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Chapter

Monoclonal Antibodies Generation: Updates and Protocols on Hybridoma Technology

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Abstract

Since its inception in 1975, the hybridoma technology revolutionized science and medicine, facilitating discoveries in almost any field from the laboratory to the clinic. Many technological advancements have been developed since then, to create these "magical bullets." Phage and yeast display libraries expressing the variable heavy and light domains of antibodies, single B-cell cloning from immunized animals of different species including humans or in silico approaches, all have rendered a myriad of newly developed antibodies or improved design of existing ones. However, still the majority of these antibodies or their recombinant versions are from hybridoma origin, a preferred methodology that trespass species barriers, due to the preservation of the natural functions of immune cells in producing the humoral response: antigen specific immunoglobulins. Remarkably, this methodology can be reproduced in small laboratories without the need of sophisticate equipment. In this chapter, we will describe the most recent methods utilized by our Monoclonal Antibodies Core Facility at the University of Texas–M.D. Anderson Cancer Center. During the last 10 years, the methods, techniques, and expertise implemented in our core had generated more than 350 antibodies for various applications.

Key words Monoclonal antibodies (MAbs), Hybridomas, Immunization, Subcloning, Purification, Functional antibodies

1 Introduction

> *"The hybridoma technology was a by-product of basic research. Its success in practical applications is the result of unexpected and unpredictable properties of the method. It represents another example of the enormous practical impact of an investment in research which might not have been considered commercially worthwhile, or of immediate medical relevance."* Dr. Cesar Milstein, who shared the Nobel Prize in Physiology or Medicine 1984 for the discovery of Hybridoma technology, pronounced these words during his lecture of the award ceremony.

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More than four decades after Milstein's work was published in Nature in 1975 [1], monoclonal antibodies (MAbs) have revolutionized many aspects of the scientific world, with applications in many preclinical, diagnostic, and therapeutic fields. In fact, as a class of therapeutic molecules, MAbs constitute the most significant advance in cancer therapy, resulting in cancer immunotherapy being chosen as *Science's* 2013 Breakthrough of the Year [2]. MAbs have been approved and used for treatment of patients with different diseases including cancer (e.g., anti-CD20-Rituximab [3]; anti-Her2/neu-trastuzumab [4]; anti-EGFR-cetuximab [5], etc.), migraines [6], autoimmune diseases [7], and Ebola [8]. Most recently, US Food and Drug Administration (FDA) authorized the emergency use of newly generated MAbs to treat COVID-19 [9, 10]. The discovery of the MAbs targeting immune-checkpoints, modulators of the immune system which, in healthy individuals, are crucial for self-tolerance, has positively impacted the survival rates in cancer patients [11, 12]. Moreover, MAbs became essential tools enabling basic, translational, and clinical investigators to gain insight into the roles of various molecules in normal and pathologic states. MAbs have been utilized in biochemistry, flow cytometry, cell sorting, crystallography, molecular biology, and immunoassays.

In 2017, the FDA approved the chimeric antigen receptors (CARs) in the format of two CAR-T immunotherapies [13, 14], boosting the "prestige" of MAbs. Indeed, CAR constructions relay on the utilization of a portion derived from an antibody: the single chain variable fragment (sc-Fv), in which the variable heavy (VH) and variable light (VL) domains are joined by a hinge/spacer peptide. Recently, important findings in the field of adoptive cell therapy using NK cells, such as minimal or absent alloimmune or autoimmune toxicities [15, 16], extended the exploiting of CAR to develop CAR-engineered NK cells for the treatment of various hematological and non-hematological malignancies, using unique sc-Fv constructions.

MAbs generation poses a series of major technological challenges: **First**, selection of a proper immunogen that will eventually recognize the antigen of interest. It may be necessary to target specific epitopes within an antigen to generate antibodies with the desired specificity for a particular application, such as tumor cell killing, activation, or blocking of checkpoint molecules expressed on immune cells, immunoassays that could identify cellular localization of the target, or presence of the same in soluble format in human or animal fluids, etc. **Second**, according to this central role, the identification of specific surface markers in malignant cells and the development of antibodies capable of mediating tumor ablation has also become one of the major cornerstones of cancer therapeutics research. **Third**, once a candidate antibody that satisfies the appropriate criteria has been identified, further characterization and eventual validation and/or preclinical development need to be

undertaken. Developing antibodies that meet all of these requirements requires a level of technical sophistication that is well beyond the capabilities of many basic research laboratories.

There has been an increased interest in generating unique first in class/new antibodies. TABS, a therapeutic antibody database, shows over a thousand companies stepping in the discovery of new targets or the development of new versions of existing patented antibodies, in the hope of bringing to the clinic an improved therapeutic tool that complement, or even displace, the ones already undergoing clinical trials.

Although most of these efforts are focusing in the development of MAbs as therapeutic tools for diseases such as cancer, some of them are being currently used to prevent the evolution into more advanced stages of the disease. For instance, since it has been demonstrated that tumors are promoted by chronic inflammatory diseases, blocking inflammatory IL-1 pathways by MAbs has been analyzed and recently demonstrated to prevent both primary tumors growth and metastases formation in different murine models of breast cancer [17, 18]. Interestingly, an ongoing Phase II clinical trial studies the effects of canakinumab, an IL-1 blocking antibody, in preventing lung cancer in patients who have high-risk pulmonary nodules (NCT04789681). Specifically, canakinumab may interfere with the ability of tumor cells to grow and spread, thus preventing the development of lung cancer. In another study using this antibody, a secondary analysis of the obtained data including a 5-year follow-up, revealed that canakinumab-receiving patients showed a significant dose-dependent reduction in lung cancer incidence and mortality compared with placebo-treated patients [19, 20].

