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Srinivas V. Kaveri
Jagadeesh Bayry *Editors*

Natural Antibodies

Methods and Protocols

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Preface

Natural antibodies, belonging to isotypes IgM, IgG, and IgA, were discovered nearly half a century ago. Natural antibodies are those that are detected in the serum of healthy organisms in the absence of pathology or intentional immunization. Most natural antibodies bind to one or more autoantigens and are called as natural autoantibodies. The importance of natural autoantibodies in immune regulation and in therapeutic application is now well established.

The role of natural antibodies in antigen presentation, pathogen elimination, cell survival and homeostasis, inflammation, cancer, and autoimmunity is exhaustively documented. While the basic properties of natural antibodies, origin, distribution, evolution in physiology and pathology, and functions were the subjects of thorough investigations, in parallel, a large body of highly interesting information has been generated on the methodology of isolation, identification, characterization, and quantification of natural antibodies in various situations. In this edition, we have gathered protocols from experts who have made significant contribution in this domain.

An overview of the progress in the understanding of the functions of natural antibodies is summarized by *Heinz Kohler*. Three chapters focus on the protocols for isolation of natural antibodies: while *Vogel and Horn* provide methods for purifying anti-FcεRIα autoantibodies from serum, *Schneider and colleagues* describe methods for separation of natural antibodies from human plasma, saliva, breast milk, and gastrointestinal fluid, and *Kolarova and colleagues* present protocols for purification of natural antibodies against tau protein. *Bannoudi and colleagues* detail protocols for unbiased RACE-based massive parallel surveys of human IgA antibody repertoires. In view of the role of natural antibodies in B cell survival and homeostasis, *Huo and colleagues* present protocols for the assessment of signaling events in B-1a cells, while *Mohr and Lino* narrate how microbiota influences the B1 and MZ B-cell numbers by the normal polyreactive immunoglobulins.

Certain intriguing functional properties of natural antibodies such as their anti-tumor cytotoxic activity (*Schwartz-Albiez and Dill*), hydrolysis and dissolution of their target antigens (*Meretoja and colleagues*), and the ability of natural antibodies to undergo enhanced polyreactivity (*Lecerf and colleagues*) are elaborated in subsequent chapters. Natural IgM antibodies recognizing oxidation-specific epitopes on circulating microvesicles (*Puhm and Binder*) and oxidized low-density lipoprotein and *Aggregatibacter actinomycetemcomitans* (*Wang and Hörkkö*) are discussed in the ensuing sections. Relevance of serological diagnosis of microbial antigens is discussed by *Jiménez-Munguía and colleagues* and detection of naturally occurring human antibodies against gangliosides is then narrated by *Hernández and Rodríguez-Zhurbenko*. Adenoviral vectors are the most widely used class of gene therapy vector in clinical trials. It is important to underline the role of natural antibodies in inhibiting viral vectors even in the absence of prior exposure to the virus. Thus, *Xu and colleagues* examine the protocols for evaluation of impact of natural IgM on adenovirus type 5 gene therapy vectors.

We are indebted to all the colleagues who shared time, energy, and experience for writing these protocols and to John Walker, the series editor, for his constant advice and support. We hope that the protocol series is highly helpful for laboratory approach to study the interesting features of natural antibodies.

Paris, France   *Srinivas V. Kaveri*
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Natural Antibodies: Next Steps Toward Translational Investigation

Heinz Kohler

Abstract

Natural antibodies have diverse functions in maintaining immune homeostasis and in the regulation of autoimmune response. Yet there might be other important functions of natural antibodies for human medicine such as the ability of natural antibodies to shape and induce antibody immune responses. I propose that natural antibodies, present in IVIG, could be used to prevent autoimmune reactions and to enhance the immune response to vaccination.

Key words Natural antibodies, T15, Autoimmunity, Infection, Vaccine

1 Introduction

Interest in and studies of natural antibodies have a long history [1–3]. Natural antibodies are produced spontaneously by B-lymphocytes without antigenic exposure from infection or vaccination. Natural antibodies are predominantly of IgM class. In addition, IgG and IgA natural antibodies have also been identified and validated in functional assays. Natural antibodies provide protection against disease [4, 5] and perform housekeeping to eliminate cell debris and damaging molecular species [6–9]. Natural antibodies are also drugs to treat autoimmune diseases delivered as pools from serum of healthy donors, the so-call IVIG [10]. Yet there might be other important functions of natural antibodies for human medicine.

Recently, we have speculated that levels of disease-specific antibodies in natural antibodies predict the immune response to vaccination [11]. Natural antibodies are antigen promiscuous with low affinity to different antigens including self-antigens. Natural antibodies are found in germ-free mice underscoring the finding that NAbs appear in the developing immune system without antigenic stimulation. The presence of natural antibodies in serum is under

genetic control as shown by dominant T15 anti-phosphoryl-choline antibodies in inbred mice. Lieberman and Potter have identified inbred mice with T15 expression and without T15 antibodies in normal sera [12]. Also the development of the T15 antibodies in the neonatal period is delayed and under genetic control [13]. Natural antibodies can bind, as being polyspecific, to a wide spectrum of antigens forming Ag–Ab complexes. These immune complexes are presented to T-helper cells and sensitize responding B-cells [14]. Niels Jerne [15, 16] proposed that the pre-immune antibodies make the initial contact with antigen and that this is the triggering event that leads to an antibody response.

The role of natural antibodies in shaping and inducing the antibody immune response can be used to predict the response to vaccination. This concept, however, needs to be applied in clinical trials and its potential utility for vaccine development awaits validation.

The second novel medical utility of natural antibodies stems also from the power of natural antibodies to shape and induce an immune response. Natural antibodies, used as IVIG, can suppress autoimmune reactions in a variety of autoimmune diseases [10, 17–20]. While this use of IVIG as therapeutic drug is well documented, there are no data, to my knowledge, on IVIG preventing autoimmune disease. The epidemic increase of allergic reactions in children has been contributed to lack of antigenic stimulation in modern households. Recognizing that natural antibodies shape the developing immune system in the neonatal period, supplementing the individual natural antibodies with the global collection of immunoglobulins in IVIG could enhance and broaden the process of immune repertoire formation. Therefore, IVIG given to neonate or toddlers could "prime" the immune system to control autoimmune responses, such as juvenile diabetes and asthma, in later life.

Patients receiving chemotherapy are immunocompromised and are prone to bacterial and viral infections. *Streptococcus pneumoniae* infections are common in hematological malignancy, and prophylactic vaccination is ineffective in many patients [21]. Thus, "priming" the immune system with IVIG, as outlined for infants, could increase the protective vaccination response in adult patients. In addition, IVIG could reduce the pathology caused by toxins of methicillin-resistant *Staphylococcus aureus* [22].

The use of global natural antibodies (IVIG) to prevent autoimmune disease or to enhance the vaccine response could become a new drug, coined pre-IVIG.

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Isolation of Natural Anti-FcεRIα Autoantibodies from Healthy Donors

Monique Vogel and Michael P. Horn

Abstract

Natural antibodies are defined as antibodies detected in a healthy individual without active immunization. These antibodies are specific for exoantigens, as well as for autoantigens, mostly without any pathogenic role. Most of the studies conducted with natural (auto-) antibodies have been performed using affinity purified antibodies from individual sera or polyclonal Ig-preparations such as Intravenous Ig (IVIg). For in-depth analysis of such autoantibodies affinity-purified Ig-preparations from healthy individuals are of no use, as they are oligoclonal or polyclonal. Thus, there is a need of human monoclonal autoantibodies. Human monoclonal autoantibodies can be produced from B cells isolated from humans; however, this requires the screening of a large number of antibodies to identify one among them specific to an antigen. Using the phage display technology we generated such autoantibodies against the alpha subunit of the high-affinity IgE receptor (FcεRIα). Here we describe the step-by-step protocol for the generation of such libraries and the isolation of autoantibodies by affinity panning.

Key words Natural antibodies, Anti-FcεRIα autoantibodies, IVIg, Phage display technology, Isolation

1 Introduction

The pathogenic role of natural antibodies in autoimmune disease is controversial. These antibodies are often polyreactive with a variety of both self and foreign antigens. However, the presence of such physiological autoantibodies does not generally precipitate autoimmune disease. Our investigations of autoantibodies associated with autoimmune urticaria suggest they are indistinguishable from natural antibodies found in healthy subjects.

Autoantibodies reacting with the α-subunit of the human high-affinity immunoglobulin E (IgE) receptor (FcεRIα) have been described in autoimmune urticaria [1, 2]. However, we previously reported the presence of anti-FcεRIα autoantibodies in the serum of healthy donors as well as in multidonor intravenous IgG (IVIg) preparations [3]. Thus, these anti-FcεRIα autoantibodies may

belong to the natural antibody repertoire reacting with a restricted set of self-antigens [4, 5]. In general, functional studies and in-depth characterizations of natural autoantibodies using serum or IVIg preparations are difficult to interpret due to the polyclonal nature of the respective antibody preparations, which may contain mixtures of different types (specificities) of autoantibodies. There-fore, to characterize these natural anti-FcεRIα autoantibodies and investigate their functional activity in vitro and in vivo, we gener-ated human recombinant anti-FcεRIα antibodies by repertoire cloning from a nonimmune IgM library displayed on the filamen-tous phage M13 [6, 7].

The generation of monoclonal antibodies against an exogenous antigen is a very well-established method in rodents, but also in humans, monoclonal antibodies can be generated upon immuniza-tion with the antigen and subsequent isolation of antigen-specific B cells. These cells can further be immortalized by EBV infection or by cell fusion using heterohybridoma cell lines [8]. Using antibody phage display technology, we generated human monoclonal Fab fragments against different antigens, such as Tetanus Toxoid (TTd, *see* ref. [9]), Rhesus D (RhD, *see* ref. [10, 11]), or even IgE [12]. For TTd and RhD, individuals have been actively immunized, and the B cells for generation of the libraries have been isolated thereafter. However, in order to generate monoclonal natural autoantibodies, active immuni-zation is not feasible. Thus, we use as a source of the antibody repertoire IgM-positive B cells isolated from children's tonsils.

There are mainly two different phage display systems for anti-body repertoire cloning, single-chain Fv (scFv) and Fab-display. In the scFv system, the variable regions of the heavy and light chains were cloned in one gene segment using a spacer element [13, 14], whereas in the Fab system the light chains and the CH1 domain and the variable region of the heavy chains are cloned individually into the vector. Even though scFv proteins might be easier to handle, Fab libraries have in our view one big advantage. The combination of the heavy and light chains after expressing these proteins is based on their conformation. The heavy chain–light chain combinations as such have a huge impact on the specificity as well as the affinity of the antibody [15], and furthermore, not all random VH/VL pairings are represented in a natural antibody repertoire [16]. Thus, taking these two points into consideration, the Fab display system might represent a more natural antibody repertoire compared to the scFv system.

2   Materials

2.1   Isolation of PBMC or B Cells from Children's Tonsils

1. Fresh tonsils from children under routine tonsillectomy.
2. Nunc™ petri dishes.
3. Hank's balanced salt solution (HBSS).

4. Ficoll-Paque™ PLUS. Store at 4 °C.

5. Trypan Blue stain (0.4%). Store at room temperature (RT).

6. Phosphate-buffered saline (PBS): 5.6 mM Na_2HPO_4, 154 mM NaCl, 1.06 mM KH_2PO_4, pH 7.4. Adjust the pH with HCl. Dilute in sterile water and store at RT.

7. RNase-free water.

8. PBS containing 2% (wt/vol) fetal calf serum (FCS) and 1 mM EDTA.

9. Plastic pipettes (1, 5, 10, and 25 mL).

10. Micropipettes and tips (20, 200, and 1000 μL).

11. 15- and 50-mL conical tubes.

12. Human B Cell enrichment kit (STEMCELL Technologies).

13. Hemocytometer.

14. RLT (RNeasy lysis buffer; Qiagen).

2.2 RNA Preparation

1. Purified B cells from fresh tonsils.

2. RNase-free 1.5 mL microfuge tubes.

3. RNase-free water.

4. RNase midi kit (Qiagen).

5. Sodium acetate solution (3 M, pH 5.2). Adjust pH with glacial acetic acid. Store at RT.

6. 100% ethanol.

2.3 RT-PCR Amplification of VH and VL cDNA

1. RNase-free 1.5 mL microfuge tubes.

2. 0.5 mL PCR tubes.

3. ThermoScript™ Reverse Transcriptase.

4. Oligo(dT)15 Primer, 500 μg/mL (Promega).

5. dNTP Mix, PCR-Grade, 100 mM (25 mM each dNTP) (Agilent Technologies).

6. 5× cDNA Synthesis Buffer.

7. 0.1 M DTT.

8. RNAsin.

9. 5 U/μL Herculase II Fusion DNA Polymerase (Agilent Technologies).

10. 5× Herculase II Reaction Buffer (Agilent Technologies).

11. 100 mM dNTP mix: 25 mM of each dATP, dCTP, dGTP, dTTP diluted in sterile water from 100 mM stock concentration (Agilent Technologies).

12. 96-well thermocycler.

13. MinElute Gel Extraction Kit (Qiagen).

14. 6× Gel loading dye solution (Fermentas). Store at RT.

15. TBE Buffer, 10×. Molecular biology grade (Promega).

16. 100-bp DNA ladder (Fermentas). Store at −20 °C

17. Agarose, LE, analytical grade.

18. red®—personal gel imaging system (Bucher Biotec AG).

2.4 Cloning of the Light Chain

1. Phagemid vector pMVS (3.49 kb) which has been reconstructed based on pComb3H-SS (Scripps Research Institute, La Jolla, Cal., USA); an oligonucleotide containing the myc and 6 His tags has been inserted at the XbaI site.

2. 20 U/μL *XbaI*.

3. 20 U/μL *SacI*.

4. 10× CutSmart® Buffer (New England Biolabs Inc.).

5. Agarose, LE, analytical grade.

6. TBE Buffer, 10×. Molecular biology grade.

7. 6× gel loading dye solution.

8. 100 bp DNA ladder.

9. QIAquick Gel extraction Kit.

10. 2 U/μL T4 DNA ligase and 10× T4 DNA ligase buffer.

11. Electrocompetent XL1-Blue cells with an efficiency of $>1 \times 10^{10}$ colony forming units (cfu)/μg pUC18/DNA (Agilent Technologies). Store at −80 °C.

12. *E. coli* Pulser™ Transformation apparatus (Bio-Rad).

13. 0.2 cm Electroporation Cuvettes Plus (BTX, 1 mm)

14. Super Optimal Broth with catabolite repression (SOC) medium for *E. coli* (Cold Spring Harbor Protocols).

15. Super broth (SB) medium: 30 g tryptone, 20 g yeast extract, 10 g 3-(N-morpholino)-propanesulfonic acid (MOPS). Dissolve 30 g tryptone, 20 g yeast extract, 10 g MOPS in water. Bring to 1 L total volume with H_2O, stir until dissolved, and bring to pH 7.0 with 1 N NaOH. Sterilize by autoclaving in 1 L glass bottle. Store at RT.

16. 100 mg/mL carbenicillin. Dissolve 1 g of carbenicillin disodium in 10 mL of water. Sterilize by filtration through 0.22 μm. Store at −20 °C.

17. 10 mg/mL tetracycline. Dissolve 100 mg of tetracycline hydrocholoride in 10 mL of ethanol. Store at −20 °C.

18. LB plates with 100 μg/mL ampicillin. Store at 4 °C.

19. Pasteur pipettes.

20. 50 mL Falcon tubes.

2.5 Production of Helper Phage

1. Electrocompetent XL1-Blue cells.
2. SB medium.
3. 10 mg/mL tetracycline.
4. VCSM13 helper phage (Agilent Technologies). Store at −80 °C.
5. Dimethyl sulfoxide (DMSO).
6. PEG 8000. Store at RT.
7. PBS.

