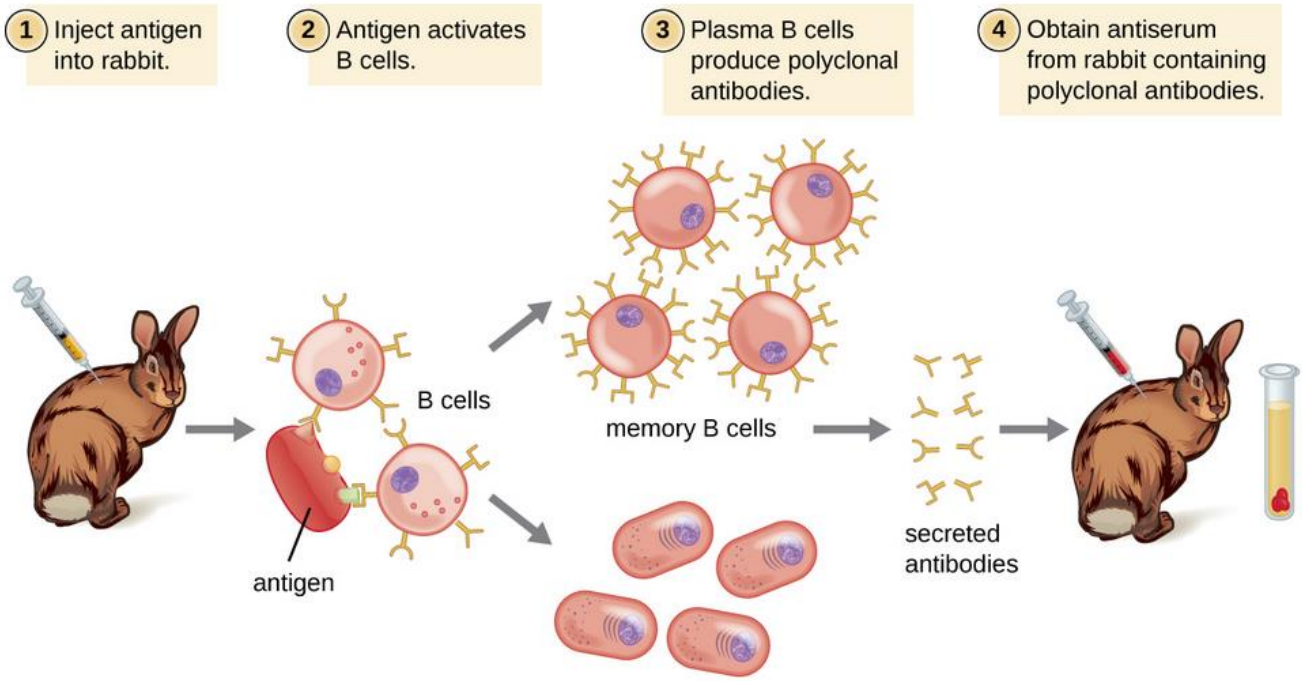


Figure 5. Diagram illustrating the process of harvesting polyclonal antibodies produced in response to an antigen.

1.5.3 Immunization protocol

Two standard protocols of 53 and 81 days can be followed; it is advisable to immunize at least two animals because the antigen response varies from animal to animal: animals are immunized with Ag in the presence of Freund's adjuvant; complete for first immunization and incomplete for recall. Injections are carried out with an intradermal and subcutaneous adjuvant.

Control samples are taken during the protocol to monitor immunoreactivity of immunoglobulins on the antigen. The time between reminders varies.

Immunoserum from animals are taken and the reactivity of the serums is tested (ELISA, IFI, WB...). The determination of the antibody titer (titration) of each sample is performed by ELISA to monitor immunoreactivity of immunoglobulins on the antigen. The final samples allow the production of 50 to 80 ml of antiserum (per rabbit).

1.6 Storage and preservation of polyclonal antibodies

These storage and preservation practices help preserve the quality, activity, and functionality of polyclonal antibodies for optimal use. They include:

- Aliquoting diagram: Shows the process of dividing antibodies into small aliquots to avoid repeated freezing/thawing cycles;
- Storage diagram at different temperatures: Shows the short-term storage conditions at 4°C and long-term storage conditions at -20°C and -80°C;
- Storage buffer and additive diagram: Illustrates storage buffers and antibody stabilizer additives such as BSA, glycerol, and sodium azide.

1.6.1 Types of polyclonal antibodies

An immune serum contains polyclonal antibodies that include:

- Immunoglobulins of different classes and subclasses;
- Several allotypes of heavy and light kappa chains;
- Light kappa and lamda chains;
- Different paratopes binding the different epitopes of the antigen molecule of different affinities.

The different types are summarized in **Table 3**.

Table 3: Types of polyclonal antibodies

Types	Objective	Examples
Research	Studying proteins and other biomolecules in research experiments	Antibodies against specific proteins
Diagnostic	diagnostic tests to detect the presence of pathogens or biomarkers in biological samples	Screening tests for infectious diseases, assessment of tumor marker levels
Therapeutic	treatment of various diseases by targeting specific antigens on pathogenic cells	Treatment of certain infections, cancers, or autoimmune diseases

1.7 Methods for characterization for therapeutic purposes

For polyclonal antibodies, characterization techniques are also crucial to assess their quality and efficacy, especially therapeutic. The following is a general overview of common techniques for characterizing polyclonal antibodies:

1.7.1 Assessment of specificity and affinity

- ***ELISA*** (Enzyme-Linked Immunosorbent Assay) to determine the ability of polyclonal antibodies to bind specifically to their antigen;
- ***Immunohistochemistry*** (IHC) is used to evaluate antibody specificity for antigens in tissue sections. Polyclonal antibodies are used to detect the presence of specific antigens in tissue samples using colorimetric or fluorescent detection;
- ***Western blot*** to confirm polyclonal antibody specificity by detecting specific proteins in a complex mixture. These proteins are separated by SDS-PAGE, transferred on a membrane, then incubated with polyclonal antibodies and revealed by colorimetric or chimiluminescence methods.

1.7.2 Analysis of purity and heterogeneity

- ***SDS-PAGE*** (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis): consists of evaluating the purity of polyclonal antibodies by separating proteins according to their size. The antibodies are loaded into a gel and separated, then visualized by staining;
- ***SEC*** (Size-Exclusion Chromatography): detects aggregates and evaluate the homogeneity of polyclonal antibodies. The separation of antibodies according to their size by passing through a column filled with porous beads;
- ***High-performance liquid chromatography (HPLC***) to analyze the purity and concentration of polyclonal antibodies (separation of proteins according to their chemical properties and interaction with the stationary phase);

1.7.3 Analysis of glycosylation

- ***HPLC*** (High-Performance Liquid Chromatography) for the separation and quantification of antibody-linked glycans;

- ***MALDI-TOF MS*** (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry): it is an identification of the glycosyle structures of the antibodies. Analysis of the mass of released glycans to determine their composition.

1.7.4 Functional tests

1. ***Neutralization*** to test the ability of polyclonal antibodies to neutralize a pathogen (virus, toxin). Antibodies are incubated with the pathogen, and neutralization capacity is measured using biological or virological tests,
2. ***Opsonization*** evaluates the ability of polyclonal antibodies to mark cells for phagocytosis. Antibodies are added to target cells, and phagocytosis by macrophages is measured.

1.7.5 *In vivo* functionality tests

1. ***Evaluation of the immune response*** to test the efficacy of antibodies in an animal model. Polyclonal antibodies are administered to animals and their effects on infection or inflammation are observed;
2. ***Pharmacokinetics and Toxicity***: to study the distribution, metabolism, and toxicity of polyclonal antibodies and to measure antibody levels in blood and tissues, and to monitor adverse reactions.

1.8 Polyclonal vs monoclonal antibodies

Polyclonal antibodies preparation is not as cumbersome as monoclonal antibodies. Monoclonal antibodies are produced by a single B cell line and specifically recognize one type of epitope. However, polyclonal antibodies are produced by several different B cell lines and each one recognizes a particular epitope present on an antigen. Hybridomas are used to produce monoclonal antibodies, whereas polyclonal antibodies are produced by animals that have developed immunity to an antigen injected into them (**Figure 6**).