Our laboratory of Monoclonal Antibody Facility (MAF) at the University of Texas MD Anderson Cancer Center is actively participating in this endeavor. MAF developments are based on hybridoma technology, designing custom immunizations using peptides [21, 22], proteins [23], cells over expressing the target protein [24–26], or whole different species cells for projects involving marker discovery [27]. The methods and protocols described in this chapter are the most currently used by our laboratory. However, the state of the art and expertise has to be acquired to modify some steps and improve the yield in individual projects, creating in some cases unique designs in order to achieve the desired goals.

2 Materials

Here we describe the materials and equipment needed for the procedures in all sections. Materials for tissue culture need sterilization or process under sterile conditions, unless otherwise indicated.

2.1 Animals

1. Pathogen-free BALB/c mouse (6–8 weeks old) (Jackson Labs stock No. 000651 or similar Charles River, etc.).
2. NZBWF1/J mice Stock No: 100008|NZB/W (6–8 weeks old).
3. Sprague-Dawley rats (NTac: SD-Tg(SOD1G93A)L26H 5–6 weeks.
4. Knockout (KO) mice for the target molecule (if available).

2.2 Cells

1. L murine fibroblast cells (parental and genetically modified to express the target) (ATTC CRL-2648).
2. SP2/0 myeloma cells.

2.3 Antigens

1. Peptide-Keyhole limpet hemocyanin (KLH) conjugated.
2. Corresponding peptide alone.
3. Proteins.
4. Engineered cells expressing the target.
5. Whole tumor cells.

2.4 Media/Reagents

1. 70% Ethanol.
2. Phosphate-buffered saline 0.01% $MgCl_2$, 0.01% $CaCl_2$ (ThermoFisher Scientific Cat. No.10010023).
3. RPMI 1640 media.
4. Fetal bovine serum.
5. Penicillin-streptomycin antibiotic solution (ThermoFisher Scientific Cat. No. 15140148).
6. Sodium pyruvate 100 mM (ThermoFisher Scientific Cat. No. 11360070).
7. MEM non-essential amino acids 100 (ThermoFisher Scientific Cat. No. 11140050).
8. 0.05% Trypsin–EDTA solution (ThermoFisher Scientific Cat. No. 25300062).
9. EDTA 1 mM in PBS.
10. Incomplete Freund's adjuvant (InvivoGen Cat. No. vac-ifa-10).
11. Glycerol tissue culture grade (SIGMA, Cat No. G5516).
12. Polyethylene glycol (PEG 1450).
13. Red Blood Cell lysis Buffer (SIGMA, Cat. No. R7757).
14. HAT supplement (ThermoFisher Scientific Cat. No. 21060017).
15. HT medium (ThermoFisher Scientific Cat. No. 11067030).

16. Rat supplement conditioned media (RSCM) (commercially available or in-home made).
17. Concanavalin A (1 mg/mL).
18. Trypan blue solution.
19. Bovine serum albumin.
20. Blocking buffer for ELISA (PBS, 1% w/v BSA).
21. Tween-20.
22. PBST buffer (PBS containing 0.05% v/v Tween -20).
23. Secondary antibody, goat/rabbit/donkey IgG antimouse, HRP conjugated (Jackson Immunoresearch).
24. 3,3′,5,5′-Tetramethylbenzidine (TMB substrate solution).
25. H_2SO_4 0.2 N.
26. Protein A (GE Healthcare MabSelect SuRe Cat 17-5438-08).
27. Tris–HCl 1 M (pH 9) buffer.
28. Glycine–HCl, 0.1 M pH 3.
29. Pierce Rapid ELISA Mouse mAb Isotyping Kit.
30. EZ Blue Gel staining reagent (G1041-Sigma).
31. Bio Rad precision Plus protein; Dual Color Standards: catalog 161-0374.

2.5 Sterile Tissue Culture Plastics Surgical Instruments PAGE

1. T75, T150 tissue culture flask.
2. 96-well flat and round bottom plates.
3. 24-well tissue culture plate.
4. 10- and 50-mL conical tubes.
5. 10-cm diameter tissue culture dish (P100).
6. Pipet and multichannel pipet.
7. 10-, 20-, 200-, 1000-μL pipet tip.
8. 50-mL reagent reservoir.
9. 1-, 5-, 10-, 25-, 50-mL sterile pipets
10. Cell strainer 70 μm.
11. Zero dead volume BD 27G 1/2 mL (insulin) and 28 G syringes.
12. 1–2 mL glass syringes with Luer-Lok tips, sterile 3-way stopcock.
13. 20- and 22-G needles.
14. Surgical instruments: pointed scissors, blunt scissor, forceps.
15. Homogenizer.
16. Fine-mesh metal screen (25 μm pore).

17. Hemocytometer.
18. Vaccu-Pette/96 multiwell pipette.
19. Corning Transtar liquid handling system.
20. Corning Transtar liquid handling system adjustable volume pipettor.
21. Corning Transtar liquid handling system disposable cartridges.
22. 96-multiwell RIA Costar plate w/o lid cat: 3369-Fisher.
23. Fisherbrand Regenerated Cellulose Dialysis Tubing.
24. 0.45-micron sterile filter membrane.
25. Novex 4–20% Tris Glycine Gel 1 mm 12 well (Invitrogen).