2.6 Cloning of the Heavy Chain and Phage Library Preparation

1. 20 U/μL *Xho*I.
2. 10 U/μL *Spe*I.
3. 10× CutSmart® Buffer.
4. Agarose.
5. QIAquick Gel extraction Kit.
6. 2 U/μL T4 DNA Ligase and 10× T4 DNA ligase buffer.
7. Electrocompetent XL1-Blue cells with an efficiency of >1 × 10^{10} colony forming units (cfu)/μg pUC18/DNA.
8. *E. coli* Pulser™ Transformation apparatus.
9. 100 mg/mL carbenicillin.
10. 5 mg/mL tetracycline.
11. LB plates with 100 μg/mL ampicillin.
12. 100 mg/mL kanamycin sulfate. Dissolve 100 mg of kanamycin sulfate in 10 mL of water. Store at −20 °C.
13. PEG 8000.
14. 12% NaCl

2.7 Library Selection on FcεRIα

1. 5 mL polystyrene immunotubes.
2. PBS.
3. Blocking solution: PBS containing 1.5 mg/mL casein.
4. PBS containing 0.1% Tween 20.
5. 0.1 M glycine–HCl, pH 2.2.
6. 2 M Tris–HCl pH 9.5.
7. SB Medium.
8. 0.05 M bicarbonate buffer pH 9.6. Prepare 0.05 M buffer with a pH of 9.6 by mixing 16 mL of 0.2 M sodium carbonate (21.2 g of Na$_2$CO3 in 1 L of H$_2$O) and 34 mL of 0.2 M sodium bicarbonate (16.8 g of NaHCO$_3$ in 1 L of H$_2$O) and dilute to a volume of 200 mL.

2.8 Selection of Specific Anti-FcεRIα Clones

1. Protran™ BA85 nitrocellulose membranes (Krackeler).
2. 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Axon lab).

3. Lysozyme buffer: 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 0.4 mg/mL lysozyme, 1 U/mL DNase I recombinant, RNase-free.

4. Blocking solution.

5. 20 μg/mL human serum albumin (HSA)-FcεRIα. A fusion protein of two moieties of the extracellular part of the alpha subunit of the high affinity IgE receptor with one moiety of HSA (Novartis AG).

6. 10 μg/mL HSA.

7. Peroxidase-labeled sheep anti-human Fab antibody (Nordic).

8. 4-chloro-1-α-naphthol.

9. SB Medium.

10. Qiagen plasmid maxi kit (Qiagen).

15. LB agar plates containing 100 μg/mL ampicillin.

2.9 ELISA with Single Phage Clones for Definition of Recognition Pattern

1. Costar assay plates (Costar).

2. 5 mM IPTG.

3. PBS containing 1.5 mg/mL casein.

4. 5 μg/mL HSA-FcεRIα.

5. 5 μg/mL HSA.

6. 50 μg/mL β-casein from bovine milk.

7. PBS containing 0.1% Tween 20.

8. PBS.

9. 3,3′,5,5′-tetramethylbenzidine.

10. 1 M H_2SO_4.

11. ELISA microplate reader (Spectra Max).

2.10 Nucleotide Sequencing

1. SB medium.

2. 100 mg/mL carbenicillin.

3. QIAprep Spin Miniprep Kit (Qiagen).

4. Sense and antisense primers (Microsynth AG).

5. V-base sequence directory (http://www2.mrc-lmb.cam.ac.uk/vbase/vbase).

2.11 Generation of Full-Length IgG for Functional Studies and Affinity Measurements

1. 25 U/μL *BssHII*.

2. 10 U/μL *SalI*.

3. 12.5 U/μL *ApaLI*.

4. 2.5 U/μL *SpeI* .

5. 10× CutSmart® Buffer (New England Biolabs).

6. QIAquick Gel extraction Kit.

7. 2 U/μL T4 DNA ligase and $10\times$ T4 DNA ligase buffer.

8. Human embryonic kidney cells 293 (HEK-293) expressing SV40 large T antigen (HEK-293T)

9. Lipofectamine® 2000 Transfection reagent (ThermoFisher Scientific).

10. Dulbecco's Modified Eagle Medium–Nutrient Mixture F-12 (DMEM–F-12) (ThermoFisher Scientific).

11. Fetal calf serum (FCS).

12. HiTrap Protein G Sepharose High Performance column (GE Healthcare Life Sciences).

13. Sodium dodecyl sulfate (SDS)–polyacrylamide gel.

14. Goat anti-human IgG (Fc specific) and peroxidase-labeled-goat anti-human IgG (whole molecule) antibodies.

15. Tissue culture (TC) flasks (Becton Dickinson).

16. 6-well TC plates (Becton Dickinson).

3 Methods

The generation of a human Fab library is summarized in Fig. 1. The single steps are depicted in detail in the following sections. The following section can also be used for the isolation of peripheral blood mononuclear cells (PBMC).
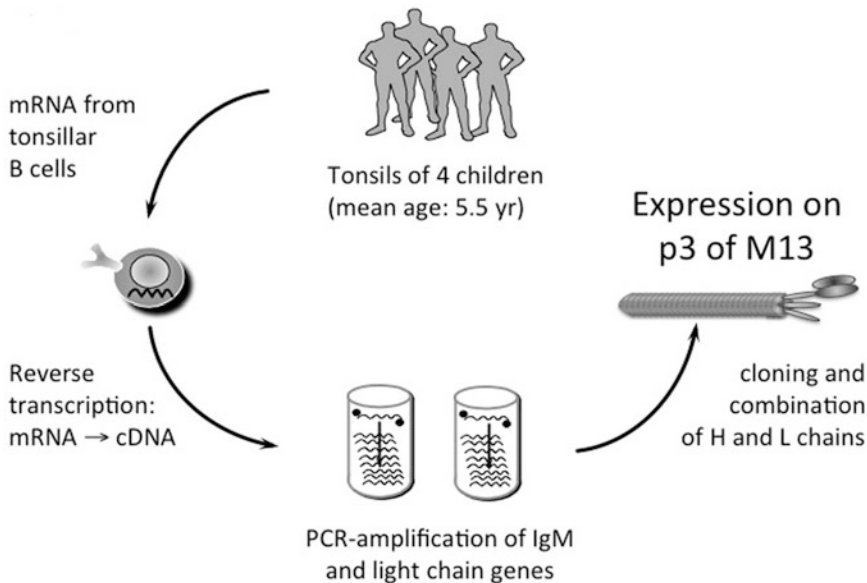
Fig. 1 Schematic illustration of the library construction. Tonsillar B cells are used as the source for the antibody library. Individual clones are amplified from the total mRNA from these cells. Light chain and Fd fragment were cloned into the phagemid vector and expressed on the surface of the bacteriophage M13

3.1 Isolation of Mononuclear Immune Cells (MNC) and B Cells from Children's Tonsils

1. Obtain fresh tonsils from children undergoing routine tonsillectomy.

2. To isolate MNC, place the tonsils in a petri dish, cut them into fine pieces with scissors or razor blades, then push out the cells with pincers into a 50 mL tube, rinse the plate with HBSS and pool with the first supernatant.

3. Let stand for 5–10 min until precipitation of the cell debris.

4. Carefully layer the supernatant over 15 mL Ficoll-Paque in a 50 mL centrifuge tube. Centrifuge at $800 \times g$ for 20 min at RT without brake.

5. Collect the mononuclear cells from interface leaving the cell pellet containing fibroblast and other cell debris.

6. Wash the cells three times with HBSS and resuspend in PBS containing 2% FCS and 1 mM EDTA (*see* **Note 1**).

7. Determine the number of viable cells by Trypan Blue staining using a hemocytometer. The expected yield is between 5×10^8 and 10^9 cells per tonsil.

8. Purify B cells by positive selection with a human CD19$^+$ selection kit. Proceed according to the manufacturer's instruction.

9. After purification, assess the number of viable cells as above.

10. To assess the loss during the procedure determine the number of recovered B cells as a percentage of the input number. Expected percentage number is between 50 and 70% with a viability of >95%.

11. After isolation of B cells, resuspend the cells in RLT Buffer (2 mL up to 5×10^7 cells and 4 mL for more than 5×10^7 up to 10^8) cells and proceed according to Subheading 3.2.

3.2 Total RNA Preparation

1. For total RNA preparation we use the RNeasy Midi kit preparation. After resuspension of the B cells in either 2 or 4 mL of RLT buffer, proceed according to the manufacturer's instruction.

2. Following the last centrifugation step, elute the RNA in 200 μL of RNAse-free water. Immediately remove a 1.5 μL aliquot and measure the absorbance at 260 nm in a NanoDrop instrument to calculate the total RNA concentration. A ratio of around 2.0 is generally considered as "pure" RNA and a concentration of 40 μg/mL RNA gives an absorbance of 1.

3. Store remaining total RNA at −80 °C for up to a year. For long-term storage precipitate the RNA by adding 0.1 volumes RNase-free 3 M sodium acetate (pH 5.2) and 2.2 volumes of 100% ethanol, vortex, and store at −80 °C.

3.3 RT-PCR Amplification of Fd (See Note 2) and Light Chains cDNA

For this step we use the primers depicted in Table 1.

1. In a RNase-free 1.5 mL Eppendorf tube, premix 2.5 μg total RNA, 1 μL of oligo(dT) (500 μg/mL), 10 mM dNTP mix, and RNase-free water up to 12 μL. Incubate for 5 min at 4 °C store on ice for at least 1 min and then collect by brief centrifugation.

2. Prepare reverse transcriptase mix by combining 4 μL of 10× Thermoscript Buffer, 1 μL of 0.1 M DTT, 1 μL of RNAsin, and 1 μL of Thermoscript™ Reverse Transcriptase, and RNase-free water up to 8 μL.

3. Add the prepared reverse transcriptase reaction mixture to the prepared RNA-Oligo(dT)-dNTP sample (12 μL), incubate at

Table 1
Primer sequences

Name	Sequences
Reverse	
IgM (CM1)	5′–GCTCACACTAGTGGCAATCACTGGAAGAGG–3
IgG$_1$ (CG1d)	5′–GCATGTACTAGTTTTGTCACAAGATTGG–3′
Cκ (CLκ)	5′–GCGCCGTCTAGAATTAACACTCTCCCCTGTTGAAGCTCTTTGTGA CGGGCGAACTCAG–3′
Cλ (CLλ)	5′–GCGCCGTCTAGAATTATGAACATTCTGTAGG–3
VH forward	
VH135	5′–AGGTGCAGCTGCTCGAGTCTGG–3′
VH135b	5′–AGGTGCAGCTCGAGCAGTCTGG–3′
VH2	5′–CAGATCACCTTGCTCGAGTCTGG–3′
VH2F	5′–CAGGTGCAGCTACTCGAGTCGGG–3′
VH4F	5′–CAGGTGCAGCTGCTCGAGTCGGG–3′
VH4G	5′–CAGGTGCAGCTACTCGAGTGGGG–3′
VH6A	5′–CAGGTACAGCTCGAGCAGTCAGG–3′
VH6F	5′–CAGGTACAGCTCGAGCAGTCAGG–3
Vκ forward	
Vκ1	5′–GAGCCGCACGAGCCCGAGCTCCAGATGACCCAGTCTCC–3
Vκ2	5′–GAGCCGCACGAGCCCGAGCTCGTGATGACCCAGTCTCC–3
Vκ3	5′–GAGCCGCACGAGCCCGAGCTCGTGATGACCCAGTCTCC–3
Vκ4	5′–GAGCCGCACGAGCCCGAGCTCGTGATGACACAGTCTCC–3
Vκ5	5′–GAGCCGCACGAGCCCGAGCTCACACTCACGCAGTCTCC–3
Vλ forward	
Vλ1a	5′–GAGCCGCACGAGCCCGAGCTCGTGTTGACGCAGCCGCCCTC––3
Vλ1b	5′–GAGCCGCACGAGCCCGAGCTCGTGCTGACTCAGCCACCCTC–3
Vλ2	5′–GAGCCGCACGAGCCCGAGCTCGCCCTGACTCAGCCTCCCTCCGT–3
Vλ4	5′–GAGCCGCACGAGCCCGAGCTCGTGCTGACTCAGCCCCCGTC–3
Vλ5	5′–GAGCCGCACGAGCCCGAGCTCGTGCTGACTCAGCCACCTTC–3
Vλ3–9	5′–GAGCCGCACGAGCCCGAGCTCGAGCTGACTCAGCCACCCTC–3
Vλ7–8	5′–GAGCCGCACGAGCCCGAGCTCGTGGTGACTCAGGAGCCCTC– 3
Sequencing primers	
pCH3H	5′–GGAGGAATTTAAAATGAAATAC–3
pCL3H	5′–GTGGAATTGTGAGCGGATAAC–3

50 °C for 60 min, followed by 5 min incubation at 85 °C. First strand cDNA can be stored for weeks at −20 °C.

4. For Fd amplification, use 2 μL of the first strand cDNA with 1.25 μL 10 μM of the eight VH forward primers (*see* Table 1) in 0.5 mL PCR tubes.

5. For light chain amplification, mix 2 μL of the first strand cDNA with 1.25 μL 10 μM of each 5 Vκ or Vλ forward primers (*see* Table 1) in 0.5 mL PCR tubes.

6. Prepare three PCR master mixes for 8 reactions of Fd, 5 reactions of kappa, and 7 reactions of lambda amplifications.

    (a) Fd master mix: In 1.5 mL Eppendorf tubes, combine 80 μL 5× Herculase Buffer, 6.4 μL of 25 mM dNTP mix, 10 μL of 10 μM of either CG1D or CM1 reverse primers, 281.6 μL of water, and 4 μL 5 U/μL Herculase polymerase.

    (b) Kappa master mix: In 1.5 mL Eppendorf tubes, combine 50 μL 5× Herculase Buffer, 4 μL of 25 mM dNTP mix, 6.25 μL of 10 μM of CLκ reverse primer, 176 μL of water, and 2.5 μL 5 U/μL Herculase polymerase.

    (c) Lambda master mix: In 1.5 mL Eppendorf tubes, combine 70 μL of 5× Herculase Buffer, 5.6 μL of 25 mM dNTP mix, 8.75 μL of 10 μM of CLλ reverse primer, 246.4 μL of water, and 3.5 μL 5 U/μL Herculase polymerase.

7. Add 47.75 μL of the master mix to the corresponding prepared first strand cDNA/forward primer.

8. In a 96-well thermocycler, use the following PCR conditions:

    95 °C for 1 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 30, 68 °C for 1 min. Followed by 72 °C for 10 min and then by cooling to 4 °C.

9. To each sample add 10 μL of 6× gel loading dye solution and separate by gel electrophoresis on a 1% (wt/vol) agarose gel in TBE buffer using a 100 bp DNA ladder as reference. The PCR band should run at 660 bp for both heavy and light chains.

10. Cut the band with a razor blade and elute DNA using Qiagen MinElute Gel extraction kit. Measure the absorbance at 260 nm and calculate the DNA concentration based on the formula that 50 μg/mL DNA gives an absorbance of 1.

3.4 Cloning of the Light Chain

The cloning of both chains is illustrated in Fig. 2. The cloning of the light chain represents the first step of the library construction. Both light chains will be first digested with *Sac*I and *Xba*I and then into cloned into vector pMVS.

