Video Article

Generation of Discriminative Human Monoclonal Antibodies from Rare Antigen-specific B Cells Circulating in Blood

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Abstract

Monoclonal antibodies (mAbs) are powerful tools useful for both fundamental research and in biomedicine. Their high specificity is indispensable when the antibody needs to distinguish between highly related structures (*e.g.*, a normal protein and a mutated version thereof). The current way of generating such discriminative mAbs involves extensive screening of multiple Ab-producing B cells, which is both costly and time consuming. We propose here a rapid and cost-effective method for the generation of discriminative, fully human mAbs starting from human blood circulating B lymphocytes. The originality of this strategy is due to the selection of specific antigen binding B cells combined with the counter-selection of all other cells, using readily available Peripheral Blood Mononuclear Cells (PBMC). Once specific B cells are isolated, cDNA (complementary deoxyribonucleic acid) sequences coding for the corresponding mAb are obtained using single cell Reverse Transcription-Polymerase Chain Reaction (RT-PCR) technology and subsequently expressed in human cells. Within as little as 1 month, it is possible to produce milligrams of highly discriminative human mAbs directed against virtually any desired antigen naturally detected by the B cell repertoire.

Video Link

The video component of this article can be found at https://www.jove.com/video/56508/

Introduction

The method described here allows the rapid and versatile production of fully human monoclonal antibodies (mAbs) against a desired antigen (Ag). mAbs are essential tools in many fundamental research applications *in vitro* and *in vivo*: flow cytometry, histology, western-blotting, and blocking experiments for example. Furthermore, mAbs are being used more and more in medicine to treat autoimmune diseases, cancer, and to control transplantation rejection[1]. For example, anti-CTLA-4 and anti-PD-1 (or anti-PD-L1) mAbs were recently used as immune checkpoint inhibitors in cancer treatments[2].

The first mAbs were produced by immunoglobulin (Ig)-secreting hybridomas obtained from the splenic cells of immunized mice or rats. However, the strong immune response against murine or rat mAbs hampers their therapeutic use in humans, due to their rapid clearance and the probable induction of hypersensitivity reactions[3]. To tackle this problem, animal protein sequences of mAbs have been partially replaced by human ones to generate so-called chimeric mouse-human or humanized antibodies. However, this strategy only partially decreases immunogenicity, while substantially increasing both the cost and the time-scale of production. A better solution is to generate human mAbs directly from human B cells and several strategies for this are available. One of them is the use of phage or yeast display. This involves displaying variable domains from a combinatorial library of random human Ig heavy and light chains on phages or yeasts, and carrying out a selection step using the specific antigen of interest. A major drawback of this strategy is that heavy and light chains are randomly associated, leading to a very large increase in the diversity of generated antibodies. Antibodies obtained are unlikely to correspond to those that would arise from a natural immune response against a particular Ag. Moreover, human protein folding and post-translational modifications are not systematically reproduced in prokaryotes or even in yeasts. A second human mAb production method is the immortalization of natural human B cells, by Epstein-Barr virus infection or expression of the anti-apoptotic factors BCL-6 and BCL-XL[4]. However, this method is applicable only to memory B cells and is inefficient, requiring screening of numerous mAb-producing immortalized B cells to identify the few (if any) mAb clones with the desired antigenic specificity. The method is thus both costly and time consuming.

A new protocol has recently been described for production of human mAbs from isolated single B cells[5]. It relies on an optimized single-cell Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for amplification of both the heavy- and light-chain encoding segments from a single sorted B cell. This is followed by the cloning and expression of these segments in a eukaryotic expression system, thus allowing reconstruction of a fully human mAb. This protocol has been used successfully starting from B cells from vaccinated donors. Cells were harvested several weeks after vaccination to obtain higher frequencies of B cells directed against the desired Ag, and thus limit the time required for screening[6]. Other fully human mAbs have also been produced from HIV^+ (Human Immunodeficiency Virus) infected patients[7] and melanoma

patients[8]. Despite these advances, there is still no procedure available that enables the isolation of Ag-specific B cells independent of their memory phenotype or frequency.

The procedure described here leads to efficient *ex vivo* isolation of human circulating B cells based on their BCR specificity, followed by the production of fully human antigen-specific mAbs in high yield and with a low screening time. The method is not restricted to memory B cells or antibody-secreting B cells induced after an immune response, but can also be applied to the human naïve B cell repertoire. That it works even starting from Ag-specific B cells present at very low frequencies is a good indication of its efficiency. The principle of the method is as follows: Peripheral Blood Mononuclear Cells (PBMC) are stained with two tetramers presenting the antigen of interest, each labeled with a different fluorochrome (*e.g.*, Phycoerythrin (PE) and Allophycocyanin (APC)), and a third tetramer presenting a closely related antigen conjugated with a third fluorochrome (*e.g.*, Brillant Violet 421 (BV421)). To enrich for antigen-binding cells, cells are then incubated with beads coated with anti-PE and anti-APC Abs, and sorted in cell separation columns. The PE^+ APC^+ cell fraction is selected, stained with a variety of mAbs specific for different PBMC cell types to permit identification of B cells, and subjected to flow cytometry cell sorting. B cells which are PE^+ and APC^+, but $Brilliant\ Violet^-$, are isolated. This step counter-selects cells which are not B cells or do not bind to the tetramerized antigen, but do bind to either PE or APC (these cells will be PE^+ APC^- or PE^- APC^+) or to the non-antigen part of the tetramers used (these cells will be $BV421^+$). B cells not highly specific for the epitope of interest are also counter-selected at this step (these cells will also be $BV421^+$). Thus, this method can purify highly specific B cells expressing B-cell Receptors (BCRs) able to discriminate between two very closely related antigens. Single specific B cells are collected in tubes and their PCR-amplified Ig cDNAs (complementary deoxyribonucleic acids) cloned and expressed by a human cell line as secreted IgG mAbs.