2.6 Equipment

1. Microscopes (inverted, phase contrast).
2. Centrifuges.
3. Water bath (37 °C).
4. Freezers (−20, −80 °C).
5. Tissue culture incubators at 37 °C with 5% CO_2.
6. Tissue culture flow hood (BSL2).
7. Spectrophotometer (plate reader).
8. Nanodrop spectrophotometer.
9. Liquid nitrogen tanks.

2.7 IACUC Protocols Approved for

1. Generation of antihuman molecules monoclonal antibodies.
2. Generation of antimouse molecules monoclonal antibodies.
3. Rat protocol for rat spleen condition medium preparation.

3 Methods

All of the following procedures have to be performed in a class 2 biosafety cabinet (BSC) under sterile aseptic techniques unless otherwise specified. All items should be cleaned with ethanol before being placed inside the BSC for processing. Use adequate personal protective equipment (PPE).

3.1 Antigen Preparation

The following protocol provides antigen (Ag) preparation of (*see* **Note**):

1. Peptide/protein Ag emulsification: only one foot per mice will be injected. Prepare doses according to the number of mice. Recommended: three mice per project.

2. L-cells expressing the target (Ag) of interest: 2 feet per mice will be injected. Prepare doses according to the number of mice. Recommended: three mice per project.
3. Synthetic peptides, short chain of minimum 20 amino acids, part of the protein against which is planned to generate the antibodies, are mainly used for IHC or WB applications, although it can be used for therapeutic antibodies generation as well [17]. Commonly used for immunization, peptides have to be conjugated to Keyhole limpet hemocyanin (KLH) which is a large immunogenic protein, very antigenic to the mammalian immune system, derived from the hemolymph of the mollusk *Megathura crenulate*, essential to trigger immune response against short peptides [28]. In addition, peptide without KLH conjugation is needed, to be used during the boosts and as the main target during the hybridoma screening. There are companies that generate the custom reagent: require good solubility, trifluoroacetate as a counter ion, and 95% purity. Require the provider to add a cysteine in the C-terminal or N-terminal for the KLH conjugation. MBS method is suggested (KLH conjugation on cysteine); therefore, the cysteine residues inside the sequence have to be protected.

Recombinant proteins, whether they are commercially available, are other source of antigen for immunization.

1. The peptide or protein (Ag) doses are emulsified using incomplete Freund Adjuvant (IFA; InvivoGen).
2. Equal volumes of peptide/protein and IFA are mixed vigorously for several minutes, using a 28-G insulin syringe, until a white emulsion with maximum stability is formed.
3. Each emulsified 20 μL dose contained 10 μg of peptide-KLH or recombinant protein.
4. On the additional boost injections, the dose of the peptide will be raised to 15 μg.
5. If necessary, and upon results on serum titration of the immunized mice, some of these last boosts could be performed with the peptide alone to enrich the immune response against this molecule.
   (a) Immunization using stable L-cells expressing the Ag on the cell surface. First, expansion of mock and (Ag)-expressing cells in tissue culture is performed to have enough cells for immunization and preparation of twenty 96-multiwell ELISA plates for cell-ELISA-based screening. Cell preparation is following described:

      Briefly rinse L-Cells (Ag-expressing) with PBS to remove all traces of serum.

Add 5 mL of EDTA 1 mM in PBS solution to T75 flask and incubate at 37 °C for 3 min or until cells are detached.

Add 10 mL of PBS and centrifuge at 222 *g* for 3 min.

Resuspend cells with 10 mL of PBS. Wash one more time with PBS.

Next, resuspend cells in 20 mL of PBS, and adjust cell density to 5 10^6 per 50 μL of PBS for one foot-pad injections. Prepare enough for 2–4 mice.

As an example, for 4 mice transfer the cells into a 50-mL tube, spin and add 150 μL of PBS, and adjust to 200 μl.

To load the zero dead volume syringes, the plunger is removed and 100 μl is added to each syringe, remove any air bubble and push the cells to the top of the needle ready for injection.

3.2 Immunization Route and Schedule

All animal experiments need to be approved by the Institutional Animal Care and Use Committee (IACUC). Animals should be monitoring according to the protocol and veterinary recommendations. Female mice are typically preferred over male mice for immunizations, due to more aggressive behavior that could compromise the animals in the cage, and are purchased from any of the commercial sources, such as Jackson Laboratories (Bar Harbor, ME), Charles River (https://www.criver.com/about-us/about-us-overview/locations?region=3601), etc. All the companies offer good quality animals, with a similar immunogenic response compared side by side. We are describing two routes of immunization: foot-pad (fp) and subcutaneous (sc).

1. Immunization is usually performed on regular BALB/c, KO, or special strain NZBWF1/J mice [29] (*see* **Note 2**), this special strain is used, when a high homology between the human target and mouse ortholog could impair the humoral response from the immunized mice and the KO is not available.
2. Before immunization, 50 μL of blood samples are collected from the lateral tail vein. The puncture is performed with a 28-G insulin needle, while mice are restrained.
3. After coagulation, serum is separated and an equal volume of glycerol is added to preserve serum samples at −20 °C.
4. A total of three serum samples are collected: on day 0 (preimmunization), day 14 (after five injections), and day 31 after completing the schedule of immunization (Fig. 1). These serum samples will be used to determine the antibody titer (immune response) during and after immunization procedure.