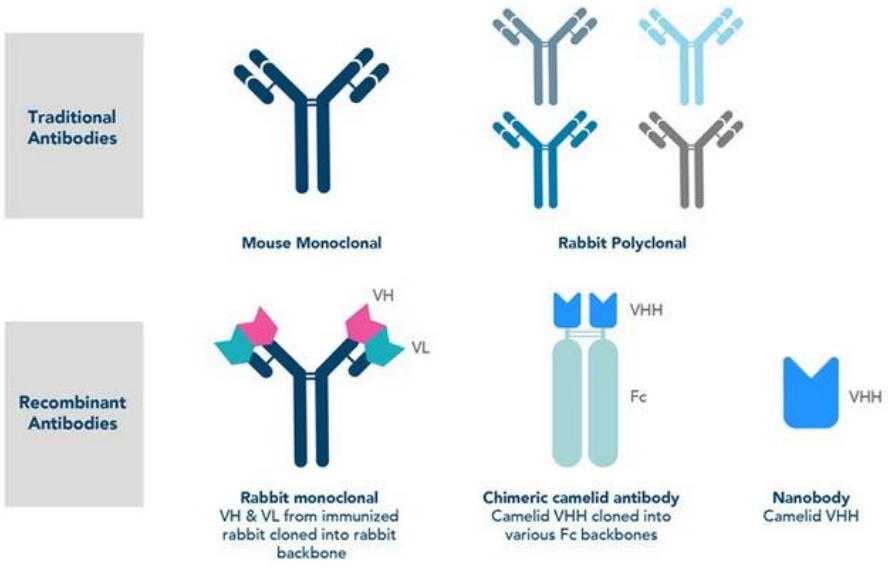


Figure 6. Polyclonal vs monoclonal antibodies.

The following table shows the major differences between polyclonal and monoclonal:

Table 4: Differences between poly and monoclonal antibodies

Feature	**Polyclonal antibodies**	**Monoclonal antibodies**
Source	Multiple B cell lineages	Single B cell lineages
Epitope recognition	Multiple epitopes	Single epitopes
Production method	Immunization of animals	Hybridoma technology
Specificity	Lower	Higher
Production time	Shorter	longer
Cost	Lower	Higher
Batch consistency	Lower	Higher
Applications	Diagnostics, antivenom	Diagnostics, therapeutics research
Avantages	Broad reactivity, cost effective	Hignt specificity, consistent
Disavantages	Batch variability, cross reactivity, Differences between different animal lots at different times, Recognition of multiple epitopes Possibility of cross-reactivity is high Difficulty in standardizing and controlling quality due to the diversity	Expensive, time consuming, Production of many specific antibodies, but may not be detected in several species, Sensitive to changes in epitopes (minor changes in conformation) can result in significantly reduced binding capacity

1.9 Applications

Polyclonal antibodies are used in many clinical trials designed to determine whether a patient is producing antibodies in response to a particular pathogen. The different applications are:

- ***Research applications by*** detection and quantification of proteins or biomarkers in biological samples, identification of specific proteins after separation by visualization of the distribution and expression of proteins in the attached tissues or cells, analysis of the properties of individual cells in suspension, including their size, their granularity, and surface marker expression;
- ***Diagnostic applications*** for screening and detection of pathogens (viruses, bacteria) in patient samples, disease diagnosis examples tests for the detection of autoimmune diseases, tumor markers;
- ***Therapeutic applications*** in the treatment of infections, Examples: antibodies against specific pathogens to treat bacterial or viral infections by administration of polyclonal antibody preparations as anti-venum or treatments for specific infections;
- ***Treatment of cancers*** using polyclonal antibodies targeting specific antigens expressed on the surface of cancer cells and use in targeted therapy to destroy tumor cells or modulate immune response;
- ***Treatment of autoimmune diseases*** by polyclonal antibodies targeting auto-antigens involved in autoimmune diseases and their uses to modulate the immune response and reduce inflammation.

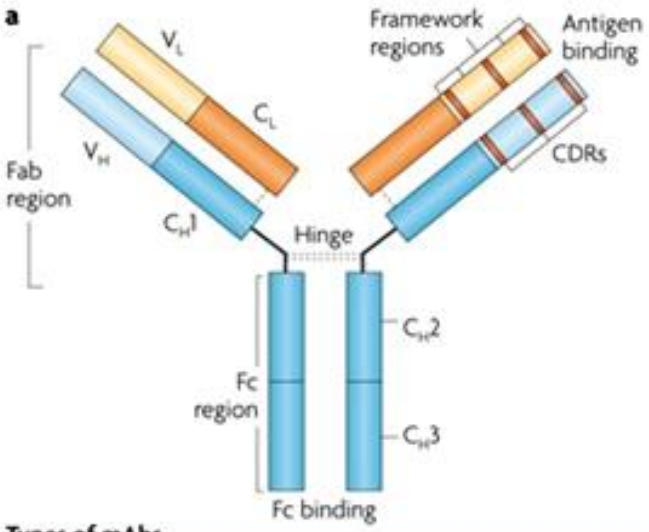
2. Production of monoclonal antibodies

The strength of the immune system depends on its ability to resist disease and harmful agents, but when the immune system is weak, an individual can be exposed to many diseases over and over again. As a result, people with weakened immune systems turn to Mab monoclonal antibodies, a treatment used to strengthen the immune system and protect it from disease.

The engineering of Mab, which are now used in the human clinic, has made it possible to develop a new generation of antibodies with optimized functional properties. These antibodies will undoubtedly lead to significant advances in the treatment of diseases for which the therapeutic arsenal is limited. However, the current cost of treatment with Mab requires new advances in production and purification, and raises the question of biosimilar molecules.

2.1 Definition

Mabs are antibodies that recognize only one type of epitope on a given antigen. They are identical and produced by the same cell (**Figure 7**). Mab are widely used in biology and medicine, as diagnostic tools or for therapeutic purposes. Mab used as medicinal treatments have an International Non-proprietary Name ending in "mab", an acronym for "antigen».



Types of mAbs

Murine	Entirely murine amino acids	'o' = mouse e.g. muromonab
Chimeric	Human constant (C) + murine variable (V) regions	'xi' = chimeric e.g. rituximab
Humanized	Murine complementarity determining regions (CDRs)	'zu' = humanized e.g. alemtuzumab
Human	Entirely human amino acids	'u' = human e.g. adalimumab

Figure 7. Schematic structure of Mab

2.2 Mab molecular engineering

Mab manipulation by genetic engineering began in the early 1980s. This led to the emergence on the market of chimeric, then humanized and finally fully human therapeutic antibodies.

2.2.1 Non-human Mab

2.2.1.1 Murine (Mouse) Mabs

Antibodies entirely of murine origin. Mainly used in research, but their clinical use is limited because of possible immune reactions in humans.

2.2.1.2 Chimeric Mabs

Chimeric antibodies biotechnology consists of isolating the DNA coding for the VH domain and the VL domain of a murine Mab and linking it to the DNA coding for the H and L constant domains of a human immunoglobulin. This produces a hybrid antibody in which the constant part is human: usually the human IgG1 constant region (γ1) and the human κ constant region (Cκ). In most cases, the specificity and affinity of chimeric antibodies remain identical to those of parental murine antibodies. Slight changes in fine specificity were, however, occasionally observed, suggesting an influence of the constant regions on the topology of the variable domains (**Figure 8**).

2.2.1.3 Humanized Mabs

The construction of humanized antibodies involves grafting the hyper variable regions of mouse antibodies onto human framework regions, in order to reduce their immunogenicity in humans. This process requires a precise choice of human domains to ensure correct antibody conformation. Protein structure modeling and analysis methods, as well as site-directed mutagenesis techniques, are used to create these humanized antibodies while preserving their antigen-binding capacity.

2.2.1.4 Human Mabs

Fully human Mabs are produced from human B cells or generated by recombinant techniques. They are used to minimize immune reactions and maximize therapeutic efficacy.

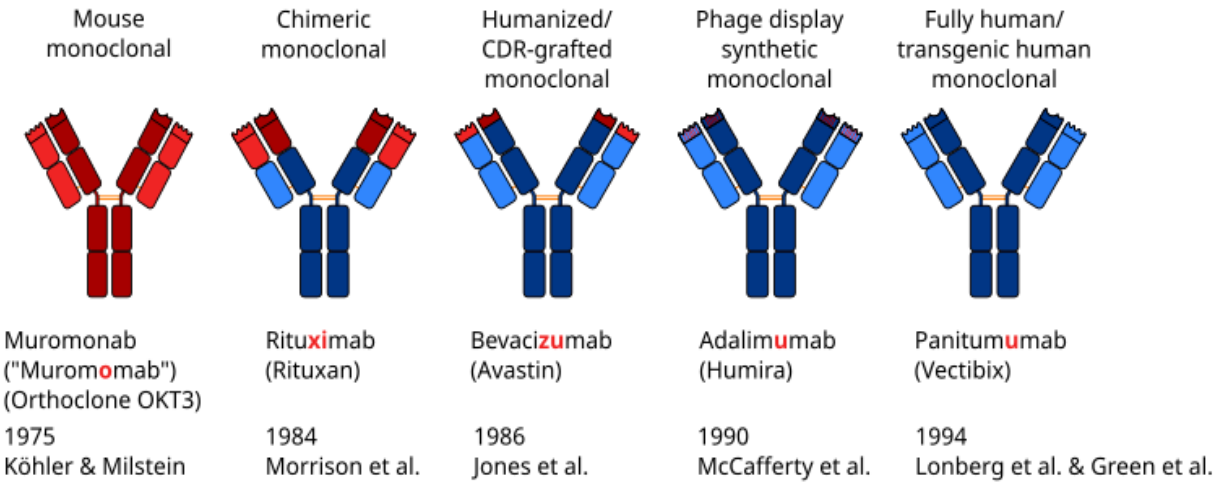


Figure 8. Type of therapeutic monoclonal antibodies

2.3 Therapeutic antibodies generation

Mabs have been classified into several types because of their different suffixes, for example (ximab), and the complexity of their nomenclature. Given the significant side-effects associated with the use of murine therapeutic antibodies, it was essential to rapidly develop other types of 'more human' antibodies to overcome these problems. Advances in recombinant DNA and protein engineering technologies have enabled the successive generation of chimeric, humanized and then fully human antibodies, giving new impetus to the therapeutic use of Mabs (**Table 5**).