As a proof of concept, this study describes the efficient generation of human mAbs, which recognize a peptide presented by a major histocompatibility complex class I (MHC-I) molecule and can discriminate between this peptide and other peptides loaded on the same MHC-I allele. Although the level of complexity of this Ag is important, this method allows (i) high yield recovery of Ag-specific mAbs; (ii) production of mAbs able to discriminate between two structurally close Ags. This approach can be extended to vaccinated or infected patients without any protocol modification, and has also already been successfully implemented in a humanized rat system[9]. Thus, this study describes a versatile and efficient approach to generate fully human mAbs that can be used in basic research and immunotherapy.

Protocol

All human peripheral blood samples were obtained from anonymous adult donors after informed consent, in accordance with the local ethics committee (Etablissement Français du Sang, EFS, Nantes, procedure PLER NTS-2016-08).

1. Isolation of Human Peripheral Blood Mononuclear Cells

NOTE: Starting material can be total human peripheral blood or cytapheresis samples. Samples should not be older than 8 h and supplemented with anticoagulants (*e.g.*, heparin).

1. Dilute blood with 2 volumes (human peripheral blood) or 5 volumes (cytapheresis sample) of RPMI (Roswell Park Memorial Institute) medium.
2. Carefully layer 35 mL of diluted cell suspension onto 15 mL of density gradient medium in a 50-mL conical tube. Make as many tubes as necessary to distribute all the diluted blood. Centrifuge at 1,290 x g for 25 min at room temperature in a swinging-bucket rotor with brake off.
3. Carefully aspirate the mononuclear cell layer at the interface between the density gradient medium and the plasma layer, and transfer the cells to a new 50 mL conical tube.
4. Fill the tube with RPMI medium, mix and centrifuge at 1,290 x g for 10 min at room temperature. Carefully remove the supernatant.
5. Resuspend the cell pellet in 20 mL of RPMI medium and centrifuge at 200 x g for 10 min to remove the platelets. Carefully remove the supernatant. Repeat this step once.
6. Resuspend the cell pellet in RPMI medium with 10% Fetal Bovine Serum (FBS).
   NOTE: Proceed directly to tetramer labeling or keep cells at 4 °C overnight at a concentration of 10^7 cells/mL.

2. Tetramer-associated Magnetic Enrichment of Ag-specific B Cells

1. Prepare Ag-tetramers by adding either PE or APC-labeled premium grade streptavidin or BV421-labeled streptavidin at a molar ratio of 1:4 to biotinylated antigen monomers. Add the appropriate amount of streptavidin-conjugate in three separate portions, adding one aliquot every 10 min at room temperature.
2. Distribute cells (up to 3 x 10^8 per tube) in 15 mL conical tubes and centrifuge at 460 x g for 5 min.
   NOTE: The following protocol is for one tube.
3. Resuspend the cell pellet in 200 µL of PBS (Phosphate Buffered Saline) with 2% FBS. Add PE-, APC-, and BV421-conjugated tetramers, each to a final concentration of 10 µg/mL. Mix well and incubate for 30 min at room temperature.
4. Prepare ice-cold cell separation buffer (SB) using PBS with 0.5% BSA (Bovine Serum Albumin) and EDTA (Ethylenediaminetetraacetic acid) 2 mM.
5. Add 10 mL of SB. Pellet cells by centrifugation at 460 x g for 5 min.
6. Resuspend cells in 500 µL of ice-cold SB. Add 50 µL of anti-PE and 50 µL of anti-APC magnetic microbeads.
7. Mix well and incubate for 20 min at 4 °C.
8. Repeat step 2.5 twice.
9. Eliminate the supernatant carefully andresuspend the cell pellet at a concentration of 2 x 10^8 cells/mL in ice cold SB.
10. Equilibrate a large magnetic column positioned on a magnet with 3 mL of SB.
11. Transfer cell suspension from 2.9 onto the top of the equilibrated column and allow it to drain completely. CRITICAL STEP: To avoid clumping of the column, pass cells through a 70 µm nylon mesh.