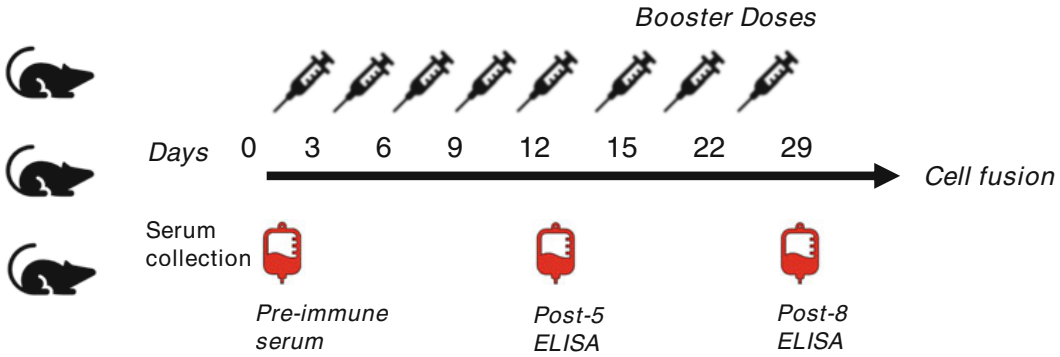

Fig. 1 Immunization schedule. Three mice are immunized every 3 days for the first five doses and then weekly for the three boost doses. Blood samples were drawn at indicated times, following the Institutional IACUC protocol

5. Three-four 6- to 8-week-old female Balb/c mice are immunized. On day 0, 50 μL pre-immune serum sample is collected from each mouse and a series of five injections are given through foot-pad route at 2-day intervals (day 0, 3, 6, 9, and 12). One to three additional weekly injections are given to boost the immune response (day 15, 22, and 29) to complete a total of maximum 8 injections, depending on serum titration results.
6. Subcutaneous immunization: a series of three injections are given through subcutaneous route every other week (day 0, 14, 28). 100 μl of the antigen, prepared as previously described, is injected subcutaneously in the dorsal left or right side of the mice. Additional injection is given to boost the immune response on day 35 completing a total of four injections. Depending on serum titration results, more boosts can be administered.

3.3 Euthanasia and Harvesting the Popliteal Lymph Nodes or Spleen

1. Following IACUC approved protocols, mice are exposed to carbon dioxide (CO_2) in the CO_2 chamber, at a flow rate of 2 L/min for 6 min.
2. Cervical dislocation is performed as a secondary euthanasia method.
3. After euthanasia, the mice are whipped with ethanol 70%, transferred to the tissue culture hood and pinned into a dissection board. Using scissors, a midline incision is made, and the fur is removed. The popliteal lymph nodes are located at the popliteal fossa and embedded in adipose tissue and are carefully excised to preserve the node structure and integrity.

Note: Only the popliteal lymph nodes draining the injected foot-pad/s are collected and placed in a petri dish with RPMI-1640 medium.

3.4 Hybridoma Generation

1. The two mice with the highest serum titers are selected for fusion while the third mouse is reserved as a backup. The following materials and media are used:
2. HAT supplement (50) [Sigma]: the mixture of hypoxanthine (5 mM), aminopterin (20 μM), and thymidine (0.8 mM) was used to prepare the HAT selection medium against unfused or self-fused SP2/0 myeloma cells. As a folic acid inhibitor, aminopterin blocks the de novo pathway for nucleoside synthesis in the SP2/0 myeloma cells, which lack the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) enzyme and, hence, lose the ability to utilize nucleotides provided by the salvage pathway.
3. RPMI-1640 medium [Sigma]: used to prepare the selection medium and washing cells.
4. Polyethylene glycol (PEG 1450): used as the fusing agent in the hybridoma technique. On the day of fusion, 1 mL vial of PEG was maintained at 37 °C until the fusion step.
5. Fetal bovine serum (FBS).
6. The selection media consists of RPMI-1640 with 10% FBS, 2% HAT supplement 50 , and 3% rat serum conditioned media (RSCM) (commercially available or in-home made).
7. Rat Supplement Condition Medium Preparation (RSCM), used as a growth supplement for the hybridomas.
   (a) Prepare 10 mL of Concanavalin A (1 mg/mL).
   (b) Euthanize rat in CO_2 chamber and aseptically harvest the spleen.
   (c) Transfer the spleen into a sterile 10-cm diameter petri dish filled with 10 mL of RPMI medium.
   (d) Gently dissociate spleen into single-cell suspension by applying a glass pestle against the fine-mesh metal screen.
   (e) Remove debris and disperse cells further by passage them through the fine-mesh metal screen.
   (f) Transfer spleen cells suspension into a sterile 50-mL conical centrifuge tube.
   (g) Centrifuge for 5 min at 300 *g* at room temperature, then discard supernatant.
   (h) Wash twice with RPMI medium.
   (i) Culture cells in RPMI medium containing 10% FBS, 1 mM sodium pyruvate, 1 Penicillin/Streptomycin, and 10 mL Concanavalin A.
   (j) Incubate at 37 °C for 48 h.
   (k) Harvest supernatant.

(l) Spin down at 670 *g* for 5 min.

(m) Filter supernatant with 0.2-μm filter.

(n) Store at −20 °C.

8. The following cells are used during the hybridoma generation experiment:

(a) SP2/0: The myeloma cell line SP2/0 is selected as a partner cell line for hybridoma generation. One week before fusion, SP2/0 murine myeloma cells are thawed, maintained in RPMI-1640 and 10% FBS and expanded to 1 10^8 cells in petri dishes. On the day of fusion, cells are collected in 50-mL tubes, centrifuged, and washed twice with RPMI medium before the fusion process.