1. For digestion, combine 1 μg of each light chain kappa and lambda with 10× CutSmart Buffer, 3.5 μL of 20 U/μL *Xba*I,
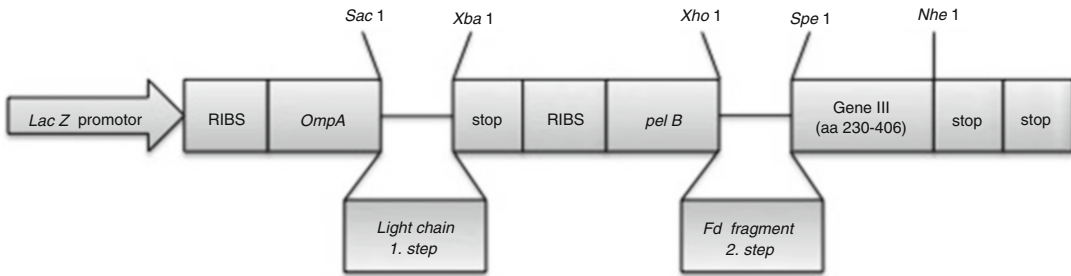
Fig. 2 Cloning site of the phagemid vector for the integration of the Fd fragment and the light chains

and 0.9 μL of 40 U/μL *Sac*I and add water to a final volume of 100 μL. Incubate at 37 °C for 90 min.

2. After inactivation of the enzyme by heating 25 min at 80 °C, purify the DNA with the QIAquick PCR Purification Kit. Resuspend the DNA in 10 μL of water and measure the absorbance at 260 nm as previously. Store at −20 °C.

3. Perform a test ligation by mixing 100 ng of *Xba*I/*Sac*I-digested pMVS vector with 33 ng of *Xba*I/*Sac*I-digested light chain, 1 μL of 10× T4 DNA ligase buffer, 1 μL of 1 U/μL T4 DNA ligase, and water to obtain a final volume of 10 μL. Prepare another test ligation using only *Xba*I/*Sac*I-digested pMVS vector. Incubate for 3 h at RT.

4. For *E. coli* transformation, we set an *E. coli* Pulser apparatus to 2.5 kV. For each ligation use 40 μL of electrocompetent cells and add 1 μL of the ligation mixture. Mix well and let stand for 1 min on ice.

5. Transfer the mixture of cells and DNA to an ice-cold electroporation cuvette and shake the suspension to the bottom. Place the cuvette into the chamber unit and pulse once. Remove the cuvette and immediately add 1 mL of SOC medium, resuspend with a Pasteur pipette and transfer to a 15 mL tube and shake at 37 °C and 250 rpm for 1 h.

6. For estimation of the library size, plate 10 and 1 μL of the culture on Luria–Bertani broth (LB) agar plates containing 100 μg/mL ampicillin plates. Incubate overnight at 37 °C. Calculate the number of independent clones that can be expected from the ligation, e.g., 50 colonies from a 1 μL aliquot would give 1×10^7 independent clones (50 colonies × 1000 (dilution factor) × 20 (fraction of ligations) × 10 (adjusted to library scale)). To perform the library ligation of light chain, the number of independent clones should be at least 1×10^7 independent clones with a background less than 10%.

7. For cloning the light chain combine 1 μg of *Xba*I/*Sac*I-digested pMVS vector with 330 ng of *Xba*I/*Sac*I-digested

light chain fragment, add 10 μL of 10× T4 DNA ligase buffer, water, and T4 DNA ligase 10 U/mL. Incubate at RT for 3 h.

8. Purify the DNA with the QIAquick PCR Purification Kit. Elute the DNA in 10 μL of elution buffer and store on ice. As for the test ligation electrocompetent *E. coli* cells are thawed, put on ice and cuvettes required for the ligation are also put on ice.

9. Add to the eluted DNA 100 μL of thawed *E. coli*, divide the sample into two cuvettes, store 1 min on ice and then electroporate at 2.5 V. Transfer the cells into a 50 mL Falcon tube. Shake at 37 °C and 250 rpm for 1 h.

10. Add 10 mL SB medium containing 20 μg/mL carbenicillin and 10 μg/mL tetracycline and shake at 37 °C and 250 rpm for 1 h.

11. To assess the number of independent transformants remove immediately after adding the 10 mL SB medium 20, 1 and 0.1 aliquots and plate on LB medium plus 100 μg/mL ampicillin. Shake at 37 °C and 250 rpm for 1 h.

12. After 1 h incubation, transfer the 10–100 mL SB medium containing 50 μg/mL carbenicillin and 10 μg/mL tetracycline. Shake at 37 °C and 250 rpm overnight.

13. Centrifuge at 3000 × *g* for 20 min at 4 °C. Remove the supernatant and prepare from the cell pellet plasmid DNA using Qiagen plasmid maxi kit. Elute DNA in 50 μL and measure the absorbance at 260 nm as previously. Store at −20 °C.

3.5 Production of Helper Phage

1. Streak out *E. coli* strain XL1-Blue onto an LB tetracycline Plate and incubate overnight at 37 °C.

2. Pick a single colony and inoculate into 10 mL of SB medium with 10 μg/mL tetracycline and incubate at 37 °C until $OD_{600} = 0.3$ (approx. 2.0×10^8 cfu/mL).

3. Then two alternatives for proceeding.
   Add helper phage at a multiplicity of infection (MOI) of 20:1 phage to bacterial cells($=4.0 \times 10^{10}$ pfu/10 mL).Add a single plaque of M13K07 helper phage from a fresh plate to the 10 mL culture.

4. Incubate at 37 °C for 30 min without shaking.

5. Transfer to 100 mL of SB (LB) medium containing 10 μg/mL tetracycline and 70 μg/mL kanamycin and incubate at 37 °C with vigorous aeration (225 rpm) for 8 h.

6. Heat the culture to 65 °C for 15 min and then centrifuge at 2794 × *g* for 15 min to discard cell debris.

7. Titer the helper phage produced (Titer should be 1×10^{11}–1×10^{12} pfu/mL).

8. Store at 4 °C (*see* **Note 3**).

9. If the titer is too low, then precipitate the phage by adding 4% of PEG and 3% NaCl. Dissolve by swirling and precipitate the phage on ice for 30 min.

10. Centrifuge at 14,145 × *g* for 20 min at 4 °C. Resuspend the phage pellet in 2 mL of PBS, centrifuge for 5 min at 16,000 × *g* and store the supernatant at 4 °C long-term storage.

3.6 Cloning of the Heavy Chain and Phage Library Preparation

The cloning of the heavy chain depicted in Fig. 2 is followed by the phage preparation using the helper phage VCSM13 helper phage.

1. For cloning, the Fd fragment is first digested with *Xho*I and *Spe*I by mixing 1 μg of Fd DNA with 10 μL of 10× CutSmart Buffer, 3.5 μL of 20 U/μL *Xho*I, and 0.5 μL of 40 U/μL *Spe*I and add water to a final volume of 100 μL. Incubate at 37 °C for 90 min.

2. After inactivation of the enzyme and purification of the DNA (purification as above for the light chains, *see* Subheading 3.4) clone into the *Spe*I/*Xho*I-digested pMVS containing the light chains. For ligation and transformation proceed as previously for the light chains.

3. After transformation and incubation in 1 mL of SOC for 1 h at 37 °C transfer the culture to 100 mL SB containing 20 μg/mL carbenicillin and 10 μg/mL tetracycline. Remove aliquots as before to determine the size of the library.

4. Then shake for 1 h at 250 rpm at 37 °C. After increase the concentration of carbenicillin to 50 μg/mL by adding 3 μL of 1 mg/mL carbenicillin. Shake at 37 °C and 250 rpm for an additional 1 h.

5. Add 1 mL of VCSM13 helper phage (10^{12} pfu) and transfer to 100 mL of SB medium containing 50 μL of 100 mg/mL carbenicillin and 70 μL of 10 mg/mL tetracycline. Continue shaking for 1 h at 37 °C and at 250 rpm.

6. Add 70 μL of 100 mg/mL kanamycin and continue shaking at 37 °C, 250 rpm overnight.

7. Centrifuge at 3000 × *g* for 20 min at 4 °C. Keep supernatant and cell pellet. With the cell pellet carry out phagemid isolation using Qiagen plasmid maxi kit.

8. Elute DNA in 50 μL and measure the absorbance at 260 nm as previously. Store at −20 °C.

9. For the phage preparation, add to the supernatant 33 mL of 16% PEG 8000/12% NaCl. Mix and let stand 30 min on ice. Centrifuge at 15,000 × *g* for 30 min at 4 °C.

10. Discard the supernatant and resuspend in 2 mL PBS containing 1.5 mg/mL of casein and 1.5 mg/mL of HSA and store at 4 °C.

11. Transfer to microfuge tube and centrifuge at 16,000 × g for 5 min.

12. Remove the supernatant and store on ice for panning on the same day. For long-term storage, add 1 vol. of glycerol and store at −20 °C.

3.7 Library Selection on FcεRIα

This step is performed on immobilized human recombinant FcεRIα and carried out during six rounds of panning. After each round of panning, specific binders are eluted and amplified by infection of *E. coli*. The procedure is illustrated in Fig. 3.

1. For each round of biopanning, coat one immunotube using 4 mL of 20 μg/mL FcεRIα or 20 μg/mL HSA in 0.05 M bicarbonate buffer. Incubate overnight at 4 °C or 1 h at 37 °C.

2. Remove the coating solution, fill up the tube with blocking solution. Mix on a rotator tube at 37 °C for 1 h.

3. Shake out the blocking solution, add 4 mL of the amplified phage preparation in 1% BSA to the HSA-coated tube and incubate at RT for 2 h.

4. Transfer the phage solution to the FcεRIα-coated tube and incubate at RT for 2 h.
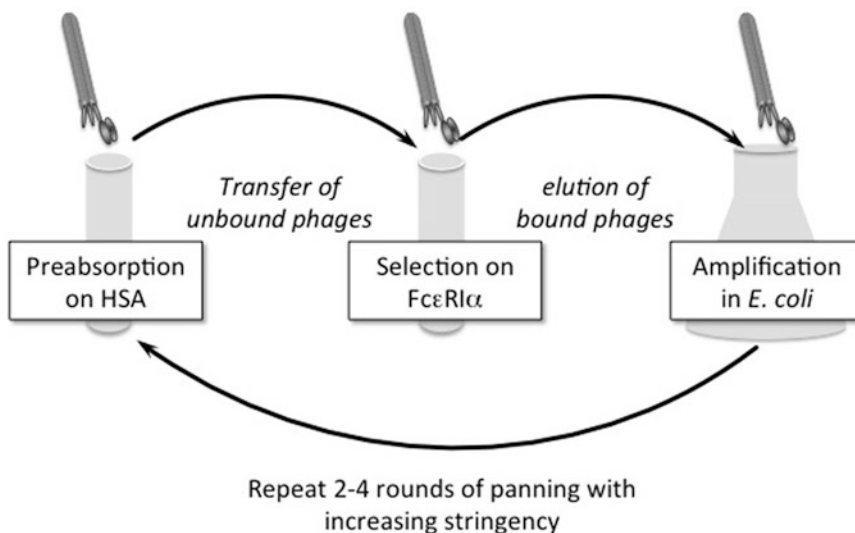
Fig. 3 Schematic illustration of the biopanning procedure for the selection of FcεRIα-specific Fab phages. The total phage population is preabsorbed on HSA, the carrier protein of the used recombinant FcεRIα. Then, the nonbinding phages are transferred to FcεRIα. After washing off the nonspecific phages, the bound phages are eluted by acid treatment and amplified in *E. coli*

5. Remove the phage solution, wash nonbinding phages with 10×5 mL PBS containing 0.1% Tween 20 and 10×5 mL PBS.

6. Wash the tubes with 3×5 mL PBS and elute the remaining phages for 15 min with 500 µL of 0.1 M glycine–HCl and neutralize with 30 µL of 2 M Tris pH 9.5.

7. Pool all eluted phages and incubate for 15 min at room temperature with 4 mL of *E. coli* XL-1 cells ($OD_{600} = 1$) for infection. Amplify and precipitate the phages as described above (*see* Subheading 3.6).

8. For output tittering remove aliquots of 10, 1, and 0.1 µL, add to 100 µL of SB medium and plate on LB plates containing 100 µg/mL of ampicillin. Incubate at 37 °C overnight.

9. For input tittering, prepare 10^{-7}, 10^{-8}, and 10^{-9} NaCl dilutions of phage in SB medium.
Add 5 µL of each dilution to 50 µL of XL1-Blue cells ($OD_{600} = 1$). Incubate 15 min at RT and then plate on LB plates containing 100 µg/mL of ampicillin. Incubate overnight at 37 °C.

3.8 Selection of Specific Anti-FcεRIα Clones

1. If the phage panning reveals an enrichment of phage titer after six rounds of panning, streak clones from 50 colonies on LB agar plates for replica-plating. After 6 h growing at 37 °C, put nitrocellulose filters, soaked in 5 mM IPTG on the plates and bacteria. Incubate overnight at 30 °C.

2. Freeze the filters for 15 min at -80 °C followed by 15 min incubation in chloroform area.

3. To digest bacteria, incubate each filter in 5 mL lysozyme buffer for 2×1 h.

4. Block each filter with 5 mL of blocking solution for 1 h at RT.

5. Incubate overnight each nitrocellulose filter on a tabletop shaker with FcεRIα (10 µg/mL), 10 µg/mL HSA or peroxidase-conjugated sheep anti-human Fab antibody (1/1000 in blocking solution). After washing with 3×10 mL PBS–Tween, incubate the filters previously incubated with FcεRIα and HSA, respectively, with peroxidase-conjugated sheep anti-human albumin (1/1000 in blocking solution) for 2 h at room temperature.

6. Develop the filters with 4-chloro-1-α-naphthol. Reclone the positive clones on LB plates containing 100 µg/mL of ampicillin and isolate DNA from 100 mL cultures using Qiagen plasmid maxi kit according to the manufacturer's instructions.

3.9 ELISA with Single Phage Clones for Definition of Recognition Pattern

If the colony screening reveals positive clones, these should be produced as single phage clones and analyzed by ELISA for recognition pattern.

1. For the production of single Fab phage (Phab) clones, inoculate single colonies in 20 mL of SB medium and incubate overnight at 37 °C.

2. Dilute the cultures 1/100 into 100 mL of fresh SB medium and incubate at 37 °C until $OD_{600} = 0.4$. Add 1 mL of VCSM13 helper phage in 20 fold excess and precipitate the phages as described above (*see* Subheading 3.6).

3. To determine the recognition pattern of the Fab phage clones, coat RIA/EIA plates with the following antigens in 0.05 M bicarbonate buffer as follows: 5 μg/mL of FcεRIα, 5 μg/mL of HSA, and 50 μg/mL of casein. Incubate overnight at 4 °C followed by incubation with blocking solution for 2 h at 37 °C.

4. After discarding blocking buffer, incubate Fab clones in three-fold dilutions (1/3–1/6561) for 4 h at 37 °C. As negative control, use phagemid phage supernatants containing no Fab fragments.

5. Wash plates with 5× PBS–Tween 20 and 5× PBS and then incubate plates with peroxidase-conjugated rabbit anti-phage antibodies for 2 h at 37 °C.

6. Shake out the detecting antibody solution. Wash the wells as before and add 50 μL of HRP substrate solution (TMB) and incubate for 5–20 min at RT.

7. Stop the reaction with 50 μL/well 1 M H_2SO_4. Determine the absorbance at 450 nm with an ELISA microplate reader.

3.10 Nucleotide Sequencing

If the ELISA revealed specific phage clones, they should be analyzed by DNA sequencing after phagemid preparation.

1. Prepare ten tubes containing 5 mL of SB medium supplemented with 50 μg/mL of carbenicillin.

2. Inoculate each tube with one colony from a master plate containing the bacteria corresponding to the positive clones. Shake at 37 °C at 250 rpm overnight.

3. Centrifuge at 3000 × *g* for 20 min at 4 °C. Discard the supernatant and resuspend the pellet in 250 μL of Qiagen buffer P1 from the QIAprep Spin Miniprep Kit. Continue with the phagemid preparation according to the protocol supplied by the QIAprep Spin Miniprep Kit. Elute DNA in 50 μL of elution and store at −20 °C.

4. For sequencing use senseand antisense primers (*see* Table 1). After sequencing, compare heavy and light chain sequences with the V Base Sequence Directory.