12. Rinse the tube which contained the cells with 3 mL of ice cold SB and transfer the buffer directly onto the column (in case of filtration, rinse the 70 µm nylon mesh).
13. When the buffer has completely drained into the column, add another 3 mL of ice cold SB to the column.
14. Repeat step 2.13 for another 3 mL wash.
15. Remove the column from the magnet and place over a 15-mL conical collecting tube.
16. Add 5 mL of ice cold SB onto the top of the column and immediately flush the cells out of the column using the plunger. Repeat this step once.
17. Centrifuge the collected cells (enriched relevant tetramer positive cell fraction) at 460 x g for 5 min and proceed to additional antibody staining.
    NOTE: Pool collected cells from all tubes, if appropriate.

3. Staining of Ag-specific Human B Cells and Cell Sorting

1. Resuspend the cell pellet with a cocktail of anti-human antibodies against CD3 (final dilution: 1:20), CD19 (1:20), CD14 (1:50), CD16 (1:50), and 7AAD (1:1000) in a final volume of 100 µL of PBS with 2% FBS. Incubate for 30 min at 4 °C.
2. Add 10 mL of PBS to cells, centrifuge at 460 x g for 5 min, and eliminate the supernatant. Repeat this step once. Resuspend the cell pellet in 200 µL of PBS, filter onto 70 µm nylon mesh.
3. Proceed to sort specific B cells at the single cell level on a cell sorter cytometer with a 100 µm-nozzle and a pressure of 20 psi without exceeding 1,000 events/s[9].
   NOTE: The gating strategy used is shown in **Figure 1**. Cells were first gated on $CD14^-CD16^-7AAD^-$ cells to exclude monocytes, NK, and dead cells (not shown in **Figure 1**). Then $CD19^+$ B cells were selected before gating on double stained cells by PE and APC relevant Ag-tetramers. Non-specific B cells were excluded by gating on BV421 negative cells (unstained by irrelevant Ag-tetramer).
4. Collect single B cells into 8-strip PCR tubes previously filled with 10 µL of 1x PBS and 10 units of RNAse Inhibitor and placed on a rack for 96 microtubes.Immediately freeze the PCR tubes at -80 °C.
   NOTE: The sort masks chosen on the cytometer instrument are yield mask: 0, purity mask: 32, and phase mask: 0. This single-cell deposit could be adjusted to a 96- or 384-well PCR plate if needed. Single B cells must be frozen as quickly as possible and can be left at -80 °C for several weeks, if needed.

4. Single Cell RT-PCR

1. Lyse cells by directly heating the frozen samples in PCR strips at 70 °C for 5 min in a dry bath.
2. Transfer strips to ice and proceed to reverse transcription (RT) by adding 10 µL per tube of a 2x master mix buffer containing 1 mM dNTP, 25 µg/mL oligod(T) primers, 5 µM random hexamers, 20 units of RNAse inhibitor, and 400 units of reverse transcriptase.
   NOTE: After cell thawing, RT must be performed quickly to avoid RNA degradation.
3. Incubate at 25 °C for 5 min to allow random hexamers to hybridize, then incubate for 1 h at 50 °C, and finish with an incubation at 95 °C for 3 min to stop the reaction.
4. Proceed with two rounds of nested PCR amplification for each of the regions encoding for variable heavy (vH) and kappa (vLκ) or lambda light chains (vLλ).
   NOTE: Alternatively, RT samples can be frozen at -20 °C and kept for one week.
   1. For the first round of PCR, add 3 µL of cDNA for a 40 µL final volume containing 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 2.5 units of DNA Polymerase, and 200 nM of outer primers (see **Figure 2** and **Table of Materials** for primer sequences).
      NOTE: Composition of outer primers mix. For heavy chain amplification: 4 forward primers (5'LVH mix) and 2 reverse primers (3'CµCγ mix). For light kappa chain amplification, 3 forward primers (5'LV| mix) and 1 reverse primer (3'Cκ543-566). For light lambda chain amplification, 7 forward primers (5'LVl mix) and 1 reverse primer (3'Cl).
      NOTE: Primers were designed by Tiller *et al.* for amplification of all the heavy and light chain family genes[5].
   2. Apply the following cycling conditions: 94 °C for 4 min, followed by 40 cycles of 30 s at 94 °C, 30 s at 58 °C for VH and VLκ (60 °C for VLλ), and 55 s at 72 °C, with a final elongation step at 72 °C for 7 min.
   3. For the second round PCR, use 3 µL of the first amplification product for a final volume of 40 µL containing 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 2.5 units of DNA polymerase, and 200 nM of inner primers containing restriction enzyme sites for cloning into expression vectors (see **Figure 2** and **Table of Materials** for primer sequences).
      NOTE: Composition of inner primers mix. For heavy chain amplification: 9 forward primers (5'AgeIVH mix) and 3 reverse primers (3'SalIJH mix). For light kappa chain amplification, 9 forward primers (5'AgeIV| mix) and 3 reverse primers (3'BsiWIJκ mix). For light lambda chain amplification, 6 forward primers (5'AgeIVl mix) and 1 reverse primer (3'XhoICl).
      NOTE: Primers were designed by Tiller *et al.*, for amplification of all of the heavy and light chain family genes[5].
   4. Apply the following cycling conditions: 94 °C for 4 min, followed by 40 cycles of 30 s at 94°C, 30 s at 58 °C for VH and LCκ (60 °C for LCλ), and 45 s at 72 °C with a final elongation step at 72 °C for 7 min.
5. Identify positive wells by migrating 5 µL of PCR products of vH, vLκ and vLλ on a 1.5% agarose gel (500 bp products for variable heavy chain encoding segments and a 350 bp product for variable light chain encoding segments).
6. Purify the remaining 35 µL PCR products remaining from positive wells using ultrafiltration membranes designed for purification of PCR products.
7. Elute PCR products with 60 µL of H_2O.