(b) Murine lymphocytes from lymph nodes (LN): These cells are collected from the excised popliteal lymph nodes. These lymph nodes are dissected with scissors and forced through a sterile stainless-steel strainer, to collect the cells in RPMI medium. The collected lymphocytes are centrifuged (210 *g* for 3 min) and washed twice with RPMI medium before the fusion process.

(c) Splenocytes are collected following similar procedure used to prepare RSCM: Transfer aseptically harvested spleen into a sterile 10-cm diameter petri dish filled with 10 mL RPMI medium. Gently dissociate spleen into single-cell suspension by applying a glass pestle against the fine-mesh metal screen. Remove debris and disperse cells further by passage them through the fine-mesh metal screen. Transfer spleen cells suspension into a sterile 50-mL conical centrifuge tube. Centrifuge for 5 min at 300 *g* at room temperature. Discard the supernatant. Lyse red blood cells by suspending the pellet in 10 mL ammonium chloride solution (0.83% NH_4HCL, pH 7.4). Incubate for 10 min at room temperature. Add 20 mL of RPMI medium to stop lysis reaction, and centrifuge for 5 min at 300 *g* (2 times).

(d) Both SP2/0 and murine lymphocytes (from LN or spleen) are counted to assess viability using cell counting hemocytometer and Trypan Blue.

(e) Cells are combined in a ratio of 1:2 (Sp2/0 to lymphocytes) in a 50-mL tube, and centrifuged at 210 *g* for 5 min to pack the cells together and prepare them for the fusion.

(f) After discarding the supernatant and loosening the cell pellet, 1 mL prewarmed (37 °C) PEG is added to the tube while swirling for 45 s.

(g) The fusion reaction is stopped by adding 24 mL prewarmed (37 °C) RPMI medium.

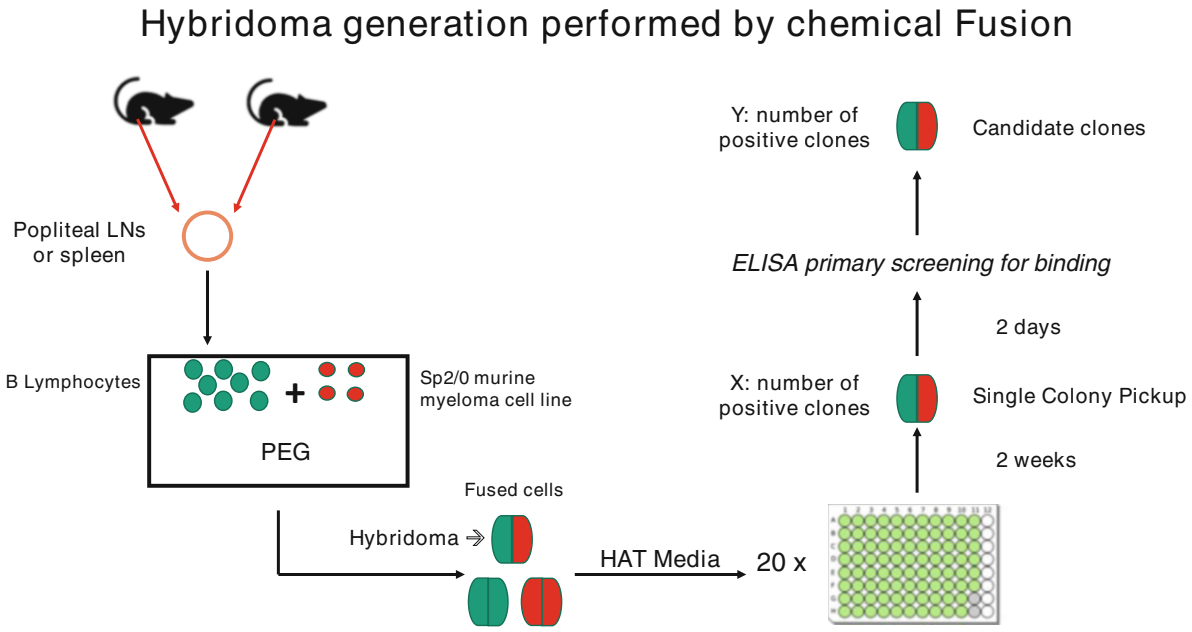

Fig. 2 Hybridoma generation by chemical fusion. Two immunized mice with highest serum titers are selected for fusion. Popliteal LNs or spleen) are excised and murine lymphocytes are collected and combined with SP2/0 cells as the fusion partner. After fusion with PEG, hybridomas are selected with HAT media and positive clones, specific for the target, are chosen with an ELISA screening. Additional subcloning guarantee clones stabilization of MAb candidates

(h) Fused cells are centrifuged at 210 *g* for 5 min, supernatant is discarded, and the cell pellet is loosened by gentle tapping and transferred to the selection medium.

(i) The hybridoma cell suspension is distributed into twenty 96 tissue culture multiwell plates (150 μl/well) and incubated at 37 °C, 5% CO_2. The fusion process is represented in Fig. 2.

3.5 Hybridomas Colony Pickup

1. Seven days after fusion, ELISA is performed to detect positive wells containing hybridoma colonies.
2. Upon results, the procedure of "single colonies pickup" (SCP) is performed in two phases at 1-week interval.
3. Well-demarcated colonies are identified, collected with 2-μL pipette and transferred into 96-multiwell plate containing hypoxanthine and thymidine (HT)—selection media.
4. These colonies are incubated at 37 °C and 5% CO_2.