Table 5: Simplified nomenclature of the different categories of Mabs

Type	**Suffix**	**% Human**	**Examples**
Murines	-momab	0	Muromomab (Orthoclone®)-IbridomomaB (Zevalin®)
Chimeric	-ximab	60-70	Infliximab (Remicade®) Ituximab (Mabthera®)
Humanized	-zumab	>90	Trastuzumab (Herceptin®)-Bévacizumab (Avastin®)
Human	-mumab	100	Adalimumab (Humira®)

Mabs nomenclature of is very complicated. This nomenclature is very interesting because it allows us to classify them and the molecule at the origin of these antibodies; Mabs have a completely different nomenclature from other common molecules. However, we do get other molecules, the names are often derived from chemical origins such as the word endings -alol and -olol are for beta-blockers (**Table 6**).

Table 6: Mabs Classification and properties

Categories	Properties	**Examples**
Murine antibodies (o-mab)	Injection leads to rapid production of human anti-mouse antibodies (HAMA) directed against murine Mabs	Muromomab
	Recognised as non-self antigens causing resistance to treatment, allergy or even anaphylactic shock	Ibridomomab
Chimeric antibodies (xi-mab)	Consisting of two parts: a human Fc fragment and a murine antigen-binding fragment (Fab)	Infliximab
	An increase in the half-life of the Ac, from less than 20 hours with murine Ac to several days, approaching the 21 days of endogenous human IgG	Rituximab
Humanized antibodies (zu-mab)	Induce far fewer anti-mouse immune responses (HAMA) than chimeric Mabs	Trastuzumab
	Humanized Mab directed against a tumor (in this case breast cancer)	Bévacizumab
Human antibodies (u-mab)	Improved tolerance profile compared with humanized antibodies	Adalimumab

2.4 Mabs biological functions

Mabs mimic the immune system's ability to recognise and attack foreign cells or molecules that threaten health. The biological functions of monoclonal antibodies are varied and depend on their interaction target:

1. ***Antigen recognition and binding***: Mabs are highly specific in their ability to recognise antigens. Their Y structure enables them to bind specifically to specific regions of antigens. This antigen-antibody interaction is essential for identifying and neutralizing pathogens such as viruses, bacteria or tumour cells;
2. ***Activation of the immune system***: once bound to their target antigen, Mabs can activate the immune system in different ways. For example, they can trigger a local inflammatory response, recruit other immune cells or activate the complement system, which plays a key role in destroying foreign cells;
3. ***Neutralization of pathogens***: Mabs have the ability to neutralize pathogens by preventing their entry into host cells or blocking their function. For example, in the case of viruses, antibodies can bind to the viral surface protein and prevent it from binding to cell receptors by inhibiting infection;
4. ***Marking and detection of cells and molecules***: Mabs can be used as specific probes to mark and detect specific cells or molecules. This property is widely exploited in biomedical research and medical diagnostics, where monoclonal antibodies are used in techniques such as immunohistochemistry, immunofluorescence and immune-precipitation;
5. ***Targeted therapy***: Mabs are also used as therapeutic agents specifically targeting tumour cells or molecules involved in specific diseases. For example, certain monoclonal antibodies are designed to bind specifically to receptors on the surface of cancer cells, thereby inhibiting their growth and proliferation.

2.5 Mechanisms of action of Mabs

Understanding the mechanisms of action of Mabs is crucial to their clinical application and future development. These mechanisms include specific binding to target antigens, activation of the immune system, inhibition of cell proliferation, induction of apoptosis and blockade of signalling pathways (**Figure 9**).

Each mechanism contributes to the therapeutic effect of monoclonal antibodies in different diseases. For example, in the treatment of cancer, monoclonal antibodies may target proteins responsible for tumour growth or block cancer cell survival signals. This section highlights the importance of understanding these mechanisms to improve the efficacy and safety of Mabs as a treatment.

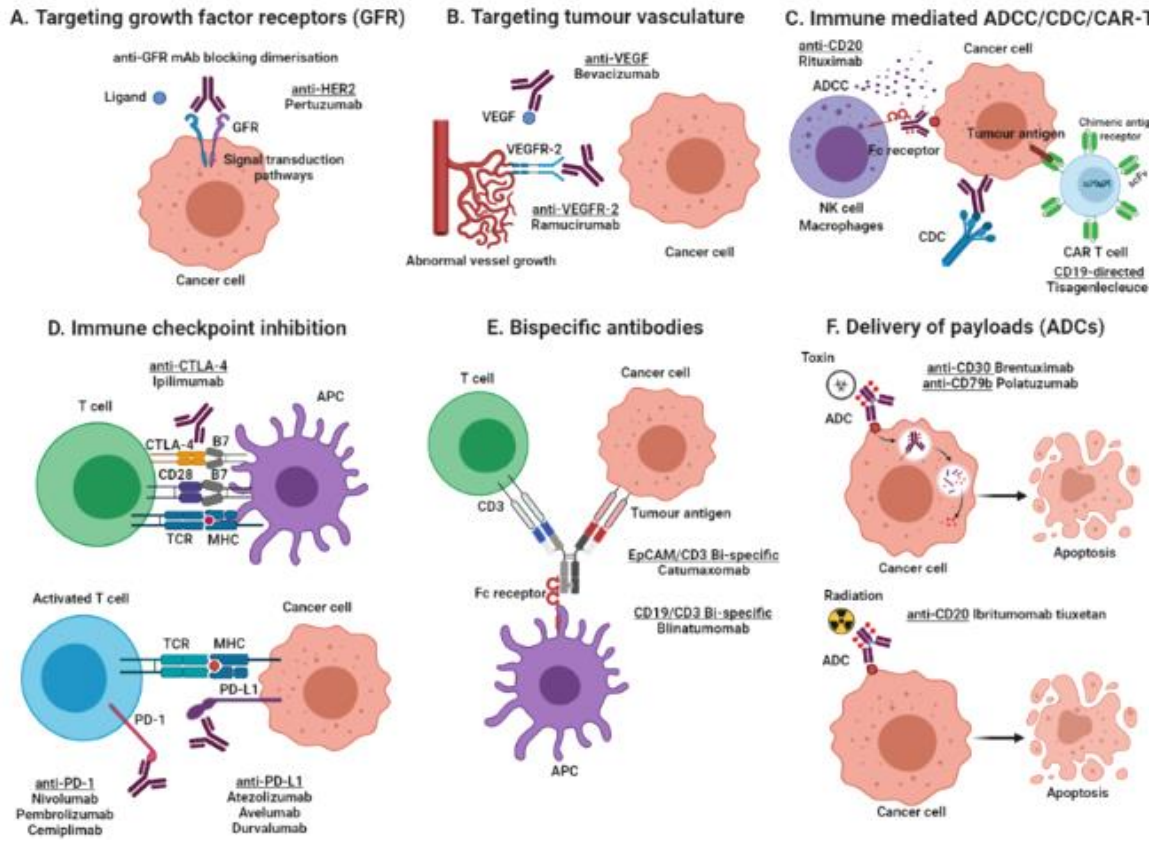


Figure 9. Mechanisms of action of monoclonal antibody-based products.
(**A**) Targeting growth factor receptors, blocking the binding of an activating ligand and inhibiting receptor homo- and hetero dimerization; (**B**) Targeting of tumour vasculature receptor or its ligands inhibiting angiogenesis; (**C**) Induction of apoptosis by recruitment of immune effector cells (ADCC) or activation of the complement cascade (CDC), and the use of antibody-based molecules to engineer T lymphocytes (CAR T cells); (**D**) Immune checkpoint inhibition by blockade of the PD-1/PD-L1 axis or CTLA-4 inhibitory receptors, increasing cytotoxic T cell activity; (**E**) Simultaneous targeting of two antigens, one on tumour cells and one on effector T cells, by using bispecific antibodies (BITE, bispecific T-cell enhancing); and (**F**) Delivery of payloads such as toxins and radioisotopes to tumour cells.