5. Expression Cloning

1. **Prepare Inserts**
   NOTE: Digest 60 µL of each purified PCR product in a final volume of 100 µL containing the appropriate restriction enzyme buffer.
   1. For vH PCR products, add 50 units of AgeI and 50 units of SalI, and incubate for 1 h at 37 °C.

2. For vLλ PCR products, add 50 units of AgeI and 50 units of XhoI, and incubate for 1 h at 37 °C.
3. For vLκ PCR products, add 50 units of AgeI and incubate for 1 h at 37 °C. Purify digests using 96 PCR membranes and elute with 60 µL of H_2O. Digest the 60 µL eluate in a final volume of 100 µL of BsiWI restriction enzyme buffer, add 50 units of BsiWI, and incubate for 1 h at 55 °C.
4. Inactivate the enzymes by heating at 80 °C for 15 min.

2. **Preparation of Expressing Vectors**
NOTE: vH PCR products are cloned in an expression vector (HCγ1) containing the constant heavy chain Cγ1 of IgG1. vLκ PCR products are cloned in an expression vector (LCκ) containing the constant light chain Cκ. vLλ PCR products are cloned in an expression vector (LCλ) containing the constant light chain Cλ.
    1. Perform the following digestions:
        1. Mix 1 µg of expression vector HCγ1 with 10 units of AgeI and 10 units of SalI endonucleases in a total volume of 20 µL of restriction buffer recommended by the manufacturer, and incubate for 1 h at 37 °C.
        2. Mix 1 µg of expression vector LCκ with 10 units of AgeI restriction enzyme in a total volume of 20 µL of restriction buffer recommended by the manufacturer, and incubate for 1 h at 37°C. Add 10 units of BsiWI restriction enzyme, and incubate for 1 h at 55 °C.
        3. Mix 1 µg of expression vector LCλ with 10 units of AgeI and 10 units of XhoI endonucleases in a total volume of 20 µL of restriction buffer recommended by the manufacturer, and incubate for 1 h at 37 °C
    2. Heat each digestion mixture at 65 °C for 10 min to inactivate the restriction enzymes.
    3. Perform ligation of 5 µL of digested PCR product with 2 µL (100 ng) of corresponding linearized expression vector in a total volume of 20 µL of 1x DNA ligase buffer with 1 unit of T4 DNA-Ligase by incubation overnight at 4 °C.
    NOTE: Alternatively, ligation is performed for 3 h at 4°C.

3. **Cloning**
    1. Electroporate 1 µL of the 20 µL ligation mixture into 45 µL of homemade electrocompetent TOP10 *E. coli* (Current Protocols in Molecular Biology, section 1.8.4-1.8.8).Immediately add 500 µL of 2X YT medium and incubate in a water bath at 37 °C for 30 min. Spread transformed bacteria on 2x YT ampicillin plates. Incubate overnight at 37 °C.
    2. For each transformation, screen eight colonies by PCR.
        1. Set up a reaction premix as follows on ice: 45 µL of 5x PCR Buffer, 12 µL of $MgCl_2$ (25 mM), 4 µL of dNTPs (from a stock containing 10 mM of each), 10 µL of forward primer (stock 10 µM) hybridizing to the vector sequence (primer Ab-vec-sense), and 10 µL of reverse primer (stock 10 µM) targeting the constant heavy or light chain region (see **Table of Materials** for primer sequences). Make up to a final volume of 200 µL with H_2O. Then add 4 µL of DNA polymerase enzyme (5 U/µL).
        2. Distribute 25 µL of reaction premix per 8-strip PCR tube on ice.
        3. Use a sterile toothpick to pick up individual colonies. Streak the toothpick onto a 2x TY ampicillin plate to constitute a replicate plate and then dip it into a PCR reaction tube.
        4. Perform the screening PCR under the following cycling conditions: 94 °C for 10 min, followed by 25 cycles of 30 s at 94 °C, 30 s at 55 °C, and 50 s at 72 °C.
        5. Identify positive bacteria by migrating 5 µL of the PCR products on a 1.5% agarose gel.
        NOTE: Positives colonies give a PCR product around 850 bp for the heavy chain vector and 600 bp for the light chain vector.
    3. Inoculate four positive colonies from the replicate plate into 2 mL of LB medium and incubate overnight at 37 °C with shaking at 200 rpm.
    4. Extract plasmids from liquid cultures using a plasmid purification kit, as indicated by the manufacturer.
    5. Verify correct insertion of variable domains by Sanger sequencing of the plasmids with the Ab-vec-sense primer.
    NOTE: Plasmids with the correct insert (encoding the HC and the corresponding LC) are to be cotransfected into 293A cells for secretion. The specificity of small scale-produced mAbs is assayed by ELISA. Then, large scale production is performed if applicable.