At the MAF, we count with a ClonePix 2 Mammalian Colony Picker robot (Molecular Devices) (*see* **Note 4**) that automates part of the generation of MAbs, accelerating discovery by selection of positive clones, instead of using manual procedures. Briefly, the hybridomas generated upon the chemical fusion are cultured on plates without well divisions, in semisolid HAT media containing

methyl cellulose (ClonaCell Hy Medium D| Hybridoma Selection & Cloning| STEMCELL Technologies) available from different vendors. The media contains a fluorescent antimouse secondary antibody that allows the robot to select proper colonies based on size and fluorescent intensity, instructed by the operator, following manufacturer recommendations and acquired expertise. Since this equipment is quite sophisticate for small laboratories with the ultimate goal of generating their own MAbs, our single-colony pickup (SCP) technique, described under Subheading 3.5, renders equal quality of isolation, when the high-throughput MAb generation is not a priority. The ELISA screening in the case of automated collection of clones is performed 7 days after the colony isolation.

3.6 ELISA Screening

1. To select the hybridomas capable of producing specific monoclonal antibodies against the antigen, ELISA is performed (*see* **Notes 2**, **5** and **6**).
2. Preparation of peptide and recombinant protein ELISA plates: Plates are coated with a 100 μL coating buffer, containing the recombinant protein or peptide forms with/without KLH in a concentration of 1 μg/mL, and kept overnight at 4 °C.
3. Preparation of Cell-ELISA plates: To prepare cell coated multiwell plates, expansion of L-cells expressing the antigen (or mock control) in T150 tissue culture flasks is needed.
   (a) For each plate, 15 10^6 cells are required. Detach cells using 1 mM EDTA in PBS and wash twice with sterile PBS.
   (b) Finally resuspend cells with PBS containing 0.01% of $MgCl_2$ and 0.01% of $CaCl_2$. 40 μL of cells are distributed in each well (96-well plate RIA).
   (c) The cells are dried overnight at room temperature in the tissue culture hood, under sterile conditions.
   (d) Next day, the plates are stored at −20 °C.
   (e) The day of the Cell-ELISA test, plates are placed 1 h at room temperature and are immediately rehydrated by adding 200 μL of PBS for 10 min at room temperature.

The steps showed below are shared in protein/peptide and Cell-ELISA.

1. The ELISA plates are washed three times with PBST and blocked with 100 μL of ELISA blocking buffer.
2. After 1-h blocking at room temperature, 50 μL supernatant from each colony is added to individual wells as the primary antibodies.

3. Blank wells with secondary antibody only are performed in triplicates. Incubation proceeds for 1 h followed by three washes.
4. 50 μL of secondary antibody, goat IgG antimouse, HRP conjugated (1:2000) is added to each well and incubated for 1 h.
5. After washing four times, the substrate solution (TMB) is added to each well and incubated for 30 min.
6. The reaction is stopped with H_2SO_4 0.2 N and the OD absorbance values are read at 450 nm.

3.7 Selection and Confirmation of Hybridoma Clones

1. The positive hybridomas are transferred to 24 MW plates containing HT medium, and incubate for 4–7 days at 37 °C, 5% CO_2.
2. Next, 2 mL supernatant is collected from each clone for further characterization of the monoclonal antibodies.
3. For ELISA dilution assay, ELISA plates are prepared as previously described. Here, supernatants from hybridoma clones are used as primary antibodies (undiluted, 1/15, 1/225, and 1/3375).
4. The clones with the highest OD 450 nm signal are selected to proceed to the next screening assays.
5. Immediately after, the candidate hybridomas are identified, the clones are checked under the microscope to detect viable clones and marked to facilitate identification to perform the second ELISA screening.
6. Further screening assays could include immunohistochemistry (IHC), Western blot (WB), flow cytometry (FACS), and potential blocking or agonist functions (*see* **Notes 7** and **8**).

3.8 Hybridoma Subcloning

1. The hybridoma clones are expanded in 24-well plates with HT-based media for 3 days.
2. With a 70% confluency and viability of more than 90%, cells are counted (using hemocytometer), diluted with HT-based media to a concentration of 5 cells/mL, and lastly added 200 μL/well to a 96-well tissue culture plate, using a multichannel pipet.
3. The hybridomas are cultured for 7–10 days at 37 °C, 5% CO_2.
4. An ELISA screening is performed as described, to identify single high antibody producer clones.
5. The clones showing absorbance with the highest OD signal are maintained and transferred into a 24-well plate.
6. Two days later, HT-based medium is substituted with RPMI-FBS 10% and the clones are transferred to T25 tissue culture flask.
7. Further expansion into T75 is performed for the purpose of monoclonal antibody purification.

3.9 Purification and Concentration of the Monoclonal Antibodies

1. After expansion and incubation of the selected hybridomas, 300 mL of the MAb-rich supernatants is collected, centrifuged, and filtered through 0.45-micron membrane.
2. MAbs are purified using Protein A affinity chromatography method.
3. After purification, the columns are washed, and antibody supernatant is neutralized with 1 M Tris–HCl (pH 9) buffer.
4. The captured antibodies are eluted from the columns, using elution buffer (0.1 M glycine–HCl, pH 3) and the concentration are measured using the Nanodrop.
5. Next, overnight dialysis in PBS using the recommended dialysis tubing is performed to concentrate and preserve the antibody activity in a neutral buffer.
6. The concentrated antibody is collected and stored at 4 °C, ready for further characterization and cell assays.
7. Typically, in our laboratory, we do not add preservatives to the purified antibody, because the commonly used preservatives interfere with functional assays, for instance, if the planned application is to detect an antibody with a blocking or agonist activity.