3.11 Generation of Full-Length IgG for Functional Studies and Affinity Measurements

For further characterization of affinities and epitope specificity, specific Fab clones are expressed as full-length IgG using an integrated vector system [kindly provided by Dr. A. Bradbury, Trieste, Italy, for details *see* ref. 6].

1. Amplify the VH regions using for amplification forward and reverse primers hybridizing to the 5′ end the VH region: 5′-TCC ACA G*GC GCG* CAC TCC GAG GTG CAG CTG CTC GAG TCT GG-3′ and the 3′ end of the FR4: 5′-ACC ACG GTC ACC GTC TCC TCA GGT GAG TCC T*GT CGA C*GG ATC CA-3′.

2. For cloning of the VH regions the restriction sites of *BssH*II are used at the 5′ end and *Sal*I at the 3′ end of the forward and reverse primers, respectively. For the light chains, the sequence of the Cκ constant region in the vector is replaced by the cDNA coding for the whole λ light chains using the following primers: Cλ reverse: 5′-AAA GT*A CTA GT*C TAC TAT GAA CAT TCT GTA GGG GCC AC-3′, Vλ forward: 5′-TAT CC*G TGC AC*T CCA ATT TTA TGC TGA CTC AGC CCC ACT CTG TG-3′. For the cloning in the light chain, backbone plasmid the *ApaL*I and *Spe*I sites are used, which are located at the 5′ end and 3′ end of the Vλ forward and Cλ reverse primers, respectively.

3. After cloning, transfect both plasmids (heavy chain–light chain, 7:3, total 10 μg/2×10^6 cells) into HEK-293T cells with Lipofectamine 2000 reagent.

4. Propagate HEK-293T cells in DMEM–F12 medium supplemented with 2% of FCS at 37 °C in 5% CO_2. For transformation, seed 2×10^6 cells/well in 6-well TC plates and grow overnight at 37 °C. Prior to transformation, exchange the medium with 2.5 mL/well of serum-free medium.

5. Mix plasmid DNA (heavy chain–light chain, 7:3, total 10 μg/2×10^6 cells) in 250 μL serum-free medium with 15 μL Lipofectamine 2000 reagent diluted in 250 μL serum-free medium and incubated at room temperature for 20 min. After incubation add the mix to the cells and incubate overnight at 37 °C in 5% CO_2. After overnight incubation replace medium with DMEM–F12 medium supplemented with 2% FCS and transfer the cells to TC-Flasks (Becton Dickinson) and culture for 5 days.

6. Purify the antibodies in the cell supernatant on protein G sepharose columns and check the purity on a 9% SDS–polyacrylamide gel.

7. Determine the concentration of the purified IgG by sandwich ELISA using two goat anti-human IgG Abs.

8. The purified full-length anti-FcεRIα IgG can be further tested for their anti-FcεRIα specificity in an ELISA as described in

Subheading 3.9. The purified human monoclonal autoantibodies can then be used for the in vitro characterization as well as for functional studies [6].

4 Notes

1. The solution should be Ca^{2+} and Mg^{2+} free.

2. Fd fragment: variable and first constant domain of the Ig heavy chain.

3. Add DMSO to 7% for storage at $-80\,^{\circ}C$.

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Isolation of Antibodies from Human Plasma, Saliva, Breast Milk, and Gastrointestinal Fluid

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Abstract

Different protocols are required for the collection and isolation of antibodies from various body sites. For the sample collection factors to be considered include anatomic or physiological particularities. Secretory fluids such as saliva, gastrointestinal fluid, or breast milk may contain degrading enzymes that potentially affect the integrity of isolated antibodies. While the isolation of IgG from plasma is a common and often-described procedure, here we focus on methodological approaches to isolate antibodies immunoglobulin A (IgA) or IgM from plasma or secretory fluids. These protocols shall facilitate research on natural and induced antibodies.

Key words IgA, IgM, Secretory antibodies, Antibody collection, Antibody isolation, Saliva, Gastrointestinal fluid, Breast milk, Humoral immunity

1 Introduction

Different immunoglobulins classes are variably distributed among anatomical compartments including distinct tissues, the body surface, or the circulation. Immunoglobulin G (IgG) is the most frequent type of antibodies in the blood, whereas IgA represents the largest fraction among all antibodies in the human body. IgA also represents the predominant class of antibodies at mucosal surfaces, thereby acting as a first line defense against an enormous amount of pathogens and potential invaders [1].

The isolation of immunoglobulin is prerequisite for their analysis and functional characterization, which has resulted in considerable insights into humoral immunity, and natural antibodies in particular. Besides a role in immune defense, plasma-derived natural or induced IgG antibodies have been reported to exhibit various immunomodulatory functions [2–12], which may also contribute

to the anti-inflammatory effects of high-dose treatment with intravenous immunoglobulin (IVIG) [13–17]. High-throughput screening of plasma IgG has gained considerable attention in the last few years [18], and has revealed insights into immunological repertoires in health and disease that are relevant for the prevention, diagnosis, and treatment of diseases [19–22].

While the isolation of IgG from plasma is a commonly performed procedure, the collection and purification of antibodies from other body sites are often more challenging. Secretory fluids, such as breast milk, saliva, or gastrointestinal fluid, contain a variety of lipids and proteases [23–25], which potentially influence the half-life of antibodies and affect the purification effectiveness during affinity chromatography. As a consequence, protocols for the isolation of antibodies from secretes need to include critical protease inhibition and delipidation steps in order to prevent the degradation of antibodies [26]. Here, we provide protocols that we have successfully used for the efficient collection and purification of secretory IgA antibodies. Furthermore, a protocol for IgA and IgM isolation from human plasma is provided. These protocols are intended to facilitate experimentation on IgA and IgM, which may lead to novel insights into the role of these antibodies in immune defense or modulation [27, 28].

2 Materials

2.1 Solutions

1. Equilibration buffer: Phosphate-buffered saline (PBS).

2. Wash/rinse buffer: PBS.

3. Elution buffer: 0.1 M glycine, dissolved in H_2O, pH adjusted to 3 using NaOH or HCl.

4. 0.5 M Tris: Tris base dissolved in H_2O. pH adjusted to 8.0 with HCl.

5. Packing solution: 150 mM NaCl (0.9% NaCl) in H_2O.

6. Gastrointestinal lavage (GIL) fluid recovery buffer: 1 mg/mL soybean trypsin inhibitor (STI) and 0.5 M EDTA dissolved in PBS.

7. Phenylmethylsulfonyl-fluoride (PMSF): PMSF 0.1 M, dissolved in 95% ethanol.

8. BSA–NaN_3 buffer: 0.2% sodium azide and 10 mg/mL bovine serum albumin (BSA) dissolved in PBS.

2.2 Human Immunoglobulin Source Material

1. Human plasma (pooled, also commercially available from for example Sigma-Aldrich®) (*see* **Note 1**).

2. 10 mL of gastrointestinal lavage fluid from the ileocaecal region (obtained by coloscopy), either pooled or from individual donors.

3. Human breast milk, obtained from voluntary donors and stored below −18 °C (*see* **Note 2**).

4. Human saliva, obtained from voluntary donors and stored below −18 °C.

2.3 Affinity Matrix	1. CaptureSelect™ IgA Affinity Matrix (ThermoFisher) with a specified capacity of >8 mg IgA per mL of resin, for IgA-specific affinity chromatography.
	2. CaptureSelect™ IgM Affinity Matrix (ThermoFisher) with a specified capacity of >2.5 mg IgM per mL of resin, for IgM-specific affinity chromatography.
2.4 Other Equipment	1. cOmplete ™ Mini (protease inhibitor cocktail, Roche).
	2. Salivette ® saliva collection device (Sarstedt).
	3. Standard lab equipment for liquid handling, centrifuges, and preparative liquid chromatography system.

3 Methods

3.1 Affinity Chromatography Purification of IgA from Human Plasma

3.1.1 Resin Slurry Preparation for Chromatography Column Packing

1. The IgA affinity resin is supplied as a slurry (~50%) in 20% ethanol. For column packing, transfer sufficient volume of resin slurry from the original to an appropriate container for exchange of the 20% ethanol solution with packing solution. After the resin has settled in the 20% ethanol solution by gravity, remove the ethanol supernatant. Then resuspend the resin by gentle agitation, in a volume of packing solution corresponding to the volume of the removed supernatant. After resettlement of the resin, repeat twice supernatant removal and resuspension in fresh packing solution (*see* **Note 3**).

3.1.2 Column Packing

1. In the present example, pack a column with 25 mm inner diameter to a resin bed height of 51 mm, resulting in a resin bed volume (= CV, column volume) of 25 mL.

2. Start with the column locked in place and being in level. Close the bottom outlet that is not yet connected to the chromatography system and keep the column top open. The top inlet adapter is not yet in place, but connect it to the system and prime with packing solution.

3. Open the bottom outlet and deliver from top 50 mL of prepared resin slurry to the column. Ideally, the column inner volume is large enough to receive the full 50 mL of slurry in one fast go. If not, slow down the slurry delivery according to the outflow rate at the outlet. As soon as the slurry is delivered completely, close the outlet. The resin settles by gravity, without flow.

4. Check the bed height of the gravity-settled resin—in our column example it should be 53–54 mm at this stage (51 mm at the end of the packing procedure). You may add or remove resin with the help of a disposable plastic pipette: resuspend at least the top 1 cm of the settled resin during the pipetting step, and let the resin settle again.

5. Generate a flow of packing solution at 15 mL/min (184 cm/h) through the inlet adapter that has been primed and made free of air. Fill the column from the top with packing solution flowing from the inlet adapter. When the column is full to the edge, reduce the flow rate to 3 mL/min and put the inlet adapter in place at the very top of the column, avoiding any air trapping. Simultaneously, open the column outlet to allow for flow through the column.

6. Bring the inlet adapter down in the column close to the resin bed and lock its position. Increase the flow rate to 15 mL/min. When the resin bed height has stabilized under flow, bring the inlet adapter down to resin and completely close the head space between the adapter and resin. Relock the adapter and stop the flow. The packed column is now ready for full connection to the chromatography system.

3.1.3 Affinity Column Preconditioning

1. Fully connect the packed affinity column to the chromatography system.

2. Rinse the column with 5 CV of PBS at a flow rate of 0.5 CV/min.

3. Perform a cleaning elution step with 5 CV of elution buffer at 0.5 CV/min.

4. Rinse the column with 5 CV of PBS at 0.5 CV/min.

3.1.4 Affinity Column Loading

1. Equilibrate the column with 5 CV of PBS at 0.5 CV/min. Monitor pH, conductivity, and A_{280} recordings of the chromatography system—stable values should be reached: pH ~7.4, conductivity ~ 15 ms/cm, and A_{280} ~ 0 mAU.

2. Load 150 mL (6 CV) of human plasma on the column at 0.2 CV/min (5 mL/min).

3. Wash column with 1 CV of PBS at 0.2 CV/min.

4. Wash column with 4 CV of PBS at 0.5 CV/min.

3.1.5 Affinity Column Elution

1. Elute the column with 4 CV of elution buffer at 0.5 CV/min and collect elution fractions of 0.5 CV each.

2. Adjust pH of individual or pooled elution fractions to 4.5–5.5, by adding 1 mL of 0.5 M Tris pH 8.0 per 20 mL of eluate.

3. Wash column with 10 CV of PBS at 0.5 CV/min.

4. The column is now ready for another chromatography run.

5. Repeat the steps as in the Subheadings 3.1.4 and 3.1.5.

3.1.6 Elution Fraction Processing

1. As indicated above, pool the individual elution fractions (*see* **Note 4**).

2. The pH-adjusted eluate fractions may be further processed for protein concentration and/or buffer exchange (*see* **Note 5**).

3. For periods longer than 10 days, storage of frozen aliquots is recommended.

3.1.7 Affinity Column Preservation

1. If the column is to be preserved (e.g., chromatography break of several days), rinse the column with 10 CV of 20% ethanol at 0.5 CV/min.

2. To resume chromatography with the preserved column, start with steps of Subheading 3.1.3.

3.2 Affinity Chromatography Purification of IgM from Human Plasma

The procedure for IgM affinity chromatography purification from human plasma is nearly identical with the procedure for IgA. Evidently, an affinity resin specific for human IgM (instead of IgA) has to be used. The IgM-specific CaptureSelect resin has a lower specific binding capacity than the CaptureSelect IgA, and the IgM content in human plasma is also lower than the IgA content. In consequence, the recommended loading of the IgM-specific affinity column is different (*see below*). Carry out all other procedure steps as described for IgA purification from human plasma (Subheading 3.1).

3.2.1 Affinity Column Loading

1. Load 125 mL of human plasma on the column at 0.2 CV/min (5 mL/min).

3.3 Collection and Affinity Chromatography Purification of Gastrointestinal IgA

1. Filter 10 mL of gastrointestinal lavage from healthy donors through a sterile gauze into a 50 mL polypropylene tube containing GIL fluid recovery buffer.

2. Centrifuge for 15 min at $1000 \times g$ at 4 °C.

3. Transfer supernatant into a 50 mL polypropylene tube containing 1 mL of 0.1 M PMSF.

4. Leave for 15 min at room temperature.

5. Centrifuge at $1000 \times g$ for 5 min at 4 °C.

6. Transfer the supernatant into a 50 mL polypropylene tube containing 1 mL of 10 mg/mL BSA and 0.2% NaN_3.

7. Proceed to affinity chromatography following the same steps as described under Subheading 3.1 (*see* **Note 6**) or store the samples at −20 °C.

8. For column packing in the present example, a column with 10 mm inner diameter is packed to a resin bed height of 10 mm, resulting in a CV of 0.8 mL.

9. For affinity column loading, load 20 mL (25 CV) of prepared GIT lavage (0.05 mg IgA per mL of GIL fluid preparation) on the column at 0.5 CV/min (0.4 mL/min). Wash column with 5 CV of PBS at 0.5 CV/min.

3.4 Collection and Affinity Chromatography Purification of Salivary IgA

1. Collect human saliva by chewing Salivette® swabs according to the patient instructions.

2. Place each saliva-saturated Salivette® swab into the suspended insert of the Salivette® centrifugation vessel and close the Salivette® with the stopper.

3. Centrifuge the Salivette® at $1000 \times g$ and 10 °C for 2 min.

4. After centrifugation, transfer the clear supernatant (= recovered saliva) from the Salivette® centrifugation vessel to an appropriate container.

5. Add one tablet of cOmplete ™ Mini per 10 mL of saliva.

6. Sterile-filter the saliva through a 0.22 μm filter.

7. Proceed with the freshly prepared saliva to chromatography following the same steps as described under Subheading 3.1 (*see* **Note 7**) or store the saliva as frozen aliquots.

8. For column packing in the present example, a column with 25 mm inner diameter is packed to a resin bed height of 7 mm, resulting in a CV of 3.4 mL.

9. For affinity column loading, load 375 mL (110 CV) of the prepared human saliva on the column at 0.5 CV/min (1.7 mL/min). Wash the column with 5 CV of PBS at 0.5 CV/min.

3.5 Affinity Chromatography Purification of IgA from Human Breast Milk

1. Human breast milk donations can be stored at −25 °C.

2. To prepare the samples for affinity chromatography, the breast milk samples have to be delipidated. Centrifuge $350 \times g$ thawed milk in a swing-out rotor at $12,200 \times g$ and 15 °C for 60 min. After centrifugation, penetrate the thick fat layer with a Pasteur pipette and carefully transfer the underlying delipidated milk from the centrifugation tube to an appropriate container.

3. Proceed to affinity chromatography. The procedure for IgA affinity chromatography purification from delipidated human breast milk is almost the same as from human plasma as described under Subheading 3.1 (*see* **Note 8**). For affinity column loading, load 300 mL of delipidated human breast milk on the column at 0.2 CV/mL (5 mL/min).