6. Production of mAbs

1. **Small scale production for specificity checking**
    1. The day before the transfection, seed 15,000 Human embryonic kidney 293A (HEK 293A) cells in 200 µL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS per well in 96-well plates. Incubate overnight in a CO_2 incubator (5% CO_2) at 37 °C.
    2. Cotransfect 293A cells in DMEM medium containing 10% FBS using a linear polyethylenimine derivative as transfection reagent.
    NOTE: Perform the transfection in triplicates. The following protocol is for one well of a flat bottom 96-well plate.
        1. Dilute 0.5 µL of DNA transfection reagent into 10 µL of 150 mM NaCl. Dilute 0.125 µg of vH and 0.125 µg of vL expressing vectors into 10 µL of 150 mM NaCl. Vortex each dilution for 10 s.
        2. Add 10 µL diluted DNA transfection reagent into the 10 µL DNA solution. Vortex 15 s and incubate for 15 min at room temperature. Add the 20 µL mix drop-wise on 293A cells, and mix by gently swirling the plate.
    3. Replace the medium 16 h after transfection and culture cells for 5 days in serum-free medium.
    NOTE: Use serum-free medium to avoid serum Igs contaminating the produced antibodies.
    4. Eliminate cells and debris by centrifugation at 460 x g for 5 min.
    5. Harvest supernatants by aspiration with a multi-channel pipette and transfer them into a V-bottom 96-well plate.
    6. Coat relevant Ag overnight at 4 °C in 100 µL per well of reconstituted ELISA/ELISPOT coating buffer 1x at a final concentration of 2 µg/mL in 96-well ELISA plates. See examples in [9].
    7. Block wells with 10% FBS DMEM medium for 2 h at 37 °C
    8. Add 50 µL of supernatants of transfected 293A cells from step 6.1.5, and incubate for 2 h at RT.

9. Add 100 µL of an anti-human IgG Ab conjugated to horseradish peroxidase (HRP) enzyme at 1 µg/mL and incubate for 1 h at room temperature. Add 50 µL chromogenic substrate (TMB), and incubate for 20 min.
10. Read optical densities at 450 nm on a spectrophotometer.
    NOTE: The specific mAbs revealed by ELISA assay can be further produced at large scale and purified on protein A column presenting affinity for human IgG.

2. **Large scale production and purification of specific mAbs**
    1. The day before the transfection, seed 6 x 10^6 Human embryonic kidney 293A (HEK 293A) cells into 175 cm^2 flasks containing 25 mL DMEM supplemented with 10% FBS. Incubate overnight in a CO_2 incubator (5% CO_2) at 37 °C.
       NOTE: Cells should be 70% confluent when transfected.
    2. Cotransfect 293A cells in DMEM medium containing 10% FBS using a linear polyethylenimine derivative as transfection reagent.
        1. Dilute 50 µL of DNA transfection reagent into 450 µL of 150 mM NaCl. Dilute 10 µg of vH and 10 µg of vL expressing vectors into 500 µL of 150 mM NaCl. Vortex 10 s each dilution.
        2. Add diluted DNA transfection reagent to the DNA solution. Vortex 15 s and incubate for 15 min at room temperature. Add the 1 mL mix drop-wise to the cells, and mix by gently swirling the flask.
    3. Replace the medium 16 h after transfection and culture cells for 5 days in serum-free medium.
    4. Harvest medium by aspirating with a 25-mL pipette.
    5. Eliminate cells and debris by centrifugation at 460 x g for 5 min.
    6. Purify the antibodies using a 1 mL column coated with protein A on a fast protein liquid chromatography (FPLC) system at 4 °C.
        1. Equilibrate the protein A sepharose column with 20 mM phosphate buffer (pH 7).
        2. Filter the cell culture medium from 6.2.5 using a 1.22 µm filter, then a 0.45 µm filter.
        3. Load the filtered medium onto the column.
        4. Wash with 20 mM phosphate buffer (pH 7) and elute with a 0.1 M citrate buffer (pH 3.5).
        5. Read optical densities at 280 nm on an absorption spectrophotometer and immediately neutralize protein-containing fractions with 1 M Tris buffer (pH 9).
    7. Dialyze purified Ab overnight at 4 °C in a cassette with a 3.5K molecular weight cutoff against PBS buffer (pH 7.4).
    8. Sterilize by 0.2 µm filtration and control purity by size-exclusion chromatography, as indicated by the manufacturer.