2.6 Mabs production

Two main techniques are used in most Mabs producing laboratories:

1. Hybridoma technique ;

2. Epstein Barr Virus (EBV) technique;

3. Specific antibody-producing B cells are isolated from the blood or tissues of immunized donors. This may include human or animal B cells. B cells are then cloned or amplified to test their ability to produce specific antibodies. Cell sorting techniques such as flow cytometry are used to select the cells that produce the most specific antibodies. The antibody genes from the selected B cells are cloned and expressed in mammalian cells for large-scale production;

4. Technologies for transgenic mice genetically modified to include segments of human immunoglobulin genes, enabling them to produce human antibodies when immunized against an antigen. Mice are immunized with the antigen of interest, thereby producing human antibodies. B cells from these mice are isolated and hybridoma can be created for long-term antibody production.

The first two techniques will be explained in detail.

2.6.1 Hybridoma technique

Hybridoma techniques are used to produce monoclonal antibodies. There are several key stages in this process (**Figure 10**).

2.6.1.1 Protocol

1. ***Immunization of an animal***, often a mouse, is immunized with the target antigen;

2. ***Cell preparation***: B cells containing antibodies are harvested from the animal's spleen or bone marrow;

3. ***Cell fusion***: the B cells are fused with myeloma cells to form hybridomas. Once the animal has been immunized, it is sacrificed, the spleen is removed sterilely and then dilacerated. A homogenous cell suspension of splenocytes was mixed with the myeloma cells in a ratio of 1:5 to 10 splenocytes. After centrifugation, the cell pellet is resuspended in the fusion agent either by physical fusion: Electrofusion or chemical fusion using the chemical agent Poly Ethylene Glycol (PEG).

4. ***Hybridoma selection***: hybridomas are cultured in a selective medium that kills non-fused cells. Fused cells are gently resuspended in selective culture medium for hybridoma growth

(RPMI - 1640, 10-15% fetal calf serum, supplemented with 10-5M aninopterin and 10-3M hypoxanthine). The cells were distributed in multi-well plates so that there was only one cell per well. Selection is carried out on HAT medium: Hypoxanthine Aminopterine Thymidine (substrate for HGPRT enzyme) where only hybridomas develop.

5. ***Cloning***: individual hybridomas are isolated to obtain cells producing a specific antibody. As soon as growth is sufficient, the hybridoma cultures producing the desired antibody must be propagated, preserved and cloned. Two methods are used to obtain a clone from a cell:

- Either in Agarose: the cells divide and form foci on the agarose gel that look like small spheres. As the medium is semi-solid, these small balls can be removed using a pipette and placed in a culture well plate after dilution.

- Or by limit dilution, where cloning is carried out directly in a microtiter plate. This method is the most widely used in the lab because it is inexpensive.

6. ***Screening***: the hybridomas are tested for their ability to produce the desired antibody. The positive culture wells contain a whole series of hybridomas secreting different antibodies. The hybridoma cell line that actually produces the monoclonal antibody must be isolated before it is cultured. Hybridomas producing specific monoclonal antibodies are selected by testing antibody activity in the culture supernatant. The methods used are radioimmunology (RIA), enzyme-linked immunosorbent assay (ELISA), haemagglutination and indirect immunofluorescence. These tests are generally carried out between 9 and 11 days after fusion.

7. ***Expansion and production***: selected hybridomas are cultured in large volumes to produce Mabs specific to a given antigen.

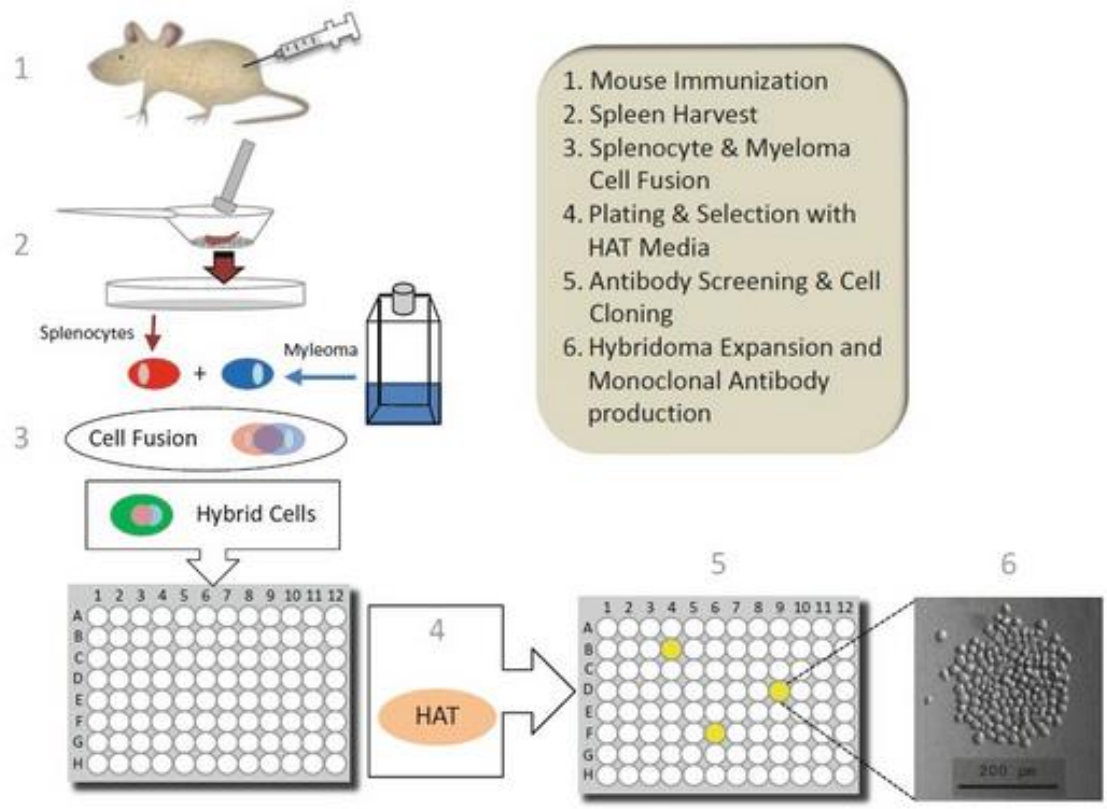


Figure 10. Schematic of hybridoma technology illustrating steps involved in the generation of a Mab producing hybridoma cell line.

8. ***Mass production of Mabs***: the next goal is to get the selected hybridoma to proliferate under the best possible conditions in order to produce enough antibodies. Large-scale production of monoclonal antibodies is based on hybridoma culture, either *in vivo* or *in vitro*.

In vivo production: the ascites process involves first injecting pristane (mineral oil) intraperitoneally or subcutaneously into a mouse or rat, causing irritation of the abdominal cavity but not yet ascites.

In vitro production: using cell culture for various applications, it is necessary to produce larger quantities of antibodies. For *in vivo* imaging, a few hundred microgrammes of antibody per patient are required. For in vivo therapy, several hundred milligrams are required per patient. In vitro production is based on growing hybridomas in defined culture media.

2.6.1.2 Advantages and disadvantages of hybridoma technique

One of the main advantages of hybridomas is their ability to produce Mabs continuously and in large quantities. These are extremely specific, making them valuable for medical,

diagnostic and research applications, particularly in the treatment of certain diseases such as cancers and autoimmune diseases, as well as in the development of accurate diagnostic tests. In addition, hybridomas enable long-term production of these antibodies without the need to re-immunize animals, simplifying and stabilizing the production process.

However, hybridoma technology also has its drawbacks. The process of creating hybridomas is complex and can be expensive, requiring specialized facilities and skilled personnel. In addition, although hybridomas can theoretically produce antibodies indefinitely, their long-term stability can sometimes be a problem, with the risk of mutation or loss of productivity over time. Furthermore, the use of myeloma cells, which are of human or animal origin, raises ethical and safety issues, particularly concerning the risks of contamination by pathogens.

2.6.2 Production of Mabs using the EBV technique

2.6.2.1 Phage display technique

The construction of combinatorial libraries of human VH and VL regions expressed on the surface of phages, a method known as "phage display". This approach involves the expression of peptides on the surface of filamentous phages and the generation of recombinant scFv or F(ab) fragments binding a VH region and a VL region in *E. coli*.