4   Notes

1. No source material processing is required prior to affinity chromatography. Store below $-18\ ^\circ$C.

2. Delipidation of milk is important prior to affinity chromatography.

3. The recommended packing solution is 150 mM NaCl (0.9% NaCl in water) with a conductivity similar to the mobile phase during chromatography. In a milieu of higher conductivity than 20% ethanol (in water) the resin beads form a more compact resin bed, which is preferred for column packing.

4. With priority on purity or maximal concentration, the main fraction(s) of the elution peak may be selected. If yield is important, a pool of all peak fractions may be appropriate.

5. Isolated human immunoglobulins show good stability in PBS up to a protein concentration of 50 mg/mL.

6. The procedure for IgA affinity chromatography purification from human GIL fluid is basically the same as from human plasma. The IgA concentration in GIL fluid, however, is much lower than in plasma. Furthermore, the available amount of GIL IgA might be limited. Therefore, an affinity column with reduced resin bed volume (and IgA capacity) and, to cope with the large load volume (relative to the CV), loading at increased flow rate is appropriate, as described below. All other procedure steps can be carried out as described for IgA purification from human plasma.

7. The procedure for IgA affinity chromatography purification from human saliva is basically the same as from human plasma. The IgA concentration in human saliva, however, is about $15\times$ lower than in plasma, and the amount of saliva IgA is limited. Therefore, an affinity column with reduced resin bed volume (and IgA capacity) and, to cope with the large load volume (relative to the CV), loading at increased flow rate is appropriate, as described below. All other procedure steps can be carried out as described for IgA purification from human plasma.

8. The IgA concentration in human breast milk is lower than in plasma, with 0.45 mg/mL about 40% of the plasma concentration. Therefore, the recommended affinity column load volume is higher. All other procedure steps can be carried out as described for IgA purification from human plasma.

Acknowledgments

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Purification of Natural Antibodies Against Tau Protein by Affinity Chromatography

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Abstract

Natural antibodies are now widely studied for their therapeutical potential. Therefore, their isolation and subsequent characterization is desired. Here, we describe an isolation method for natural anti-tau antibodies from human plasma by utilization of affinity chromatography with epoxy-activated copolymer resin. The evalution of isolation efficiency and avidity of isolated antibodies is decribed by modified indirect ELISA assay.

Key words Human plasma, Immobilization, Immunopurification, Immunoreactivity, Intravenous immunoglobulin, Natural antibodies, Tau protein

1 Introduction

It is now well accepted that natural autoantibodies are ubiquitous in human serum [1]. Their occurrence is influenced by age, gender, and disease [2]. The titers of these antibodies are remarkably stable over time [3, 4]. Numerous natural antibodies reactive with different neuronal proteins have been detected both in the serum of patients with Alzheimer's disease (AD) and control subjects. These facts increase general interest in revealing the relevance and therapeutic potential of natural autoantibodies [5–10]. The natural antibodies from intravenous immunoglobulin products containing IgG molecules pooled from several thousand healthy donors are now widely studied [11–20]. Some techniques focusing on purification and characterization of antibodies were described in the previous series of Methods in Molecular Biology [21–23]. However, we aim to describe the isolation of natural antibodies from human plasma against protein associated with AD using solid-phase affinity chromatography. For this purpose, we utilized solid support with epoxy groups on the surface that can easily react with an amine, thiol or hydroxyl groups of protein and form very stable covalent bond [24–26] and thus expose various epitopes of protein

to the antibodies. Moreover, epoxy groups are stable at neutral pH that is convenient for long storage periods [25]. Immobilization on epoxy-activated copolymer resins is a process where physical adsorption of protein on the hydrophobic surface has to occur to allow closer contact between reactive groups. This interaction is facilitated by high ionic strength buffers [27]. Subsequently, previously adsorbed protein is covalently attached to the support [25, 28, 29]. Finally, free epoxy groups are neutralized by either binding of amino acids or solutions containing amino groups. The elution of natural antibodies reactive with the antigen (in this case tau protein) on the column is performed at low pH. Although an exposure of monoreactive antibodies to low pH elution buffer may cause the change in their binding properties [30], we did not observe the change in reactivity of natural tau-reactive antibodies after exposure to low pH [11]. The isolation efficiency was assessed by ELISA assay as well as the avidity of the natural antibodies. Avidity of natural antibodies was described to be low [31, 32]. Hereinafter, except the isolation of natural antibodies, we describe one method of avidity estimation using ammonium thiocyanate modified according to Pullen, 1986 [33].

2   Materials

Prepare all solutions using redistilled water and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials.

2.1   Immunosorbent Preparation

1. Binding buffer: 0.1 M NaH_2PO_4–NaOH, 1 M NaCl pH 9.2. Weigh 3.58 g of $Na_2HPO_4 \cdot 12H_2O$ and 5.84 g of NaCl on separate weighing boats and transfer quantitatively to a 100 mL glass beaker. Add water to a volume of 90 mL. Mix and adjust pH with NaOH (*see* **Note 1**). Make up to 100 mL with water.

2. Inactivation solution: 0.2 M ethanolamine–HCl, pH 8.5–9. Pipette 0.12 mL of 99% ethanolamine (16.5 M) to 8 mL of water in a glass beaker. Mix and adjust pH with HCl (*see* **Note 2**). Make up to 10 mL with water.

3. Labiomer 300 resin (epoxy-activated copolymer of 2-hydroxyethyl methacrylate and ethylene dimethacrylate, lyophilized powder, 50 μm bead) (Labio). Alternatively, use Fractogel® EMD Epoxy (Merck), Eupergit® C (Rohm Pharma Polymers), or TOYOPEARL® AF-Epoxy-650 M (TOSOH Bioscience).

4. Empty Econo-Pac Chromatography gravity flow column (14 cm length, 1.5 cm diameter, 20 mL, Bio-Rad).

5. Pierce™ BCA Protein Assay Kit.

6. Microtubes (1.5 and 2 mL).

7. Antigen: Prepare recombinant human tau protein (1–441 aa, the longest isoform) according to Hromadkova et al. [11]. Use freshly dissolved antigen.

8. Sodium azide (*see* **Note 3**).

2.2 Antibody-Purification

1. Phosphate buffered saline (PBS): Weigh 8 g NaCl, 0.2 g KH_2PO_4, 0.2 g KCl, 2.9 g $Na_2HPO_4 \cdot 12\ H_2O$ on separate weighing boats and transfer quantitatively to a 1000 mL graduated laboratory bottle with cap. Add water to a volume of 950 mL. Mix and check pH; it should be at pH 7.4. Make up to 1000 mL.

2. Elution buffer: 0.1 M glycine–HCl, pH 2.6. Weigh 0.75 g of glycine and transfer to a 100 mL glass beaker. Add water to a volume 90 mL. Mix and adjust pH with HCl (*see* **Note 2**). Make up to 100 mL with water in a volumetric flask.

3. Titration solution: 1 M Tris. Weigh 12.11 g of Tris base and transfer to a 100 mL volumetric flask. Add water to a volume 100 mL.

4. pH test strips.

5. Amicon® Ultra 15 mL filters 30 K (30 kDa molecular weight cutoff, Merck Millipore).

6. Storage solution: 50% glycerol (*see* **Note 4**).

7. 96-well microplates (Gama group, Czech Republic).

8. Washing buffer: 0.1% bovine serum albumin (BSA) in PBST. Follow instructions for preparation of PBS and add 1 mL of 100% Tween 20 detergent. Then add water to a volume of 1000 mL. Weigh 1 g of BSA in a 1000 mL beaker and add 1000 mL of PBST. Mix properly.

9. Blocking buffer and diluent for ELISA: 1% BSA in PBST. Weigh 1 g of BSA in a 100 mL beaker and add 100 mL of PBST. Mix properly.

10. F(ab′)$_2$-goat anti-human IgG (Fc specific, highly cross-adsorbed/horseradish peroxidase conjugate) (Novex, Life Technologies).

11. 2 M sodium acetate–CH_3COOH, pH 5.5: Weigh 16.4 g of sodium acetate and transfer to a 100 mL glass beaker. Add water to a volume 90 mL. Mix and adjust pH with acetic acid (CH_3COOH) (work in fumehood). Make up to 100 mL with water in the volumetric flask.

12. 3,3′,5,5′-tetramethylbenzidine   (TMB):   6   mg/mL. Weigh 120 mg and dissolve in 20 mL of dimethylsulfoxide (*see* **Note 5**).

13. TMB substrate (TMB+, 1 plate/11 mL): Mix 0.55 mL of 2 M sodium acetate-CH_3COOH, pH 5.5, 0.18 mL of 6 mg/mL TMB, 11 μL of 3% H_2O_2 (3% H_2O_2: 10 μL of 30% H_2O_2 + 90 μL of water), and 10.3 mL of water. Prepare fresh solution just before using. Alternatively, use 1-Step™ Ultra TMB-ELISA Substrate Solution ready-made mix (Thermo Fisher).

14. 1 M H_2SO_4: Add 5.6 mL of 96% H_2SO_4 to 90 mL of water and make up to 100 mL.

15. 4 M NH_4SCN: Weigh 3.045 g of NH_4SCN in a glass beaker and add water to final volume of 10 mL.

2.3 Natural Antibodies

1. Intravenous immunoglobulin product: Immune Globulin Intravenous (Human) Flebogamma DIF (5 g/100 mL) (Grifols Biologicals Inc.) (*see* **Note 6**).

2. Human plasma samples: Prepare at least 3 mL of plasma pool with total protein concentration around 100 mg/mL (approximately 0.5 mL of plasma per subject). Estimate the protein content with BCA assay according to manufacturer's instructions.

3 Methods

3.1 Preparation of Immunosorbent for Antibody Purification

Always make sure that the prepared resin with bound antigen does not dry out. Do not use buffers containing amino groups when working with epoxy-activated resin.

1. Add 3 mL of lyophilized epoxy-activated Labiomer resin to a 20 mL column (1.5 × 14 cm). You can try the coating efficiency of different available resins using BSA.

2. Place a cap on the base of the column.

3. Dissolve antigen in binding buffer to final concentration 1–5 mg/mL (*see* **Note 7**). If the antigen is in a solution, make sure that the buffer does not contain any amino groups (desalt the antigen if necessary). Free amino groups would be coupled to the resin, which would result in lower coupling efficiency.

4. Add antigen solution to the resin in the maximum ratio 5 mg of protein per 1 mL of resin and mix gently on a rotator overnight at 4 °C.

5. Allow the column to drain into a 50 mL tube. Collect the flow-through fraction (F-T) with the unbound protein.

6. Estimate the protein concentration in the F-T. The coupling efficiency can be determined by comparing the peptide concentrations of the F-T to the starting preparation of antigen.

7. If necessary repeat the **steps 4–6** for sufficient antigen coating of the resin with freshly prepared antigen or F-T.

8. Wash the column with 50 mL of binding buffer (*see* **Note 8**).

9. Inactivate free epoxy groups with 0.2 M ethanolamine overnight at 4 °C while rotating.

10. Equilibrate the column with 20 mL of PBS and store in PBS containing 0.05% (w/v) sodium azide at 4 °C.

3.2 Purification of Natural Antibodies

1. Equilibrate the stored affinity column containing bound antigen with 20 mL of PBS to remove sodium azide.

2. Spin the plasma pool and IVIG product at $16{,}000 \times g$ to remove insolubles. Dilute 3 mL of human plasma pool or IVIG with PBS to a final volume of 10 mL (*see* **Note 9**).

3. Pipette plasma solution onto the resin in the column, allow to flow 0.1 mL/min through the column (for 1.5–2 h) and collect the F-T into a 50 mL tube.

4. Wash the column with 50 mL of PBS to remove unbound natural antibodies (*see* **Note 10**).

5. Elute the antigen-reactive antibodies from the column with 10 mL of elution buffer. If possible, check the elution by UV detector connected to the column to minimize the elution volume.

6. Neutralize eluted antigen-reactive antibodies immediately with drops of 1 M Tris to obtain pH 7–8. Check the pH with pH test strips.

7. Transfer the natural antigen-reactive antibodies to Amicon® Ultra 15 mL filter 30 K. Spin the filter at $2000 \times g$ in swing-out rotor at 4 °C for 5 min repeatedly (*see* **Note 11**) to obtain 2 mL of concentrated antibody solution.

8. Estimate the concentration of purified antibodies spectrophotometrically at absorbance 280 nm in parallel to PBS buffer as a blank for IgG from IVIG product or by BCA assay for antibodies obtained from crude plasma pool (*see* **Note 12**).

9. Add glycerol to antibody solution to final concentration 50% in PBS and store the antibodies at −40 °C.

10. Neutralize the column with 50 mL of PBS and store in PBS containing 0.05% sodium azide.

11. Check the isolation efficiency of natural antibodies using an ELISA assay. Dilute the antigen in coating buffer to obtain concentration 4 μg/mL. Coat 50 μL of antigen solution onto the well of the microplate and incubate overnight at 4 °C. Leave every third column of the plate without coating for blank control (*see* **Note 13** and Fig. 1).

	1	2	3
A	A+, 1° Ab+, 2° Ab+	A+, 1° Ab+, 2° Ab+	A−, 1° Ab+, 2° Ab+
B	A+, 1° Ab+, 2° Ab+	A+, 1° Ab+, 2° Ab+	A−, 1° Ab+, 2° Ab+
C	A+, 1° Ab+, 2° Ab+	A+, 1° Ab+, 2° Ab+	A−, 1° Ab+, 2° Ab+
D	A+, 1° Ab+, 2° Ab+	A+, 1° Ab+, 2° Ab+	A−, 1° Ab+, 2° Ab+
E	A+, 1° Ab+, 2° Ab+	A+, 1° Ab+, 2° Ab+	A−, 1° Ab+, 2° Ab+
F	A+, 1° Ab+, 2° Ab+	A+, 1° Ab+, 2° Ab+	A−, 1° Ab+, 2° Ab+
G	A+, 1° Ab+, 2° Ab+	A+, 1° Ab+, 2° Ab+	A−, 1° Ab+, 2° Ab+
H	A+, 1° Ab−, 2° Ab+	A+, 1° Ab−, 2° Ab+	A−, 1° Ab−, 2° Ab+

Fig. 1 The proposed distribution of samples on the plate measured by ELISA for natural antibodies. The rows *A–G* in the columns 1 and 2 represent positive samples, row *H* stands for negative control: effect of antigen with secondary antibody. Rows *A–G* in the third column represent blank controls for occurrence of nonspecific signal. The cell 3H shows effect of secondary antibody alone on the obtained signal. The final results should be interpreted by substraction of blank and negative control for each sample. Legend: A+: antigen-coated well; A−: noncoated well (without antigen); 1° Ab: primary antibody; 2° Ab: secondary antibody

12. Wash the plate once with washing buffer (0.25 mL/well) and block with 0.2 mL/well of blocking buffer for 2 h.

13. Serially dilute the initial plasma pool or original IVIG product, the F-T and eluted antibodies as follows: 1:100, 1:300, 1:900, 1:2700, 1:8100, 1:24,300, and 1:72,900 by 1% BSA in PBST.

14. Wash the plate once with washing buffer and add 0.1 mL of diluted antibodies per well. Run the analysis in duplicates and with a blank control (*see* proposed scheme in Fig. 1). Add 0.1 mL of 1% BSA in PBST to coated and noncoated wells as a negative control. Incubate 2 h.

15. Wash the plate five times with washing buffer and add 0.1 mL/well of 1:10,000 diluted secondary antibody conjugated to HRP for 30 min.

16. Wash the plate five times with washing buffer and incubate 0.1 mL/well of freshly prepared TMB+ substrate for 30 min in the dark. Shake the plate in this step using a shaker for microplates to evenly distribute the substrate and product of the enzymatic reaction.