Representative Results

Starting from PBMC from healthy donors, this project presents the generation of human mAbs, which recognize the peptide $Pp65_{495}$ (Pp65, from human cytomegalovirus) presented by the major histocompatibility complex class I (MHC-I) molecule HLA-A*0201 (HLA-A2). These mAbs can discriminate between this complex and complexes representing other peptides loaded onto the same MHC-I molecule.

PBMC were stained with HLA-A2/Pp65-PE, HLA-A2/Pp65-APC, and HLA-A2/MelA2-BV421 tetramers as described in the above protocol. After immunomagnetic cell enrichment of PE- and APC-tetramer positive cells, the eluted cells were stained with additional mAbs. On the flow cytometry cell sorter, magnetically enriched cells were first gated on viable $CD14^-CD16^-CD3^-CD19^+$ singlets (B cells). **Figure 1** shows the gating strategy used to isolate B cells expressing BCRs able to discriminate between HLA-A2/Pp65 and other related HLA-A2/peptide complexes. Selection of B cells of interest was performed after gating on HLA-A2/Pp65 PE^+ and HLA-A2/Pp65 APC^+ double-positives, to exclude fluorochrome specific B cells that were singly stained PE^+ or APC^+. Finally, highly specific B cells were identified by gating on HLA-A2/MelA2-BV421 negative cells. This allows the identification of B cells expressing BCRs able to bind HLA-A2/Pp65, in a peptide and HLA-A2-dependent manner, discriminating between these B cells and those directed against β2 microglobulin, biotin, HLA-A2, or those which do not discriminate between peptides in the MHC binding groove. All these latter cells will be BV421 positive cells. As previously shown and further documented with other types of Ag, this exclusion strategy is more important due to the increases in the discriminative ability of the B cells for the Ag[9].

Once single specific B cells were sorted, cDNAs encoding for heavy and light Ig-chains were amplified by RT-PCR. Pairs of heavy and light chain coding segments were obtained in about 50% of single B cells tested (**Table 1**). Variable domain sequences were then cloned into expression vectors containing corresponding constant domain sequences (heavy constant 1 and light constant κ or λ). Human embryonic kidney cells (HEK, 293A cells) were cotransfected with heavy and light chain vectors. The secreted mAbs of IgG1 isotype were harvested from the culture supernatants of 293A cells 5 days after transfection (See **Figure 3** for the global strategy of fully human mAbs production). This successfully produced one HLA-A2/Pp65 specific antibody starting from 3 single B lymphocyte cells yielding pairs of heavy and light chain coding segments (**Table 1**). ELISA assays clearly demonstrated that this mAb was both MHC- and peptide-dependent for its binding to HLA-A2/Pp65 complexes (**Figure 4A**), and several milligrams of mAb were readily produced for further analysis (*e.g.*, affinity analyses, functional assays). Its binding affinity, determined by surface plasmon resonance (SPR), was about 7 x 10^{-6} M (**Figure 4B**).

Thus, this article describes a combination of sensitive and efficient methods allowing i) detection of relevant Ag-specific B cells, even when present at very low frequencies in the blood of healthy donors and ii) the generation of highly discriminative human mAbs.

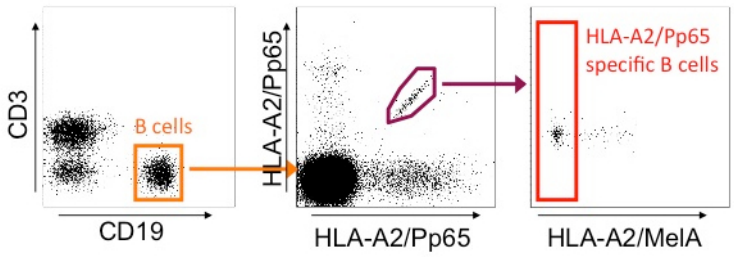

Figure 1: Detection of HLA-A2/Pp65 specific B cells from human PBMC.
3 x 10^8 PBMC were stained with HLA-A2/Pp65-PE, HLA-A2/Pp65-APC, and HLA-A2/MelA2-BV421 tetramers. After immunomagnetic cell enrichment of PE- and APC-tetramer positive cells, the eluted cells were stained with additional mAbs. On a flow cytometry cell sorter, cells were gated first on viable singlet $CD14^-CD16^-$ lymphocytes (not shown), then on $CD19^+CD3^-$ cells. Then, B cells stained with both HLA-A2/Pp65-PE and HLA-A2/Pp65-APC tetramers were gated. The HLA-A2/MelA-BV421 tetramer was used to exclude B cells that did not recognize HLA-A2/Pp65, in a peptide and MHC-dependent manner.