Antibody repertoires are created using rearranged V genes in vivo or synthetic V genes constructed *in vitro*. The cDNAs encoding the variable domains are synthesized by RTPCR, formatted to encode antibody fragments and cloned into phages or phagemids. Despite their complex construction, these libraries can be used to generate high-affinity antibodies by directed or random mutation (**Figure 11**).

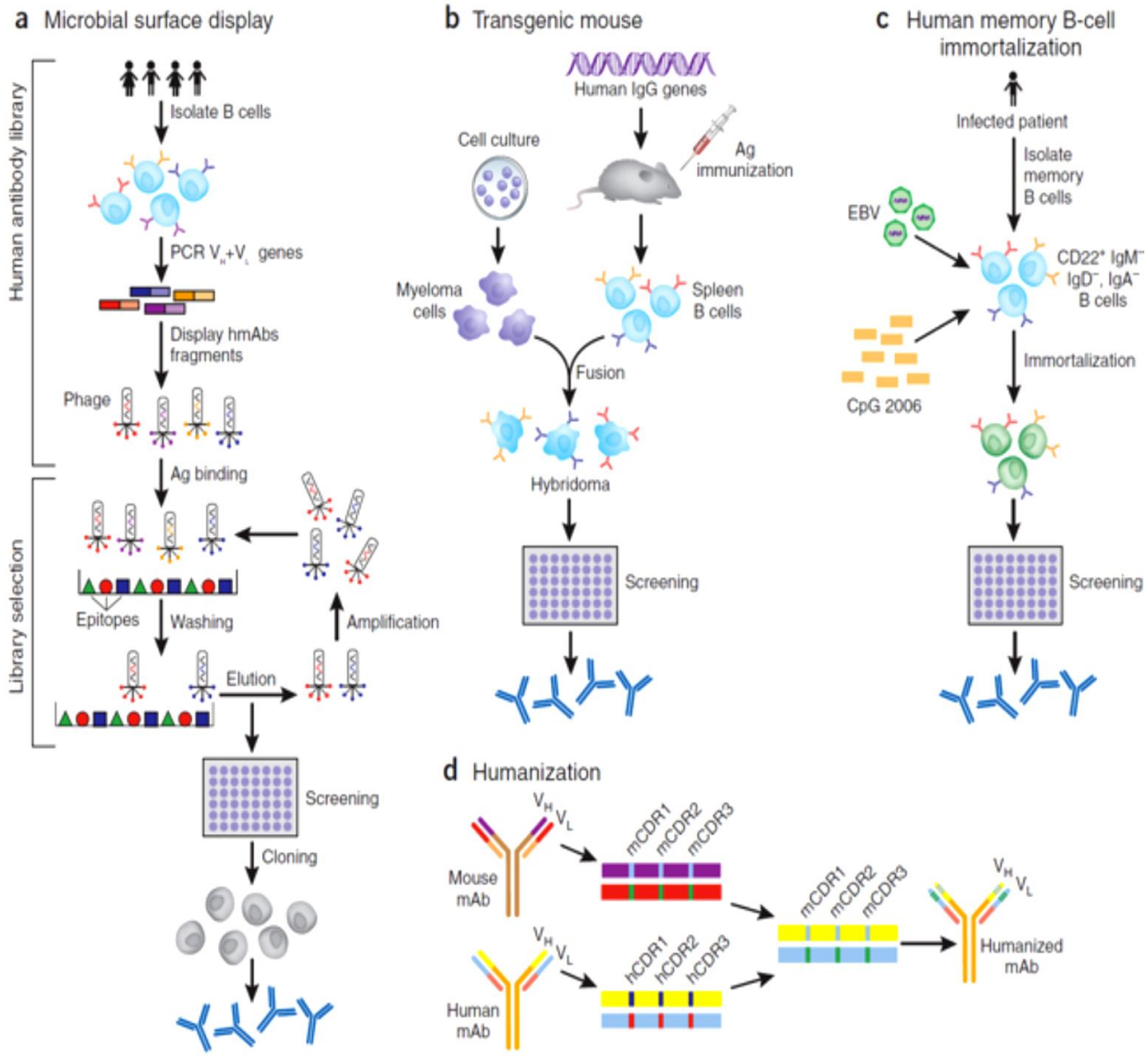


Figure 11. Phage display technique.

(a) The phage display technique is taken as an example of all the methods based on the expression of antibody fragments on the surface of microorganisms (phages, bacteria, yeasts, mammalian cells) or ribosome. A library of antibody fragments (scFv, Fab) is built up from human B lymphocytes from naive or immunized individuals, by cloning the variable regions of the different antibodies expressed by LB. The library is then expressed by phages that are selected for their interaction with the target of interest by ELISA. The target-specific phages are then amplified, isolated and the genes encoding the VH and VL regions are inserted into an expression vector suitable for the production of whole antibodies. The target-specific, fully human antibody is then expressed by the chosen cell line. (b) Human antibodies can be produced from transgenic mice in which the endogenous genes encoding immunoglobulin have been replaced by those encoding human immunoglobulin. After immunizing these mice with the antigen, human antibodies can be obtained using the classic hybridoma technique described by Köhler and Milstein. (c) The final method for obtaining human antibodies involves the immortalization of memory B lymphocytes derived from individuals exposed to the antigen. Memory B cells are isolated from peripheral blood leukocytes and immortalized using Epstein Baar virus (EBV) in the presence of CpGs. Transformed antibody-secreting cells are then selected and isolated by limiting dilutions, leading to the production of fully human monoclonal antibodies. (d) Antibody humanization is often achieved by CDRs grafting, which involves transferring the hypervariable regions of a murine antibody to a human antibody backbone, if possible with FR regions homologous to those of the original murine antibody.

2.6.2.2 Stages

1. Cell immortalization

B cells immortalization by EBV involves the involvement and diversion of cellular transcriptional factors thanks to the latency proteins of this virus. EBV can only multiply in vitro in B cells, where it induces lymphoblastic transformation and immortalization.

2. Production of EBV

Epstein-Barr viruses (EBV) are produced by the B958ATCC cell line, a wild Marmouset strain. These viruses are essential for transforming or immortalizing human B-cells. B95-8, a line of Marmouset monkey B cells transformed by the EBV virus, releases the virus into the culture medium. After centrifugation, the culture supernatant is recovered and used to transform or immortalize human B-cells. B95-8 cells are cultured under specific conditions, with several passages performed until an optimal concentration is reached. The culture supernatants containing the viruses are collected, filtered and distributed in cryotubes. These, once frozen at -80°C, are referred to as Transfokits and can be used later following a specific defrost protocol (**Figure 12**).

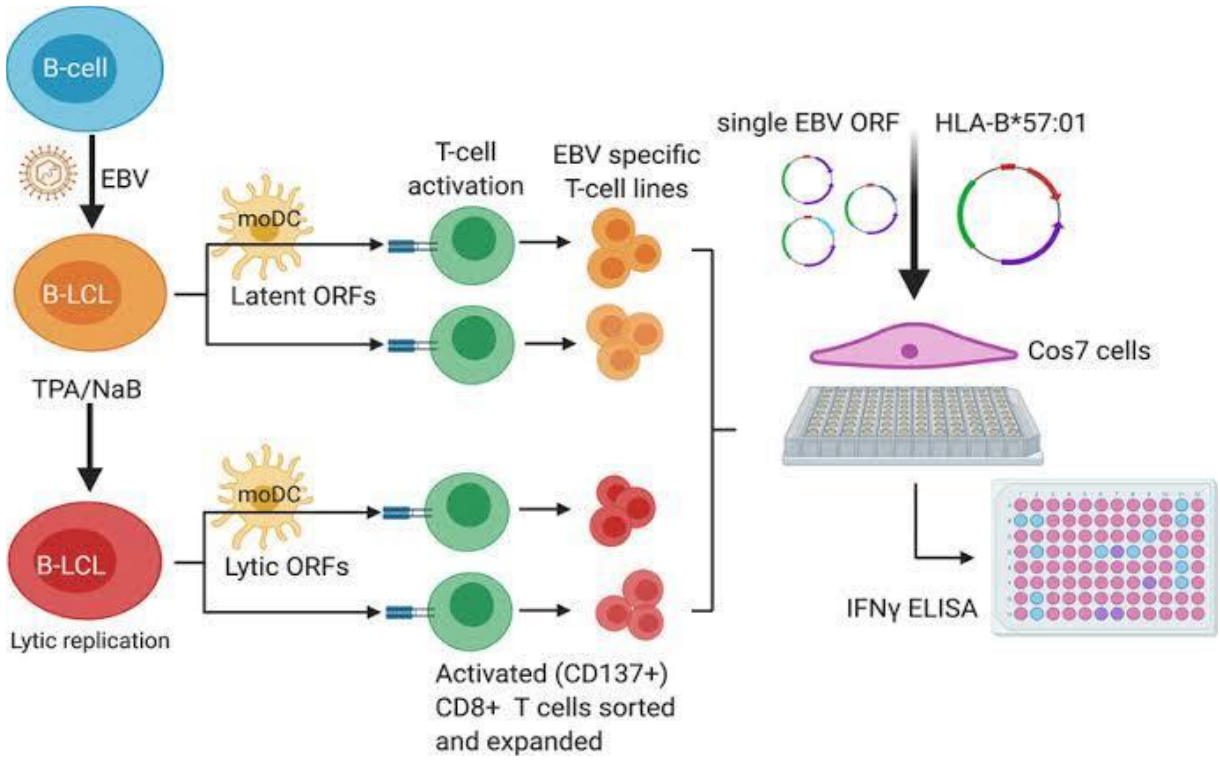


Figure 12. EBV production.