17. Stop the reaction with 0.1 mL/well of 1 M H_2SO_4.

18. Read the absorbance with the plate reader at 450 nm and 620 nm as a reference wavelength (*see* **Note 14**).

19. Plot the obtained data as absorbance at wavelength 450 nm depending on the logarithm of antibody dilution using dose–response curve. The Hill slope will give you information about the affinity of antibodies in the appropriate fraction for the antigen. The bigger the Hill slope, the higher the affinity of antibodies.

20. Determine the avidity index of natural antibodies using chaotropic agent ammonium thiocyanate (NH_4SCN). The
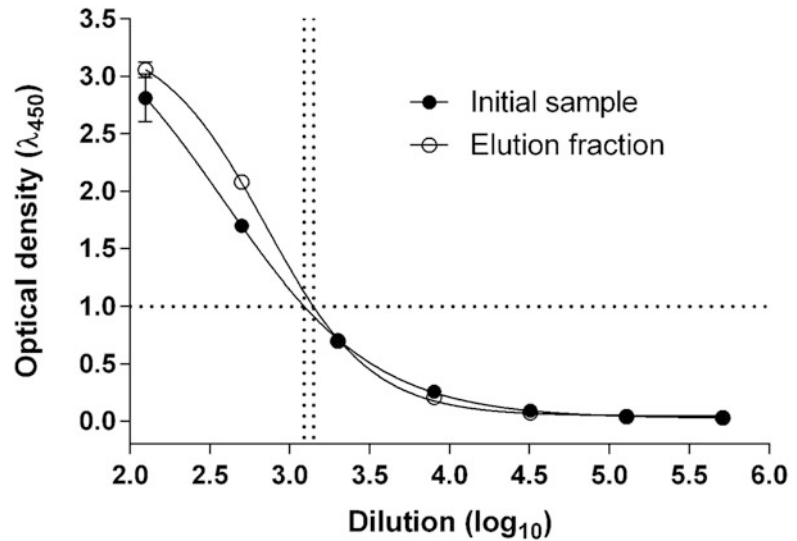
Fig. 2 Ilustration of data fitting using GraphPad Prism software and interpolation of dilution factors for OD value 1

experimental conditions are similar as for measurement of isolation efficacy by ELISA till the **step 12** of this procedure.

21. Choose dilutions of initial fractions (plasma pool or IVIG product) and elution fractions with similar OD signal (*see* Fig. 2). Dilute the samples with diluent to the final volume of 2.5 mL.

22. Wash the plate once with washing buffer and add 0.1 mL of diluted samples per well. Run the analysis in duplicates and with blank control. Incubate for 2 h.

23. Prepare NH_4SCN in PBS within the following molarity range: 2.1 M, 1.8 M, 1.5 M, 1.2 M, 0.9 M, 0.6 M, and 0.3 M using 4 M stock solution. Use PBS alone to obtain 100% signal intensity (0 M of NH_4SCN) of the sample.

24. Wash the plate five times with washing buffer and add 0.1 mL/well of NH_4SCN for 5 min.

25. Wash the plate five times with washing buffer and add 0.1 mL/well of 1:10,000 diluted secondary antibody conjugated to HRP for 30 min.

26. Wash the plate five times and incubate 0.1 mL/well of freshly prepared TMB+ substrate for 30 min in the dark. Shake the plate in this step using the shaker for microplates to evenly distribute the substrate and product of the enzymatic reaction.

27. Stop the reaction with 0.1 mL/well of 1 M H_2SO_4.

28. Read the absorbance with the plate reader at 450 and 620 nm as a reference wavelength (*see* **Note 14**).
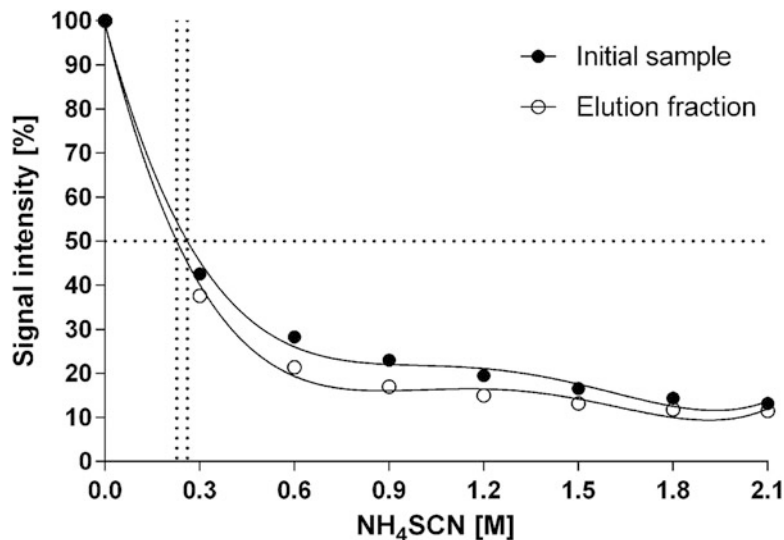
Fig. 3 Ilustration of data plot for avidity index measurement in GraphPad Prism software. Curve was fitted using fourth polynomial order with interpolation of unknowns from the curve

29. Avidity index is defined as a molar concentration of thiocyanate solution that causes the decrease of 100% signal intensity (0 M NH_4SCN) on the value 50%. Plot the data as signal intensity in % depending on the molarity of NH_4SCN and use fourth order polynomial to fit the curve in GraphPad Prism software (equation: $Y = B0 + B1 \times X + B2 \times X^2 + B3 \times X^3 + B4 \times X^4$). Interpolate the 50% signal from the curve (*see* Fig. 3). Alternatively, calculate the 50% reduction from the linear regression eq. ($Y = a + bX$) of logarithmically transformed values of % signal intensity.

4   Notes

1. Prepare 4 M NaOH and titrate the solution using drops of NaOH till reaching the desired pH. Be careful when preparing NaOH as dissolution of solid NaOH in water is highly exothermic reaction in which a large amount of heat is liberated, posing a threat to safety through the possibility of splashing.

2. Concentrated HCl (12 N) can be used at first to narrow the gap from the starting pH to the required pH. From then on it would be better to use a diluted HCl (e.g., 1 N) with a lower ionic strength to avoid a sudden drop in pH below the required pH.

3. Sodium azide is very hazardous in case of skin contact (irritant). It is also hazardous in case of ingestion, inhalation and to the aquatic environment.

4. Glycerol is a highly viscous liquid, and pipetting of precise volume can be difficult. Cut the end of the tip and slowly pipette the desired volume of glycerol. Rinse the tip properly to transfer all glycerol to the solution.

5. TMB is not stable in aqueous solutions. Store at 4 °C in the dark. Hazardous material with acute toxicity (oral, dermal, and inhalation), category 4.

6. Spin the solution of natural antibodies at $16,000 \times g$ in fixed-angle rotor for 5 min to remove any insolubles before applying onto affinity column.

7. We store purified tau protein as a precipitate under 55% methanol at -40 °C. Mix the precipitate and remove 200 μL of suspension. Spin the suspension at $16,000 \times g$ for 5 min at 4 °C. Discard the supernatant and let the pellet dry for 5 min. Dissolve the precipitate in 200 μL of ice-cold PBS on the ice. Let the solution sit for 10 min and then spin as previously. Transfer supernatant if pellet forms to a new tube and estimate the concentration by BCA assay. Use the antigen immediately as tau protein is highly prone to aggregation. You may run electrophoresis to check the protein; if necessary, treat the protein solution with 6 M urea to obtain monomers of tau protein. Dialyze the protein solution in PBS. If your protein of interest is not stable at high pH, you may couple the antigen to the resin in pH 7 using PBS buffer, but usually, the coupling efficiency is lower.

8. It is critical to avoid using any buffer containing amino groups (i.e., Tris-buffered saline).

9. In case of dilution of plasma sample or cross-interaction of albumin with the assay, you may remove albumin from crude plasma pool by precipitation of IgG using 50% (w/v) ammonium sulfate overnight at 4 °C. The final precipitate contains IgG. Dissolve the precipitate after removing the supernatant in PBS. Let it sit for 10 min and spin at $16,000 \times g$ for 5 min to remove any insolubles.

10. Rinsing the column with PBS will remove antibodies specific to other antigens; if you are interested in antibodies with higher affinity/avidity for antigen of interest, you may rinse the column with 20 mL of 1 M NaCl followed by 50 mL of PBS. The isolation yield will be probably lower, though.

11. From our experience, the concentration of proteins and antibodies in centrifugal concentrators due to the fast decreasing volume of buffer results in irreversible precipitation. Therefore, the concentration should be carried stepwise. Spin the concentrator with antibody solution for 5 min at $2000 \times g$ in swing-out rotor and 4 °C, mix the solution with a pipette as the antibody molecules tend to be pulled to the bottom of the filter. Repeat this procedure till you reach the desired volume.

12. The IVIG product contains 99% of IgG molecules [34–36]. Therefore, the estimation of concentration may be done spectrophotometrically at 280 nm. Use 1 mL of each purification fraction and measure the absorbance in parallel with PBS buffer as a blank. According to literature, 1 mg/mL of pure IgG has an absorbance of 1.4 at 280 nm [37]. Calculate the final concentration of IgG as absorbance of the sample divided by 1.4 and multiplied by 1 mg/mL. If the concentration of the sample is high (i.e., the absorbance has reached a value close to 3), you should dilute the sample ten times and multiply the calculated concentration with this dilution factor. For crude plasma pool without treatment with ammonium sulfate before the immunopurification procedure, use BCA assay.

13. According to our experience with measurement of natural antibodies occurring in blood, the type of microplate has an influence on the obtained results. The Maxisorp surface has a high binding affinity for hydrophilic proteins, and that results in a nonspecific signal even after thorough blocking of the wells when samples of sera and plasma are used in the assay. We recommend optimizing the method for the type of microplate used (choose Medisorp binding plates or Polysorp if the antigen is hydrophobic) and measure the blank control in parallel for each sample.

14. The absorbance at 620 nm monitors the quality of the plate bottom. It is not mandatory to measure it, but we recommend to control this variable.

Acknowledgment

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Chapter 5

Unbiased RACE-Based Massive Parallel Surveys of Human IgA Antibody Repertoires

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Abstract

For investigations of human B-cell receptor (BCR) repertoires, we have developed a protocol for large-scale surveys of human antibody heavy chain (VH) rearrangements. Here we study IgA repertoires, as more IgA antibodies are synthesized in the human body on a daily level than all other isotypes combined. In fact, IgA is secreted at all mucosal surfaces, and it is also secreted in the perspiration that coats our cutaneous surfaces. In these studies we can characterize the IgA clonal diversity of B-cell populations obtained from any donor. To recover representative repertoire libraries, we make our libraries from antibody gene transcript templates (i.e., cDNA), as these are closer reflections of the immune repertoire expressed at the antibody protein level. To avoid biases potentially introduced by upstream oligonucleotide primers that hybridize to variable region framework regions, our approach also uses rapid amplification of cDNA ends (RACE) of antibody transcripts. For exploration of human IgA responses, we have designed a duplexing antisense constant region primer that efficiently amplifies, side-by-side, heavy chain transcripts of both the IgA1 and IgA2 subclasses. By these methods we have begun to define the molecular differences in the IgA1 and IgA2 responses occurring simultaneously in different donors. These methods will be used to investigate the effects of microbial virulence factors on host defenses, during autoimmune responses, and in B-cell malignancies.

Key words IgA antibody repertoire, B cell repertoire, IgA1, IgA2, Natural antibodies, Sequencing, MiSeq

1 Introduction

The B-cell antigen receptor (BCR) system uses a range of overlaid somatic diversification mechanisms to mold and refine a relatively limited number of inherited germ line-encoded minigene segments into a repertoire of clonally distributed antigen receptors that can have exquisitely refined binding affinity and molecular specificity. It has recently been estimated that healthy adults have ~10^{11} lymphocytes, which are distributed into about 5 million clonal sets, that fill the human immune system (discussed in [1]). Throughout life there is a continuous process of neolymphogenesis with sequential

stages of de novo antibody gene rearrangement and phenotypic differentiation from precursors in the bone marrow and many clones with inherent autoreactivity are edited out. With immune maturation new antigenic exposures contribute to clonal selection and expansion, and when levels of eliciting antigen subsequently wane due to clearance, many clones then collapse, which leaves space for other clones to respond to new challenges. Hence, there is a dynamic and shifting balance between B-cell clones.

The generation of B cell clones begins in utero and we are born with a repertoire of naturally arising antibodies (NAbs) of the IgM isotype that first arise without known prior immune or vaccine exposure to populate the immune system. These antibodies have been linked to a distinct set of mature B cells, termed B-1 cells, which represent the first wave of B-cell lymphogenesis in the body [2, 3]. NAbs are postulated to prime the early immune system and to serve two complementary functional roles [4]. A subset of these antibodies recognize microbial pathogens and protect from infection [5], and may also help to maintain balance amongst intestinal commensal bacteria [6]. Studies from our group and others have shown that some NAb clonal sets, which recur even in unrelated individuals, have a refined type of autoreactivity that can enhance fundamental housekeeping functions of the innate immune system [7–10]. B-1 cell derived NAbs can augment the clearance of cells dying of apoptotic death and aid the control of overexuberant inflammatory responses [11]. Antibodies to the B-1 cell linked determinant, phosphorylcholine (PC), have been shown to correlate with protection from atherosclerotic cardiovascular disease and tissue injury in systemic autoimmune disease both in murine models and in humans [12–16].

Based on antibody gene analyses, NAbs in mice generally display germ line or near-germ line configuration gene sequences that encode for binding sites that are often highly cross-reactive, although with very specialized types of binding specificities. Furthermore, the murine repertoire of NAb-producing B cells has been shown to contain lymphocyte clones with the same antibody gene rearrangements that have been shown to often recur in different individuals [17, 18]. Indeed there is a prototypic murine anti-PC clone that has been independently isolated from peritoneal or splenic B cells by several different research labs. These anti-PC B-cell lines with identical,or near identical, antibody gene rearrangements have been designated S107, E06, and several other names as well (discussed in [19]). The clonally related anti-PC NAb set called TEPC15 (or T15) was first isolated as an IgA-secreting plasmacytoma [20]. The first structure–function studies of NAbs used amino acid sequencing based assays, which were later followed by antibody gene sequence analyses, but these methods were suitable only for small-scale surveys of relatively limited sets of B-lineage lymphocytes [18, 21–25].

Recent advances in massive parallel DNA sequencing have now enabled the sampling of immune repertoires on a much larger scale [26–31]. From each sample, libraries can be generated with thousands or even hundreds of thousands of sequences. We now have the capacity to investigate how immune repertoires differ over time, and how they can vary between individuals of different ages [32]. Studies of homozygotic twins have also illustrated how two individuals that share the same genetic inheritance can develop B cell repertoires with key differences presumably due to cumulative subtle differences in antigenic exposures and stochastic events [21, 33].

With these approaches we can investigate the repertoires of B-cell subsets at different stages of immune development, such as between immature B cell, transitional B cell subsets, and mature naïve B cells. It is also reported that there are differences between phenotypically distinct sets of mature peripheral B cells. Indeed there are differences in gene usage and diversification patterns in B-cell subsets postulated to have different functional roles in immune defenses, such as follicular recirculating B cells (i.e., B-2 cells), marginal zone B cells [30, 34], B-1 cells that have been implicated as sources of natural antibodies and terminally differentiated plasma cells that are the antibody-producing factories of the body [27, 28]. From recently developed methods for deep sequencing of BCR repertoires, there are reports that patients with autoimmune disease can have highly characteristic abnormalities, with preferential VH gene usage [35].