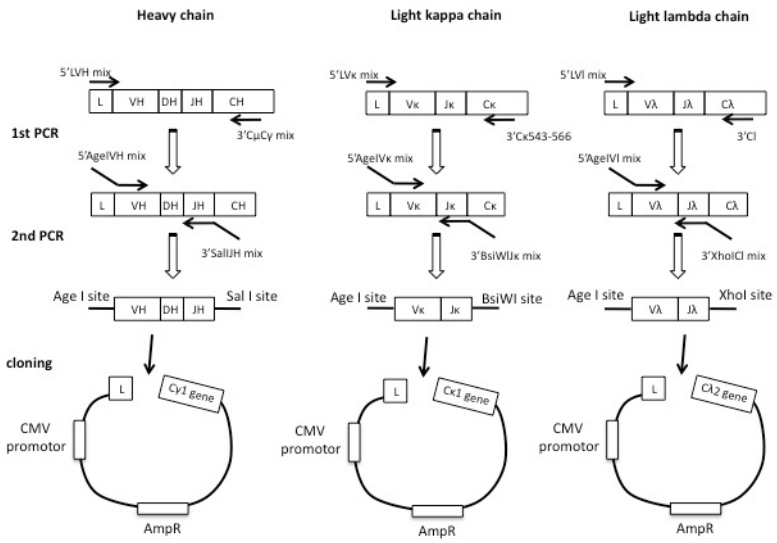

Figure 2: Strategy for amplification and cloning of Ig genes. Light and heavy Ig-chain encoding genes were amplified by nested RT-PCR. First PCRs were performed with a mix of forward primers hybridizing the leader region and reverse primers specific for constant regions of appropriate heavy, light kappa, or light lambda chains. Second PCRs were performed with primers containing restriction sites, forward and reverse primers were respectively specific for the beginning of V segments and for the end of J segments. PCR products were sequenced, digested with restriction enzymes, and cloned in expression vectors containing appropriate constant domains. CMV: cytomegalovirus promoter; AmpR: resistance gene for ampicillin.

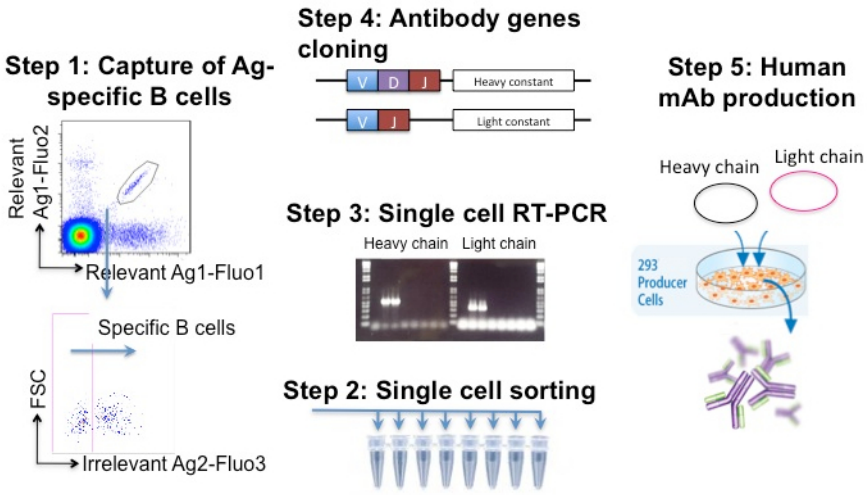

Figure 3: Overall strategy of reconstruction of recombinant human mAbs.
A tetramer-based sorting strategy allows detection of B cells of interest. Highly specific B cells were single-cell sorted. Light and heavy Ig-chain encoding segments were amplified using RT-PCR. Variable domain sequences were cloned into separate eukaryotic expression vectors in frame with gene segments encoding constant light and heavy regions. The corresponding fully human mAbs were expressed by transiently-transfected HEK 293A cells and purified from the culture supernatant. This figure was modified from Ouisse *et al.* (2017)[9].

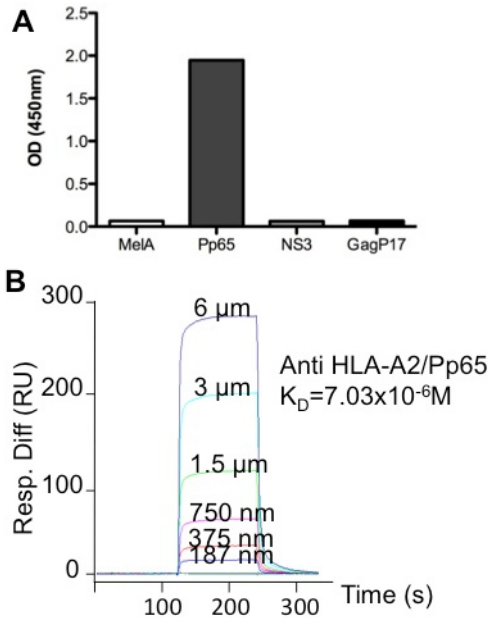

Figure 4: Characterization of a representative highly discriminative mAb against HLA-A2/Pp65 generated from human peripheral blood circulating B cells.
A) Specificity of mAb PC1.02 against HLA-A2/Pp65 tested by ELISA. Plates were coated with relevant (HLA-A2/Pp65) or irrelevant HLA-A2 complexes containing HLA-A2-restricted peptides: MelA, NS3 (HCV-1), and GagP17 (HIV-1) at 2 µg/mL, the mAb PC1.02 was added, and an anti-human IgG-HRP Ab was used for detection. Optical densities (OD) were read at 450 nm. **B)** Affinity determination of the mAb PC1.02 using Surface Plasmon Resonance (SPR) by flowing various concentrations of HLA-A2/Pp65 complexes over CM5 chip-bound mAb. This figure was modified from Ouisse *et al.* (2017)[9].