3. Fusion

This passage describes the preparation of the nourishing cells necessary for cell fusion and the production of effective antibody secreting hybridomas. The nourishing cells are obtained by irradiating PBMCs (Standfor Peripheral Blood Mononuclear Cells), or Peripheral Blood Mononucleate Cells, which are then deposited in 96-well microplates at a density of 5x104 cells per well in selective culture medium. After a few days, they form a monolayer at the bottom of the wells to accommodate cells after fusion. PBMCs are obtained from blood bags, then irradiated at a specific dose and frozen before use. At the time of use, irradiated cells are thawed and deposited on 96-well microplates.

2.6.2.3 Specific EBV-secreting hybridoma

The immortalized cells are fused with different myeloma partners to create antibody-secreting hybridomas. After centrifugation and removal of the supernatant, the fused cells are exposed to polyethylene glycol and cultured in a selective medium to promote hybridoma proliferation. Only hybrid cells with the characteristics of both mother cells are capable of proliferating under these conditions.

2.6.2.4 Advantages and disadvantages of using EBV

Among the advantages, EBV offers a high degree of financial flexibility, allowing companies to adjust their capital according to market needs and opportunities. This can facilitate investment and growth, while reducing financial risks. In addition, the EBV structure can attract investors because it allows for more dynamic resource management. However, the EBV technique also has its disadvantages. Capital variability can make financial management more complex, requiring increased monitoring and planning to avoid imbalances. Administrative costs may be higher due to the need to closely monitor capital fluctuations and comply with specific regulations. Finally, the perception of financial instability may deter some investors or trading partners from managing resources too volatile.

2.6.2.5 Mabs use

Research on the treatment of diseases is constantly evolving. A therapeutic approach that has shown great promise is the use of monoclonal antibodies. These drugs are proteins designed to target cancer cells specifically and inhibit their growth. This content provides a comprehensive introduction to the use Mabs in breast cancer treatment, and their critical importance in the fight against this disease.

- ***Mabs in diagnosis***: Antibodies are widely used for the identification of an antigen within a mixture (biological liquid, tissue slice, cell suspension...) and for the assay of antigens in solution. One of the first applications of antibodies and antigen/antibody interaction was and continues to be immuno assays for the diagnosis of disease markers (HIV, CMV) or cellular markers. Antibodies are also used in basic research as tools and tracers in techniques such as Western blot or immuno precipitation. On the other hand, antibodies have been used in medical imaging.
- ***Mabs in therapy***: These bio-drugs (recombinant proteins produced by cultured cells) have a molecular mass and mechanisms of action very different from the «conventional» drugs, usually obtained by chemical synthesis. The objective was to assess whether the specific characteristics of Mabs, in terms of structure and mode of action, have implications for their clinical development, evaluation by health authorities and long-term follow-up (**Table 7**).

Table 7: Therapeutic Mabs examples

Mabs	**Source**	**Molecular target**	**Indications**
OKT 3	Murine	CD3	Transplantation
Panorex	Murine	EpCAM	Oncology
Rituxan	Chimeric	CD20	Oncology
Simulect	Chimeric	CD25	Transplantation
Remicade	Chimeric	TNFa	Inflammation
Zenapax	Humanisé	CD25	Transplantation
Herceptine	Humanized	HER2/Neu	Oncology
Synagis	Humanized	VRS	Infectious diseases
Xolair	Humanized	IgE	Asthma, Allergy
Erbitux	Chimeric	EGF-R	Oncology
Humax - CD4	Humanized	CD4	Oncology
Avastin	Humanized	VEGF	Oncology

1. **Mabs and transplantation**: This is an antibody directed against the CD3 molecule that intervenes in the transduction of the signal to T cells after the recognition of the antigen. Paradoxically, due to retrospectively unjustified pharmaceutical hesitations the therapeutic class developed slowly. At the same time, we had learned how to genetically modify antibodies to humanize them by changing the majority of the determinants of the mouse species that produced them from human determinants. It was even possible to produce totally human antibodies using several strategies using transgenic or chimeric mice (man/mouse) or even in vitro productions (phage display). It was then an efflorescence of new antibodies used in very varied indications: always organ transplantation (anti-CD2, anti-CD4, anti-CD25, anti-CD40L).
2. **Rheumatology Mabs**: There are currently two main modes of action of therapeutic antibodies used in rheumatology. The first is to neutralize a cytokine involved in immunity or growth factor. The first effective therapeutic antibody to treat inflammatory rheumatism was an antibody against TNF-a. Other pro-inflammatory cytokines were then targeted such as interleukin (IL)- 1β with canakinumab, or IL.
3. **Mabs and immune, inflammatory and chronic diseases**: The etiology of autoimmune chronic inflammatory diseases such as rheumatoid arthritis (RA), ankylosing spondylitis (ASF), systemic lupus erythematosus (SLE), psoriasis, Crohn's disease, or multiple sclerosis (MS) remains enigmatic. Antigens responsible for these diseases are poorly defined, as is the hierarchy of their involvement. On the other hand, our detailed knowledge of the intermediate mechanisms of maintenance of inflammation and tissue destruction has made it possible to identify targets and then therapeutic molecules, and in particular Mabs antiTNFα and anti Interleukin 1 and the corresponding cytokines identified as mediators of inflammation and joint destruction.
4. **Mabs and allergy**: The first phase of allergy is a "silent" phase. It results in the production of specific IgE by B cells following initial contact with the antigen, which is then defined as allergen. The allergen is referred to as the sensitisation phase, and it will be recognized by IgE fixed on the RFcε I membrane receptors of mast cells and basophils. It binds by two distinct epitopes to two IgE on the membrane surface, creating a bridging of the IgE.

6.3. Mabs storage and conservation

Proper storage of MAB is essential to maintain their stability, activity and long-term effectiveness. Here is an overview of recommended storage conditions (**Table 8**).

Table 8 : Mabs recommended storage conditions

Parameters	**Designation**	**Conditions**
Temperature	4°C	Short periods (few days- weeks)
	-20°C à -80°C	Long periods (several months - years)
	Aliquotage	Divide Mabs into small aliquots before freezing
Buffers and preservatives	Storage buffers	PBS (phosphate-buffered saline), Tris-HCl
	Additives stabilizers	Albumine Serum Bovine (BSA) : 0,1% à 1%
		Glycerol à 50% to prevent ice crystals from forming during freezing
		Azide de Sodium (NaN_3) : 0,02% à 0,05% to prevent bacterian contamination
Aliquotage	Small volume aliquots	Avoid multiple freezing/thawing cycles
	Storage tubes	Polypropylene tubes to prevent contamination or loss of the sample
Additional considerations	Light	Mabs, especially those coupled to fluorochromes or other photosensitive molecules, must be protected from light to avoid their degradation (aluminum foil)
	Avoid freezing/thawing cycles	Repeated cycles lead to denaturation of antibodies. It is recommended to divide the antibodies into aliquots and thaw only the necessary amount
	Stability	egularly check the activity of stored antibodies by functional tests such as Western blot
Quality control	Activity verification	Regularly test the activity of stored Mabs ex. ELISA tests Quality control

2.7 Mabs characterizing methods

The methods for characterizing therapeutic Mabs are immunological tests used to detect the presence of specific antibodies, antigens or markers in an individual's immune system.

2.7.1 Affinity and specificity assessment

Affinity and specificity are achieved by several tests:

- ELISA (Enzyme-linked immunoassays) are the most frequently used.;
- SPR (Surface Plasmon Resonance) is a technique that allows the interaction between antibody and antigen to be measured in real time without the need for marking. It assesses binding kinetics (association and dissociation) and the force of interaction (affinity);
- Biacore (Surface Plasmon Resonance SPR) used to determine affinity constants and binding kinetics of antibody-antigen interactions:
- Flow cytometry Technique used to analyze the physical and chemical characteristics of cells or particles suspended in a fluid, based on their fluorescence when they pass through a laser beam. Measure the specificity of the antibody for an antigen expressed on the cell surface.