Despite the capacity of the BCR system to generate immense somatic diversity from a limited number of inherited germ line gene segments, there are now several reports showing that different adults can nonetheless have clonal sets of antibody rearrangements with highly conserved protein sequences in the somatically generated V–D–J splice site of the heavy chain complementarity determining region (i.e., public HCDR3) [33]. In particular, such evidence of convergent somatic clonal evolution and selection has been found in VH gene sequencing studies of blood B cells from unrelated patients infected by dengue, a viral pathogen [36], as well as after vaccination for influenza [37]. These convergent VH sequences may reflect in vivo antigenic selection by limited dominant sets of microbial antigens in response to infections [38], and following therapeutic vaccination [39] (reviewed in [19]). The occurrence of shared HCDR3 in the antibody genes expressed in NAbs has not yet been specifically investigated, yet should help to illuminate how other types of common antigens such as products of physiologic cell breakdown and commensal microbes, may drive B-cell clonal selection of NAb repertoires.

In our own first high-throughput BCR surveys, we investigated the effects of in vivo infusions of a microbial protein, protein L (PpL), which is produced by the human commensal anaerobic

bacteria, *Finegoldia magna,* one of the most common pathogenic species among gram-positive anaerobic cocci. Our studies were designed to study the immunobiologic properties of PpL which was known to interact with conserved motifs in V regions of B cell Ag receptor (BCR) shared by large sets of lymphocytes [29]. Therefore, we defined the properties of this candidate B-cell super-antigen by generating libraries of Vκ L chain transcripts from the spleens of control and PpL-exposed C57BL/6 mice, and the expressed Vκ rearrangements were characterized by high-throughput sequencing with the 454 instrument (Roche). A total of 120,855 sequencing reads could be assigned to a germ line Vκ gene, with all 20 known Vκ subgroups represented. In control mice, we found a recurrent and consistent hierarchy of Vκ gene usage, as well as patterns of preferential Vκ–Jκ pairing. Significantly, PpL exposure induced significant global repertoire shifts with targeted and specific reductions in the representation of Vκ genes that contain the PpL-associated putative superantigen-binding motif in each of the individual exposed mouse. We found significant targeted reductions in the expression of clonotypes encoded by 14 specific Vκ genes with the predicted PpL binding motif [29].

These investigations incorporated newly developed high-throughput sequencing technology along with in-house developed bioinformatics tools, which has been the subject of a subsequent report [40]. The inclusion of phylogenetic information in a codon-based approach can result in more accurate assignment of immunoglobulin heavy (IGH) chains to germ line V and J alleles (and to D alleles in VH regions), particularly for mutated rearrangements [40]. In our first studies of the murine response to PpL this next-generation sequencing platform provided a thorough overview of the effects on B-cell clonal diversity of exposure to a single microbial factor, which was not feasible with traditional Sanger DNA sequencing technology [29]. We are already using the improved versions of this software in more recent VH repertoire studies, as described below.

For the sampling of an immune repertoire, antibody gene amplification can be performed at the genomic level that can provide a more direct linkage to B-cell numbers. However, we and many other investigators have instead opted to make libraries from antibody gene transcript templates (i.e., cDNA), largely because we sought a closer reflection of the immune repertoire that is expressed at the antibody protein level. As a consequence of immune maturation, a single end-differentiated plasma cell can have 100- to 1000-fold the number of copies of antibody genetic information than that of a resting B cell that by comparison may make little or no contribution to the circulating antibody response (discussed in [1]).

For amplification of antibody genes, the most common approach uses panels of oligonucleotides designed to mimic V

gene leader or framework sequences. Yet there is always the risk that even a panel of degenerate primers will ligate and prime some germ line V genes more efficiently than others. Due to this concern, we have instead used an alternative approach termed rapid amplification of cDNA ends (RACE), which efficiently sidesteps this challenge by ligation of adapters onto the 5′ ends of the templates [29]. Other groups have also described methods that incorporate RACE in their repertoire studies of humans [38] and other species [41, 42].

For investigations of BCR repertoires in humans, we are currently focusing on large-scale surveys of the repertoire of VH rearrangements. The somatic origins of the HCDR3, that arise from combinatorial events of V, D, and J minigenes, together provide the greatest information regarding B-cell clonal origins. The HCDR3 also have greatest genetic and structural diversity of all subdomains within an antibody; and structure–function studies have also shown that the HCDR3 subdomain is generally the single most important contributor to antibody binding specificity [43]. During immune development, while IgM is the first isotype to be expressed, IgA antibodies are subsequently synthesized in greatest quantities, and in adults more IgA antibodies are made on a daily basis than all other isotypes combined, and IgA is also the predominant source of NAbs [44]. Human alpha H chain rearrangements are amongst the least explored parts of the B cell compartment, and these responses in particular provides an underexplored mystery of functional immunity [45].

Human IgA repertoires pose a series of inherent structural and functional dualities. Akin to most other isotypes, IgA are first expressed on B cells as membrane-associated receptors, while subsequent lymphocyte differentiation can be associated with production of IgA in secreted forms that are often dimers. Indeed, our bodies produce an estimated 3–5 g of IgA each day, exceeding all other antibody isotypes combined. Secreted IgA antibodies therefore virtually flood all of our mucosal surfaces, and we even secrete IgA in the perspiration that coats our skin [46]. Secretory IgA thereby augment the first line of defense by preventing entry into the body [47].

There is also a duality with regard to human IgA subclasses. Humans like the old world monkeys and apes (e.g., Chimpanzees, Gorillas, Gibbons) express IgA of two very different subclasses; IgA1 and IgA2, which [48] have hinge regions that convey substantial functional differences [49]. While the Fab'2 of IgA2 has a more compact overall structure, the IgA1 structure is more extended with antigen-binding arms that have generally greater flexibility that enables interactions with a broader range of antigens [50]. IgA1 and IgA2 antibodies also have different anatomic biases in the anatomic sites. In the bloodstream, most IgA are of the IgA1 subclass while IgA2 is the predominant subclass secreted along gut

surfaces [51, 52]. These two subclasses also vary in their suscepti-bility to proteases [53], with the IgA2 more stable in the harsh mucosal surface environments. IgA1 also have more glycosylation sites than IgA2 [50], Otherwise, the human alpha1 and alpha2 constant region genes are very similar in primary sequence, with only 22 amino acids difference of which 13 of these are absent from the hinge region of IgA2. In the region we are using there are only 5 nucleotides differences between these subclasses, *see* Fig. 2) in the VDJ proximal CH1 (i.e., first domains of these alpha genes) [54].

In the design of our strategy for the amplification and recovery of these alpha heavy chain rearrangements, we took advantage of this natural sequence variation through the design of a the 3′ antisense oligonucleotide primer to incorporate a very limited level of nucleotide degeneracy, which enables equal efficiency of a1 and a2 gene priming and subsequent balanced amplification of a1 and a2 rearrangements in the same mRNA sample. By this approach, we simultaneously amplify parallel IgA1 and IgA2 libraries that each has distinct and assignable part of CH1 sequences, in the same tube. As a consequence, all of the alpha Ig rearrangement amplimers from a lymphocyte sample, whether α1 or α2 heavy chain rearrangements are embedded with the same DNA sequence barcode for identification of the biologic source.

The RACE-based protocol described below provides additional potential advantages as there are additional regions from the VH locus for assigning a read to a specific clonotypic origin. These VH amplimers have longer reads than are recovered by traditional approaches as these include inherent "untranslated regions" (UTR) and leader sequences. Although these are not part of the translated amino acid sequence in the BCR expressed on the surface of a B cell, these naturally sequences occur at the 5′ end of every VH rearrangement transcript. Hence, this additional gene and gene family-specific sequence information can enable even more accurate assignments to the germ line gene segments of origin.

In our approach we apply an adaptation of a commercial RACE protocol that adds an adapter called SMARTer II® (Clontech, Mountainview CA) oligonucleotide at the 5′ of the RNA transcript, resulting in the generation of the first strand cDNA (Fig. 1) that also contains the untranslated region (UTR) and the leader sequence. These sequences which are situated between SMARTer II oligonucleotide and V region can serve as a genetic marker for the discrimination between V families, and can therefore be of great importance for the assignment of the germ line V gene segment. This adapter serves as an annealing region of the 5′ specific primer, circumventing the need for using V region specific primers (Fig. 2).
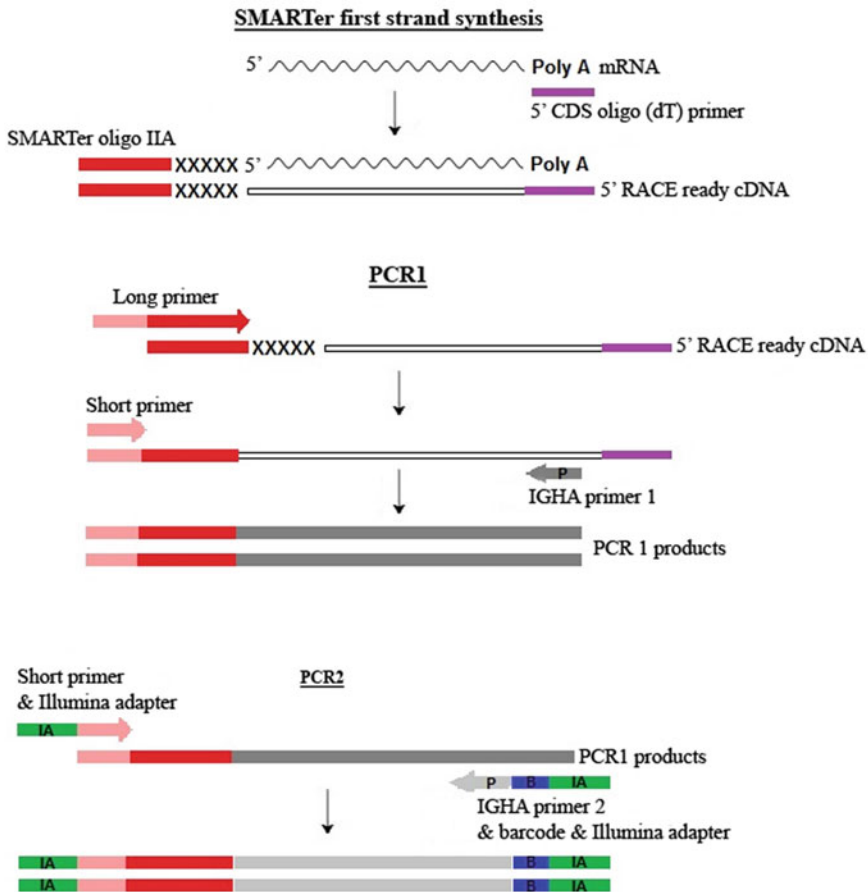
Fig. 1 Generation of IGHA libraries for sequencing on the Illumina MiSeq. In the first step, first strand cDNA is generated with a modified oligo dT primer to poly A tail of the RNA transcript, which is then extended by SMARTScribe® reverse transcriptase (RT) (Clonotech). At the end of RNA template, SMARTScribe® RT adds several nontemplated residues that anneal to SMARTer® II A oligonucleotide, which serves as a template for SMARTScribe® RT enabling subsequent amplification of the full-length RNA transcript. Subsequently, VH libraries for α chain are generated by a two-step RACE PCR protocol. PCR1 is performed with a heavy chain constant region antisense primer and the universal primer mix (UPM) as a forward primer. UPM is a mix of two primers with different length, which are 45 and 22 nucleotides long, respectively. The 23 nucleotides at the 3′ of the long primer anneal to an identical region at the 5′end of SMARTer® IIA oligo, and adds in suppression PCR inverted repeat elements ensuring the generation of libraries having SMARTer® IIA oligo only at 5′ of the cDNA, as cDNA flanked at both ends with SMARTer® IIA oligo is prevented from amplification by forming a panhandle. The short primer is present at 5× higher concentration than the long primer. Therefore, once the long primer is used up in the first rounds of PCR, the short primer takes over and anneals to the 22 bases at 5′ of the long primer for amplification in the remaining rounds of PCR. PCR2 reaction adds in Illumina adapter sequences on both ends of the transcripts to allow high throughput sequencing with the Illumina MiSeq instrument and a donor-specific barcode to enable the discrimination between donor-specific libraries after pooling and sequencing. The forward primer is composed of an adjusted sequence of the short primer and an Illumina adapter sequence, which binds to the Illumina flow cell, immobilizes DNA, and allows sequencing to occur. The reverse primer consists of a donor-specific barcode; CH1 gene-specific primer and Illumina adaptor (*see* Table 4). Our α-specific-primer amplifies both α1 and α2 libraries, permitting comparison of the heavy chain sequences in IgA1 and IgA2 responses that seem to have different structural and functional characteristics. *B* barcode, *IA* Illumina adapters, and *P* primer

IGHA-primer2

```
IgA1  5'-GTAGGGGCTGGTCGGGGTTCCAGAAGGGCGACTCGGAGacGTCGTGGGtcGgTCTACCCTTGCACCAGtAGCGgACGGACCAGGTCCCGAAGAAGGGGGTC-3'
IgA2  5'-GTAGGGGCTGGTCGGGGTTCCAGAAGGGCGACTCGGAGctGTCGTGGGggGtTCTACCCTTGCACCAGcAGCGtACGGACCAGGTCCCGAAGAAGGGGGTC-3'
```

Fig. 2 The nucleotide differences in the CH1-region of IgA1 and IgA2 that are covered by IGHA-primer2. The *underlined* nucleotides represent the sequence of the IGHA-primer2

2 Materials

1. PAXgene® Blood RNA Tubes (PreAnalytiX GmbH).
2. PAXgene® Blood RNA Kit (PreAnalytiX GmbH).
3. SMARTer® RACE 5′/3′ kit (Clontech).
4. Primers.
5. Agarose I.
6. DNA ladder.
7. Electrophoresis buffer: TAE (40 mM Tris–acetate, pH 8, 1 mM EDTA).
8. SYBR Safe DNA gel stain (Invitrogen).
9. Gel Loading Dye, Purple.
10. Agencourt AMPure XP®.
11. 3 M sodium acetate–acetic acid, pH 5.2.
12. Absolute ethanol (99.9%).
13. Glycogen.
14. Micropipettes and tips (20, 200, 1000 μL).

3 Methods

3.1 Isolation of RNA

1. Extract RNA from whole peripheral blood collected in PAX gene blood RNA tubes according to the protocol of the PAXgene® Blood RNA Kit. This system is useful not only for collection, storage, and transportation of whole blood specimens but also for stabilization of intracellular RNA (*see* **Note 1**). RNA can be stabilized for up to 50 months when blood is collected in PAX gene blood tube and stored at −20 °C or −70 °C.

2. To provide reliable results, it is essential to assess the quality of RNA before generating cDNA. We determine RNA Integrity Number (RIN) of the extracted RNA samples using Agilent 2100 Bioanalyzer. Use RNA samples with RIN equal to or higher than 7 to generate VH libraries (*see* **Notes 2–4**).

3. Use 400 ng of purified RNA in a volume that does not exceed 10 μL to generate cDNA. Concentrate the RNA samples that have a low concentration of RNA by precipitation.

3.2 RNA Precipitation	1. Measure the volume of RNA samples that need to be concentrated.

1. Measure the volume of RNA samples that need to be concentrated.

2. Add 10 µg of glycogen. Glycogen serves as a carrier for RNA and will help precipitate it and visualize it after centrifugation.

3. Add 1/10 volume of 3 M sodium acetate–acetic acid, pH 5.2 and mix well.

4. Add 2× volumes of cold absolute ethanol and mix well.

5. Place the tubes at −20 °C overnight.

6. Centrifuge at maximum speed (19,000 × g) at 4 °C for 30 min (*see* **Note 5**).

7. Carefully pipet off the supernatant.

8. Add 1 mL of 70% ethanol and mix.

9. Spin at maximum speed (19,000 × g) at 4 °C for 30 min.

10. Carefully pipet off the supernatant by first using 1000 µL pipet and then 20 µL or 10 µL pipet to get rid of as much ethanol as possible to enhance the air dry of the pellet.