	Number of PBMC	Number of PE+ APC+ cells after enrichment	Number of excluded (BV421+) cells	Number of sorted single cells	Number of analyzed wells	Number of wells with HC and LC associated (% recovery)	Number of mAbs produced	Number of specific mAbs
HLA-A2/Pp65 mAb (PC1.02)	3 x 10^8	818	117	161	7	3 (43%)	3	1

Table 1: Analysis of HLA-A2/Pp65 specific B cells.
The impact of the exclusion strategy of unspecific B cells, the yields of Ig gene amplification, and mAb production from isolated HLA-A2/Pp65 specific B cells were evaluated/measured.

Discussion

The proposed protocol is a powerful method for the generation of human mAbs directly from Ag-specific B cells circulating in the blood. It combines three important aspects: (i) the use of a tetramer-associated magnetic enrichment, which allows an *ex vivo* isolation of even rare Ag-binding B cells; (ii) a gating strategy that uses three Ag tetramers (two relevant ones and one irrelevant one) labelled with three different fluorochromes to isolate, by flow cytometry, only the B cells expressing a BCR specific for the desired Ag; (iii) the reconstruction of the corresponding recombinant mAb cDNAs by RT-PCR at the single cell level and expression of the cDNAs in human cells.

Previous studies proposed using one or two fluorescent relevant antigens to label human B cells before sorting and subsequent production of mAbs from the isolated B cells[6,7,8]. One analysis in patients with rheumatoid arthritis, and one in an autoimmune mouse model, have associated an irrelevant fluorescent antigen to characterize autoreactive B cells and determine their frequency[10,11]. As far as we know, use of a combination of two fluorescent relevant antigen tetramers and one irrelevant antigen tetramer has not been described previously for enrichment of specific B cells prior to their use for production of fully human mAbs. This optimized method allows fully human discriminative mAbs to be obtained in as little as a month, and can be performed successfully even when starting from a naïve B cell repertoire. Thus, it has none of the major drawbacks of phage display, human B cell immortalization, or other previously described molecular biology-based mAb reconstruction procedures.

This cell sorting strategy results in a high yield recovery of Ag-specific human mAbs. Pairs of heavy and light chain segments from single isolated B cells are amplified with a success rate of around 50%. Light chain segments are almost always amplified, but this is not the case for heavy chain segments. RT-PCR efficiency depends heavily on respecting the following points: i) sorted single B cells must be frozen as quickly as possible; ii) adding 30 units of RNase inhibitor and minimizing the time between taking the B cells out of the freezer and launching the RT reaction; iii) thawing all primers on ice; iv) never freezing/thawing primers more than three times; v) stocking primers for a maximum of one year.

Concerning the production efficiency of the corresponding recombinant mAbs with the desired specificity, it is about 30 - 40% of the case for pMHC specific mAbs. These particular mAbs have to recognize both the peptide and the MHC molecule, which is quite demanding, and we have previously shown that the overall yield of recovery of specific mAbs directed against more conventional antigens is superior, up to 100% for the β-galactosidase antigen[9]. It must be stressed that the choice of an appropriate Ag for the irrelevant tetramer is important to increase the specificity of the mAbs produced.

The affinity of the anti-HLA-A2/Pp65 mAb (PC1.02) described in the present article is relatively low, about 7 x 10^{-6} M, similar to the affinity of a TCR. This result was expected, as B cell isolation was performed from naïve donors. Most tetramer$^+$ B cells were IgM$^+$IgG$^-$, which reduces the probability of obtaining good Ag-binders. Nevertheless, this method can also make possible the sorting out of cross-reactive memory B cells against a desired Ag from naïve donors, because of immunological past of individuals[12]. Moreover, this method is easily applicable to vaccinated or infected patients or immunized humanized animals, as described in [9], where *in vivo* affinity maturation can increase the affinity of resulting mAbs to about 1 x 10^{-9} M. Various procedures have also been described to improve the affinity of mAbs *in vitro*, in particular through reproducing somatic hypermutation in cells expressing the antibody (reviewed in [13]).

In conclusion, we propose a versatile strategy for highly discriminative mAbs production that can be used in various types of situation, from a naïve individual to a vaccinated donor or a patient suffering from an autoimmune disease. Fully human mAbs generated in this way against a desired epitope could be useful both for basic research and immunotherapy.

Disclosures

The authors have nothing to disclose.

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