Illustration of common immunoassays: ELISA, Immunohistochemistry and Flow Cytometry are shown on **Figure 13**.

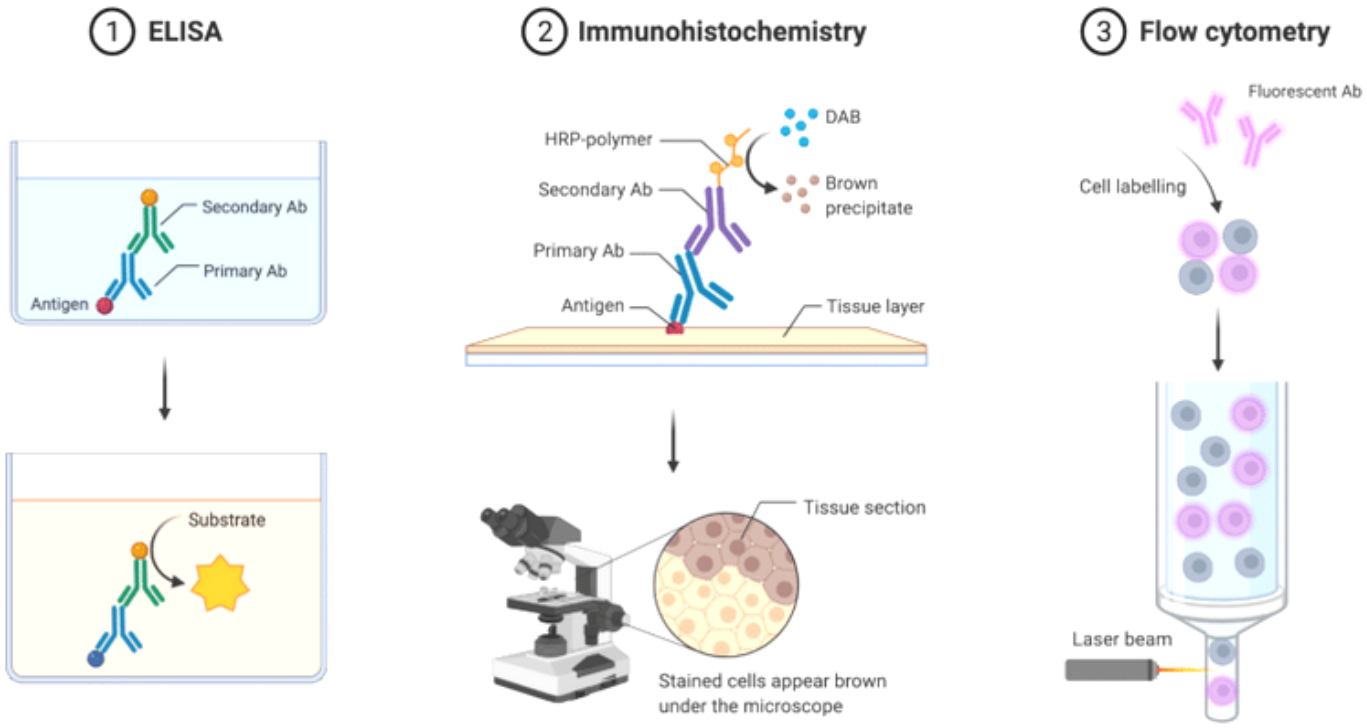


Figure 13. Affinity and specificity assessment tests.

2.7.2 Structure and stability analysis

- Size Exclusion Chromatography (SEC) separates molecules according to their size. Used to assess the purity of antibodies by detecting protein aggregates or fragments;
- Differential Scanning Calorimetry measures the heat required to increase the temperature of an antibody solution. It is used to evaluate the thermal stability of proteins. Identifies denaturation temperature (Tm) of antibodies, a key indicator of stability. A graph showing a DSC curve showing the peak of thermal denaturation, indicating the Tm of an antibody;
- Nuclear Magnetic Resonance (NMR) spectroscopy provides detailed information on the three-dimensional structure of antibodies in solution. It is based on the magnetic properties of atomic nuclei. Used to analyze the interactions between amino acid chains and chemical environment, providing an accurate picture of the structure of an antibody.A simplified diagram of an NMR spectrometer with the sample preparation steps, the magnetic field, and NMR spectrum analysis.

2.7.3 LB Screening

The process involves isolation, culture, sequencing and cloning of single B cells and screening for antibodies from selected single B cells. At the initial stage of B cell screening, several platforms are used including Berkely Lights Beacon®, Fluorescence Activated Tri Cell (FACS) and Bead or FRET technologies. It has established a Beacon platform and mature flow cytometry platform for high-throughput screening (**Figure 14**).

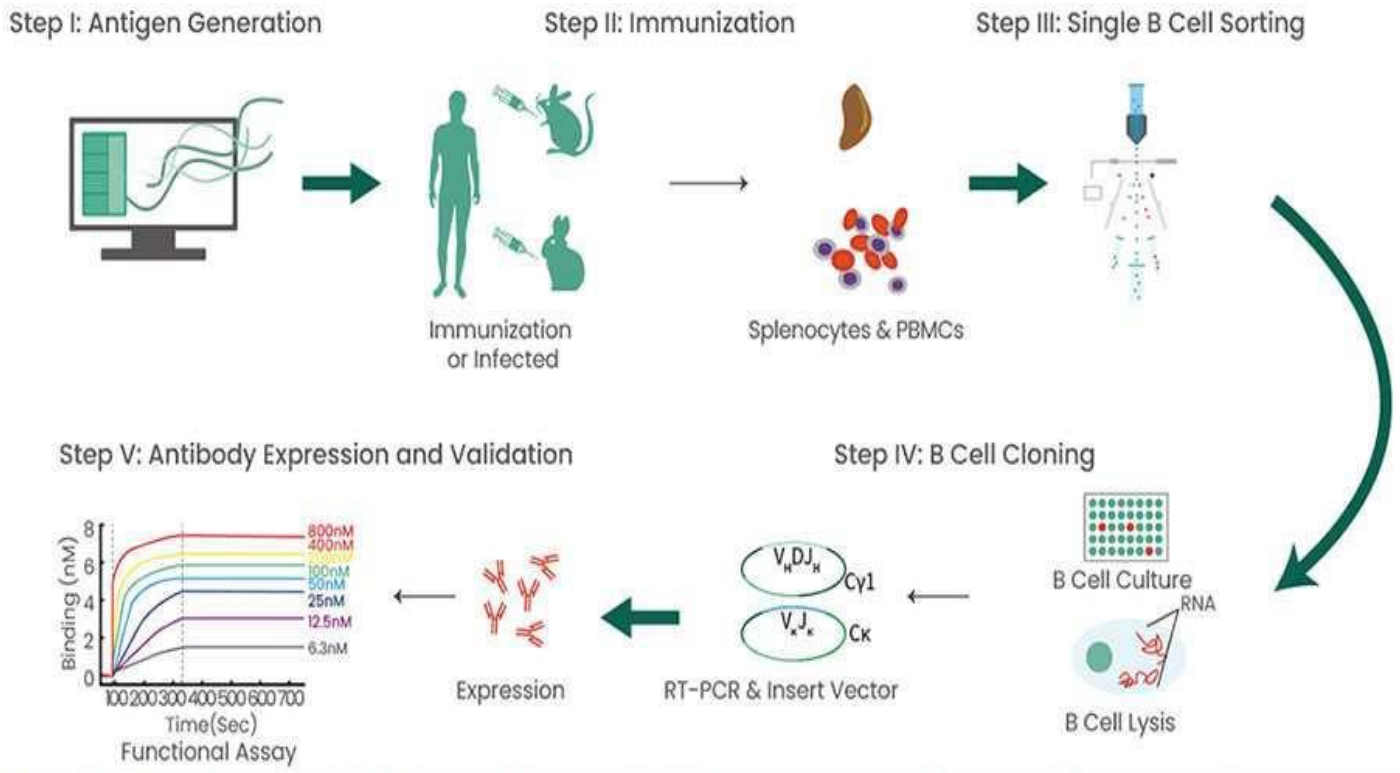


Figure 14. Screening and selection of clones according to antigen specificity and immunoglobulin class.

2.7.4 *In vitro* and *in vivo* functionality tests

In vitro tests include neutralization and opsonization tests to assess the ability of antibodies to block viral infection or to mark cells for destruction by the immune system. A schematic of a neutralization test showing target cells, antibodies, and prevention of viral infection.

For *in vivo* tests, they involve the use of animal models to assess therapeutic efficacy, pharmacokinetics and toxicity of monoclonal antibodies. An *in vivo* study diagram showing

the injection of antibodies into an animal model and the observation of effects on disease or immune response (**Figure 15**).