3.10 Antibody Isotyping

To determine the isotype of monoclonal antibodies is essential for applications in different research areas, including functional experiments using immune cells (i.e., ADCC functional mechanism), antibody internalization, and antibody drug conjugates (ADC) development.

It is recommended to use a commercial kit, such as Pierce Rapid ELISA Mouse mAb Isotyping Kit (Catalog No. 37503).

This assay uses ELISA strip-well plates with individual wells precoated with antimouse heavy-chain capture antibody (anti-IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM) or antimouse light-chain capture antibody (kappa or lambda). This approach eliminates the need to purify and immobilize an antigen for isotyping. A mouse monoclonal antibody sample applied to the wells can be isotyped within 1 h.

1. First, equilibrate TMB substrate and plate eight-well strips to room temperature. During this stage, the hybridoma supernatants are diluted 1:10 in TBS or purified antibodies are prepared at a concentration of 0.1 μg/mL in TBS.
2. Then, add 50 μL of diluted antibody sample to each well of the eight-well strip.
3. Next, add 50 μL Goat Anti-Mouse IgG + IgA + IgM HRP conjugate to each well of the eight-well strip.
4. Mix by gently pipetting and cover plate and incubate for 1 h at room temperature.

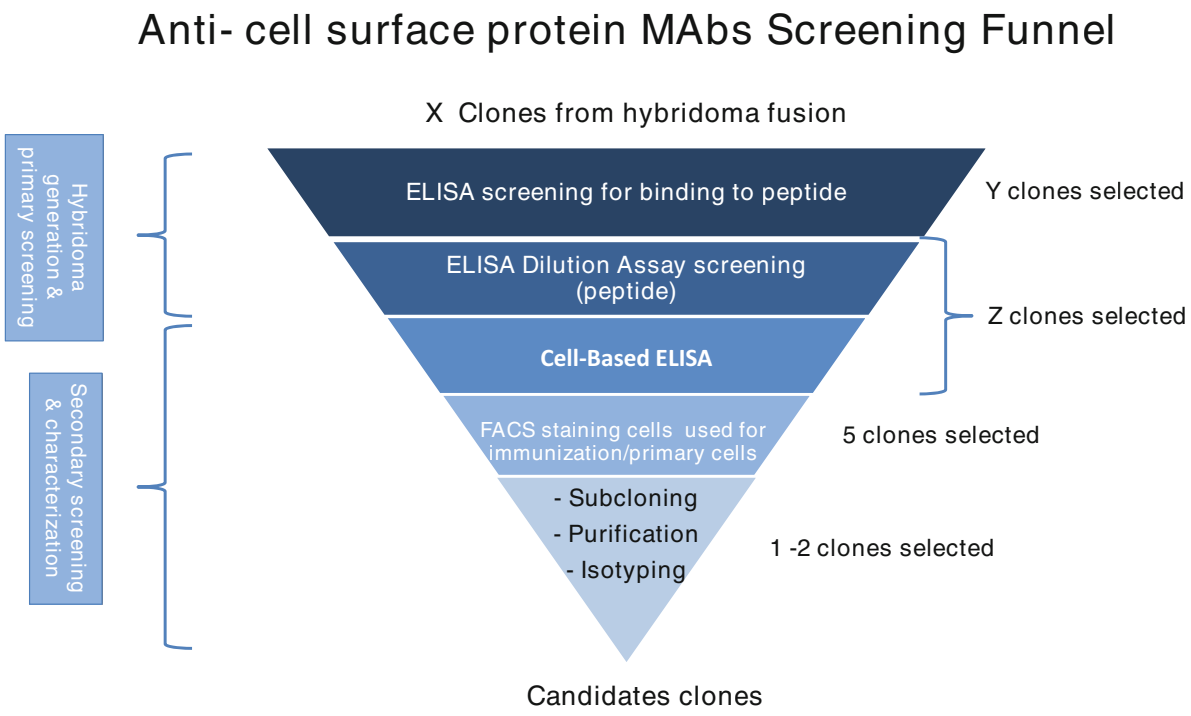

Fig. 3 Anti-cell surface protein MAb Screening Funnel. The diagram shows the various screening steps performed after the generation of the hybridomas, up to the selection of the monoclonal antibody candidate/s

5. Wash three times with Wash Buffer (250 µL/well), and tab the plate dry.
6. Add 75 µL of TMB substrate to each well. A blue positive response is visible after 1 min. Signal development and intensity vary depending on antibody concentration and isotype.
7. After 5–15 min, add 75 µL of Stop Solution.
8. Measured the plate with a spectrophotometer at 450 nm.
9. Place the plate over the template and mark location of positive response.
10. Each sample should have a positive response in one of the rows A-F (heavy-chain identification) and a positive in either row G or H (light-chain identification).

Note: Wells with the highest response (darkest color) indicate isotype and light-chain composition; lightly colored wells indicate contaminating host or myeloma antibodies.

All the screening steps from 3.6 to 3.10 are shown in Fig. 3.

3.11 Antibody Characterization by SDS PAGE

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) is used to characterize the purity and aggregates of the heavy and light chain of the monoclonal antibodies, based on their molecular weight and their electrophoretic mobility differences. Here, it is used premade Novex–Wedge well 4–20% Tris–

glycine gel 1 mm 12 well (Invitrogen), and prepare the samples as follow:

1. Prepare a prestained protein molecular weight marker to run with the samples (5–10 μL/lane).
2. Mix 3 μg of antibody in 30 μL of loading buffer (Tris–glycine SDS sample buffer—2) with a reducing agent (beta-mercaptoethanol, 2.5% final concentration).
3. Next, denature at 95 °C for 5 min.
4. Load the samples into the gel wells, then cover the top and connect the electrodes.
5. Set at 200 V and run the electrophoresis, approximately 1 h.
6. Remove the gel from the plastic plates and wash with double-distilled water.
7. Incubate with EZ Blue Gel staining reagent (G1041-Sigma) for 90 min in a shaker.
8. Next, rinse and verify the contrast with water.
9. Stop washing when staining contrast is complete.
10. Heavy and light chains, at an apparent MW of 50 kD and 25 kD, respectively, will be clearly observed compared with the MW marker lane.
11. Running a gel under nonreducing conditions will show single bands of approximately 150 kD MW.

4 Notes

1. The selection and construction of the antigens (peptides, proteins, or cells expressing the target protein) are critical for the generation of good quality antibodies. For instance, if the desired application is a Western blot (WB) method, both peptides or proteins can be used for immunization. Denature process in the WB will linearize the molecules, and, if peptides are used as immunogens, the target can be detected by the antibodies [30]. Importantly, since the subsequent selection and characterization after the ELISA screening will be performed using Mab-rich supernatants and not yet purified antibodies, the WB should be tested against the purified molecule used for immunization and not cell or tissues lysate. Moreover, if your tissues are of mice origin, using secondary antibodies antimouse immunoglobulins will show high background. Therefore, the WB against murine tissues should be performed only with purified antibodies conjugated to biotin to develop the WB with streptavidin-HRP.

2. Interestingly, Dr. Milstein, who trained Dr. Bover personally, used to emphasize that the best MAbs can be selected with the proper screening, and different traditional or innovative assays should be used for this purpose. Thus, MAbs designed for imaging, immunohistochemistry, diagnostic assays, crystallography, and preclinical or future therapeutic use can be developed.
3. When the selected target is a human molecule sharing high homology (95%) with the murine ortholog, chances of getting a significant immune response from the regular mice are low. Alternatives to the ones mentioned in this chapter (use of KO mice or NZB strain) are to develop monoclonal antibodies using other species (hamsters, rat, rabbits) that should exhibit lower homology with the human target or phage/yeast display libraries. Same consideration applies to development of anti-murine antibodies. In such case, regular mice cannot be used.
4. For the process of single clone selection, the MAF counts with a ClonePix 2 Mammalian Colony Picker robot (Molecular Devices) [31] that automates part of the generation of MAbs, accelerating discovery by selection of positive clones, instead of using manual procedures. Briefly, the hybridomas generated upon the chemical fusion are cultured on plates without wells divisions, in semisolid HAT media containing methyl cellulose (ClonaCell Hy Medium D| Hybridoma Selection & Cloning| STEMCELL Technologies) available from different vendors. The media contains a fluorescent antimouse secondary antibody that allows the robot to select proper colonies based on size and fluorescent intensity, instructed by the operator, following manufacturer recommendations and acquired expertise. Since this equipment is quite sophisticated for small laboratories with the ultimate goal of generating their own MAbs, our single-colony pick-up (SCP) technique, described under Subheading 3.5, renders equal quality of isolation, when the high-throughput MAb generation is not a priority. The ELISA screening in the case of automated collection of clones is performed 7 days after the colony isolation.
5. In the process of characterization of the MAbs, flow cytometry assay is one of the preferred methods to confirm the specificity, in particular for those antibodies generated against cell surface molecules and/or designed for therapeutic use. The following analysis can be performed with the newly generated MAbs: (a) the murine fibroblast cells used for immunization (parental, mock, and cells expressing the antigen) can be stained with the primary antibodies generated plus a secondary antimouse IgG or antitotal murine immunoglobulins—fluorochrome conjugated; (b) performing competition assay staining cells

expressing the protein using MAbs directly conjugated with a fluorochrome and preincubated with the purified target molecule, peptide or protein, or an irrelevant molecule at increasing concentrations, will aid the confirmation of the specificity. The protocols for FACS staining are published elsewhere [25, 27] and are essential in the characterization. Importantly, cells have to be detached using trypsin-free dissociating agents, to preserve epitopes that could be otherwise destroyed.

6. MAbs designed for immunohistochemistry applications could be screened by histological methods on paraffin blocks (FFPE) prepared with cell pellets of the cells used for immunization.
7. Quality control tests to ensure purity (PAGE) and specificity should be performed in accordance with Rigor and Reproducibility requirements by NIH (https://www.nih.gov/research-training/rigor-reproducibility).
8. OCTET platform for pharmacokinetic and competition determinations can be utilized for further characterization and selection of the best master hybridoma clones. Using Octet RED384 System, the kinetics of the generated MAb can be tested by measuring the interaction between the antibody and its target. Developed by FORTEBIO and based on Bio-Layer Interferometry (BLI) technology, the system can measure PK (affinity, avidity, ka and kd association and dissociation constant, respectively) and calculate the affinity constant (KD) of an antibody with a range of 1 mM to 5 pM, using the Data Acquisition software [31]. The MAb is immobilized using antimouse IgG Fc capture biosensors (AMC) and interact with its antigen added in soluble format. Biacore system is an alternative platform to generate these data of great importance at the time of moving forward to in vitro/ex vivo or preclinical development.

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