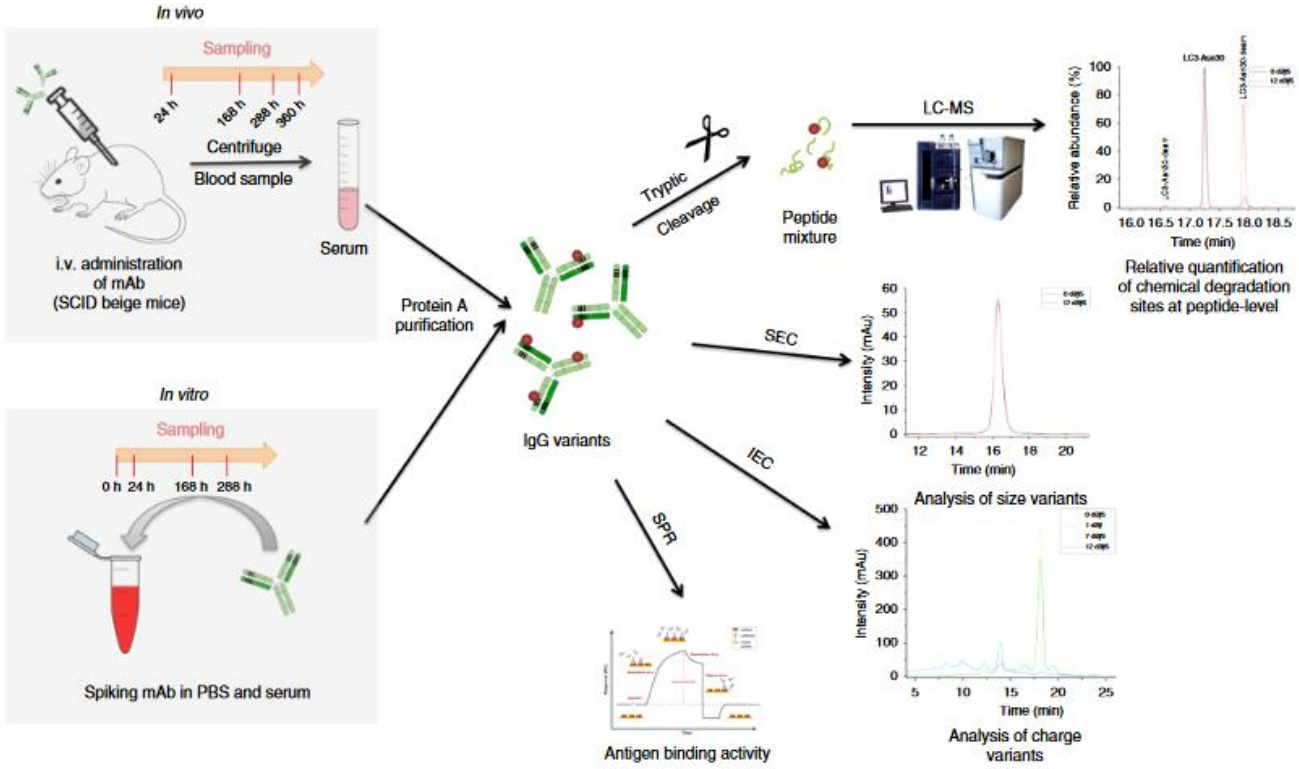


Figure 15. Mabs experimental workflow for the *in vitro* and *in vivo* characterization.

2.8 Industrial Mabs production

The optimization of the effector properties of Mabs, in addition to its interest in achieving better clinical efficiency, also has a great potential interest from an industrial and economic point of view: It could reduce the doses required for treatments and thus the cost of treatment. Several technologies can theoretically be used for the production of Mabs depending on the desired characteristics of the final product and the quantities needed to commercialize it at a regional or global scale (**Figure 16**).

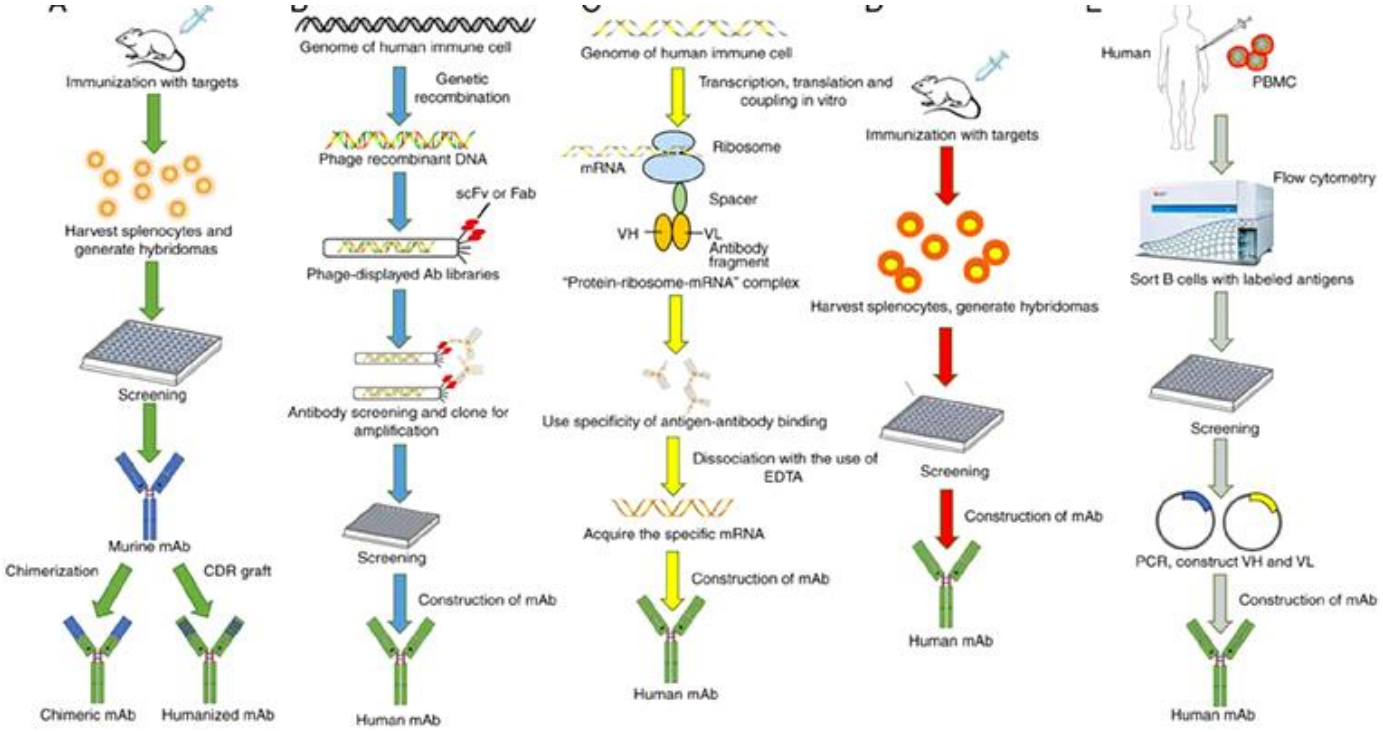


Figure 16. Approaches for the preparation of therapeutic monoclonal antibodies.

There are two main types of processes for producing the required amounts of Mabs for clinical trials and their commercialization: cell culture-based processes and transgenesis processes. In fact, the Mabs currently on the market or evaluated in clinical trials are produced in vitro in eukaryotic mammalian cells (mainly CHO and NS0). The different cell lines used (which also include YB2/0 and PERC-6) are grown in suspension in culture media without protein, especially without animal protein. Manufacturing processes are developed in pilot units and then transferred to "good manufacturing practice" (GMP) manufacturing units. All antibodies marketed to date are produced using these cell culture processes in suspended cells in bioreactors. Indeed, with regard to the production of MAB in the form of whole IgG, the production in bacteria is still not controlled: on the one hand, the production of high quantities of heterologous recombinant molecules remains toxic for the bacteria and, on the other hand, this expression does not allow the obtaining of molecules having correct functional properties due to the absence of N-glycosylation. The production in bacteria, which is inexpensive, is however well suited to the production of antibody fragments such as scFv and Fab.

Several animal species have been used for the production of Mabs: rabbits, sheep, goats, cows. Antibodies were typically produced in the milk of animals using expression vectors

containing specific promoters of mammary gland cells. This production has, however, still remained at the stage of demonstration of feasibility.

This approach allows large quantities of antibodies to be produced at very competitive costs compared with in vitro processes. However, the use of transgenic animals, in addition to the biological safety "societal" anxieties inherent in this approach, has some limitations such as the time to obtain and select transgenic animals and the purification of protein of interest from milk. In addition, these approaches require the control of different post-translational modifications of the IgG produced, specific to each species.

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