11. Place the tubes with RNA pellet on ice and let air dry for about 1 h (*see* **Note 6**).

12. Resuspend the pellet in the elution buffer supplied by PAXgene Blood RNA Kit.

3.3 Generation of First-Strand cDNA

Generate first-strand cDNA using the SMARTer® RACE 5′/3′ kit. The protocol allows the conversion of 10 ng to 1 µg of total RNA into first-strand cDNA in 20 µL reaction volume. This kit can be used to generate 5′ or 3′ first-strand cDNA. For our studies we generated 5′ first-strand cDNA.

1. Combine 400 ng of RNA present in a volume of 1–10 µL depending on the concentration of RNA with 1 µL of 5′-CDS primer A and 0–9 µL of sterile H_2O in PCR tubes.

2. Gently mix. If the sample is not in the bottom of the tube, centrifuge briefly at 14,000 × g.

3. Place the RNA-samples in the thermocycler to heat denature at 72 °C for 3 min, then cool to 42 °C for 2 min. Brief centrifuge at 14,000 × g.

4. Make buffer mix in a total volume of 5.5 µL: 4 µL 5× first-strand buffer, 0.5 µL DTT, and 1 µL dNTPs. Prepare an extra reaction to ensure sufficient volume.

5. Add 0.5 µL of RNase Inhibitor and 2.0 µL of SMARTScribe® Reverse Transcriptase to the 5.5 µL of buffer mix to make a 8 µL master mix. And mix by gently pipetting.

6. Add the 8 µL master mix to the denatured RNA prepared in **step 1**.

7. Add 1 µL of the SMARTer IIA® Oligonucleotide per reaction for a total volume of 20 µL per cDNA synthesis reaction.

8. Mix thoroughly by gently pipetting up and down, followed by brief centrifugation.

9. Incubate at 42 °C for 90 min, followed by heat denaturation at 70 °C for 10 min on PCR thermocycler.

10. Add 30 μL of Tricine–EDTA Buffer and store at −20 °C.

3.4   Primer Design

1. Design the reverse primers specific for the constant region 1 of the heavy chain following the criteria of SMARTer RACE protocol and the general rules for designing primers.

2. Generate VH-α libraries using two different reverse primers in two separate PCRs. The IGHA-primer2 for PCR2 is designed to hybridize at a 5′ region of the constant region 1, which is very close to the 3′ of the VH to produce amplicons having a size that does not exceed 600 bp and can be sequenced from both ends (2 × 300 bp) on Miseq instrument. In contrast, there was no restriction on the size of the amplicons obtained from using primer 1 in PCR1.

3.5   Rapid Amplification of cDNA Ends (RACE)

For the generation of VH libraries, we have developed a protocol consisting of two PCRs; first and second PCR using two different reverse CH-specific primers (*see* Tables 1 and 2).

Table 1
Primer sequences for the amplification of VH-α in PCR1

Primer name	Sequence
Long primer	5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′
Short primer	5′-CTAATACGACTCACTATAGGGC-3′
IGHA-primer1	5′-GCAGGGCACAGTCACATCCTGGCTGGGA-3′

Table 2
Primer sequences for the amplification of VH-α in PCR2 and sequencing on Illumina MiSeq

Primer	Sequence
Adjusted Short primer	5′-AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTCTAATACGAC TCACTATAGGGCAAGCAGTG-3′
IGHA-primer2	5′-CAAGCAGAAGACGGCATACGAGATXXXXXXAGTCAGTCAGCCCAGGCAKGCGAYGACCACGTTCCCATC-3′[a]
Read 1 primer	5′-TATGGTAATTGTCTAATACGACTCACTATAGGGCAAGCAGTG-3′
Read 2 primer	5′-AGTCAGTCAGCCCAGGCAKGCGAYGACCACGTTCCCATC-3′
Index primer	5′-GATGGGAACGTGGTCYTCGCKTGCCTGGGCTGACTGACT-3′

[a]X in the primer sequence denotes the position of the sample specific barcode sequence

3.5.1 PCR1

Universal primer mix (UPM) and CH1-specific primer (IGHA-primer1) are used as forward and reverse primer respectively to amplify the first strand cDNA.

1. Prepare a master mix for each 25 μL PCR reaction by mixing the following components: 7.75 μL PCR-grade H_2O, 12.5 μL $2\times$ SeqAmp® buffer, and 0.5 μL SeqAmp® DNA polymerase for a total volume of 20.75 μL. Make an extra reaction to ensure sufficient volume.

2. Set up PCR1 reactions in 0.5 mL PCR tubes by adding the reagents in the same order as presented in Table 3. Mix gently by pipetting up and down (*see* **Notes 7** and **8**).

3. Place the PCR tubes in a thermal cycler and run the program presented in Table 4.

4. Analyze the PCR products on 1% agarose gel. Since PCR products obtained after amplification under the PCR conditions shown in Table 4 for 26 cycles are not readily visible on the agarose gel, we amplified 5 μL of the PCR products for an additional 9 cycles under the same condition and analyzed on 1% agarose gel (Amplimers at 600–725 bp can be gel-purified to be used as template for PCR2).

Table 3
RACE PCR reactions for PCR1

Reagent	Volume (μL)
First strand cDNA	1.25
$10\times$ UPM	2.50
IGHA-primer1 (10 μM)	0.50
Master mix	20.75
Total volume	25.00

Table 4
PCR cycling condition for PCR1

	Temperature (°C)	Time (s)
Initial denaturation	98	30
26 cycles		
Denaturation	98	10
Annealing	66.1	10
Extension	72	20
Final extension	72	300

3.5.2 PCR2

PCR2 is a seminested PCR, in which PCR1 products are used as a template and a different specific primer for the constant region of the VH-α and an adjusted version of the short primer are used as reverse and forward primer respectively. In this PCR, VH libraries are prepared for sequencing on Illumina MiSeq platform. Therefore, we used primers to which Illumina adapters and sample barcode are added (Table 2; adjusted short primer and IGHA-primer2). For the design of those primers we adopted the method reported by Caporaso et al. [55]

1. Assemble the following reagents to prepare master mix for each 50 μL PCR reaction: 15.5 μL PCR-grade H_2O, 25 μL 2× SeqAmp buffer, and 1 μL SeqAmp DNA polymerase for a total volume of 41.5 μL. Prepare an extra reaction to ensure sufficient volume.

2. Dilute PCR1 product at 1:50 in PCR-grade H_2O.

3. Add the diluted PCR1 product into 0.5 mL PCR tubes together with other PCR components as shown in Table 5.

4. Run the PCR in a thermal cycler under the conditions shown in Table 6.

5. Subject 50 μL of the PCR products to gel electrophoresis and excise the bands with the right size (*see* Fig. 3a) using a sharp scalpel.

6. Purify the bands using NucleoSpin® gel and PCR cleanup, a component included in SMARTer® RACE 5′/3′ kit.

3.6 Gel
Electrophoresis

1. Make a 1% agarose gel in TAE buffer.

2. Swirl the flask to mix the agarose.

3. Bring the mixture to a boil in a microwave until the agarose solution becomes clear and all agarose particles are dissolved without bubbles.

4. Cool down the Agarose.

Table 5
RACE PCR reactions for PCR2

Reagent	Volume (μL)
PCR1 products (1:50)	5.0
Adjusted short primer	1.0
IGHA-primer 2 (10 μM)	1.0
PCR-grade H_2O	1.5
Master mix	41.5
Total volume	50

Table 6
PCR cycling condition for PCR2

	Temperature (°C)	Time (s)
Initial denaturation	98	30
26 cycles		
Denaturation	98	10
Annealing	64.6	10
Extension	72	20
Final extension	72	300
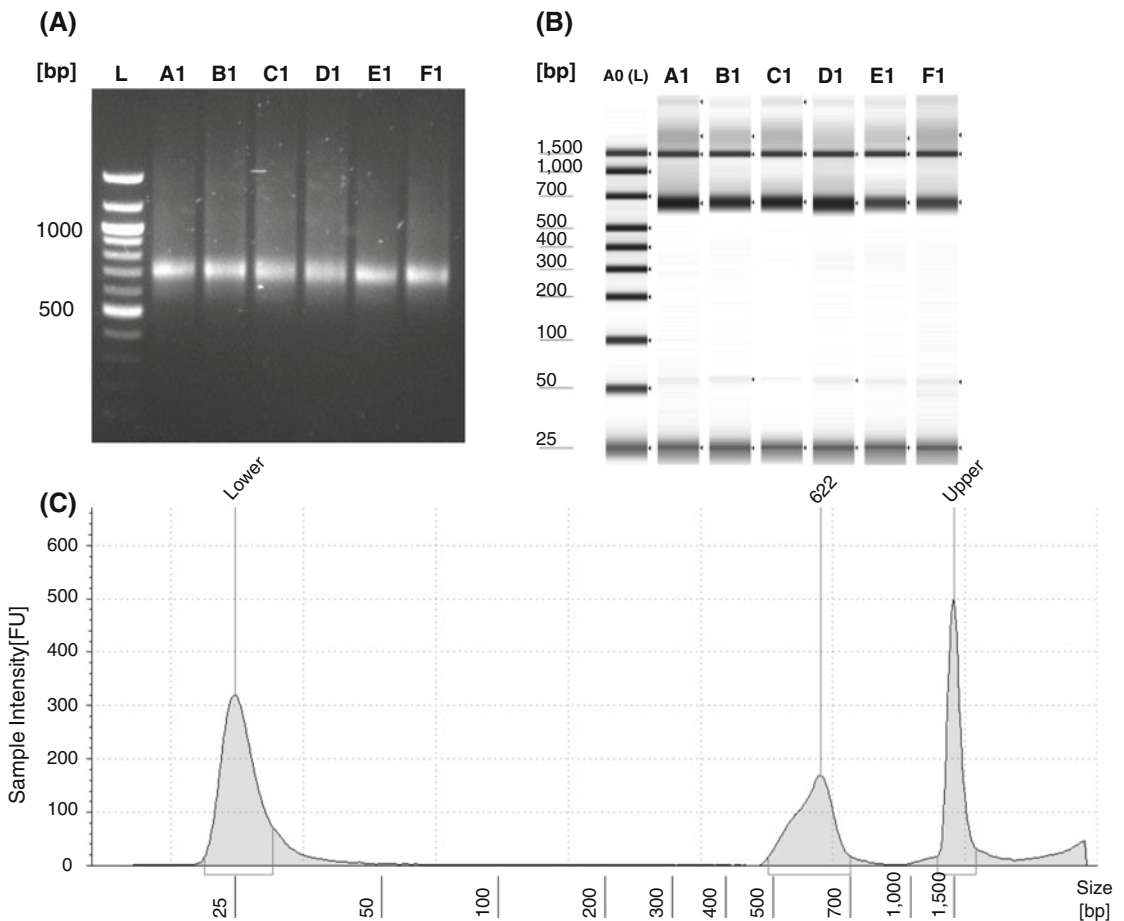
Fig. 3 Preparation of amplicons for sequencing on Illumina MiSeq instrument. (**A**) 1% Agarose gel electrophoresis of 6 amplicons after PCR2. (**B**) Analysis of the same amplicons as presented in (**A**) by electrophoresis on the acrylamide-based gel of the TapeStation to determine the concentration. (**C**) Equal molar quantities of each individual library were pooled and analyzed by gel electrophoresis on Pippin Prep system, with the band purified in the size range of 500–700 bp. At the left side and the right side are MW markers of 25 bp and 1500 bp respectively. *L* DNA ladder

5. Add 3 μL of SYBR® per 50 mL agarose solution to visualize the PCR products under the UV light.

6. Swirl the flask to ensure good mixing and pour the agarose gel in a gel tray and place the comb of choice.

7. Allow the gel to solidify.

8. Prepare PCR products by adding a loading buffer and mixing up and down.

9. Briefly centrifuge to position the PCR product sample at the bottom of the tube especially when the volume of the sample is small.

10. Put the gel tray in the electrophoresis tank with TAE buffer and remove the comb.

11. Load PCR products into the well, and load a DNA ladder to evaluate the size of the bands.

12. Run at 100 V for 1 h.

13. Expose the gel to the UV light.

3.7 Preparation of IgA Libraries for Sequencing on MiSeq

1. Purify the PCR products obtained from PCR2 to remove contaminants using paramagnetic beads (Agencourt AMPure XP®). Elute the purified PCR products in 10 mM Tris (*see* **Note 9**).

2. Analyze the purified PCR products by gel electrophoresis using High Sensitivity D1000 ScreenTape to determine the concentration of the amplicons of the right size (*see* Fig. 3b).

3. Pool the Amplicon samples at equal concentration and analyze by gel electrophoresis on Pippin Prep® system to purify the band between 500 and 700 bp.

4. Spike 10% of Phix library into the sample library to enhance the diversity and to serve as a quality control.

5. Use custom primers (*see* Table 2; read 1 primer, read 2 primer, and index primer) and Illumina primers to sequence amplicons library and Phix respectively.

6. We performed paired ends 300 bp sequencing on the VH libraries using the Illumina MiSeq instrument.

3.8 Analysis

The analysis of BCR can be divided into three steps.

1. In step 1, preprocess the data to provide barcode-sorted, quality-controlled, and paired end assembled sequencing reads.

2. In step 2, identify the germ line V, D, and J gene segments that were rearranged to generate V heavy chains and determine the population structure of the repertoire, which is shaped by clonal expansion and affinity maturation.

3. In step 3, analyze the overall BCR repertoire.

There are different ways for processing, filtering, and aligning Ig sequences to the germ line V, D, and J gene segments. Our sequencing data are analyzed according to a pipeline: IgSCUEAL that we have previously reported [29, 40]. Based on the amino acid sequence similarities, V gene families form distinct phylogenetic clades. Therefore, IgSCUEAL is designed to consider phylogenetic relationship between germ line genes. This approach is particularly useful for the clustering of rearranged VH–DH–JH sequence with an ancestral sequence. To aid analyses of the data, we have compiled the DNA sequences for the 5′ UTR (*see* Table 7) and Leaders (*see* Table 8) for human VH genes.

4 Notes

1. RNA expression profile is prone to changes that can be a result of induction of gene expression during sample handling and/or rapid RNA degradation. It is essential to obtain RNA that reflects in vivo gene expression, which can be achieved through minimizing ex vivo gene expression and preventing degradation through contamination with environmental RNases. PAX gene blood RNA tubes contain proprietary reagents that protect RNA from degradation and minimize further induction of gene expression.

2. It is essential that DNase is pipetted directly on the membrane of the PAXgene RNA spin column. Not placing DNase mix onto the membrane will lead to incomplete DNA digestion and therefore to a low quality RNA.

3. Prepare DNase mix for an extra sample to ensure all the samples get the same amount and any DNA in the RNA-sample is removed.

4. DNase provided in the PAXgene Blood RNA Kit cannot be purchased separately. An alternative is RNase-free DNAse set from Qiagen.

5. Longer centrifugation at a high speed aids in obtaining an enhanced RNA yield.

6. Air dry of the RNA pellet is done on ice, which takes longer time. Air dry at RT for 15-20 min works as well.

7. Prepare PCR reaction on ice when you have plentiful samples.

8. Add the master mix with the polymerase shortly before running PCR in thermal cycler.

9. Replacing the buffer helps in successful running of the obtained PCR products from PCR2 on the gel of the Pippin Prep® system.

Table 7
5′ UTR of human VH genes

VH1

```
5UTR-V1-46*01    AAGCATCATCCAACAACCACATCCCTTCTCTACAGAGCCCTCTGAGAGGAAAGTTCTTCACC
5UTR-V1-46*03    AAGCATCATCCAACAACCACATCCCTTCTCTACAGAGCCCTCTGAGAGGAAAGTTCTTCACC
5UTR-V1-24*01    GACCATCACACAACAGCCACATCCCTCCCTCACAGAAGCCCCC-AGAGCGCAGCACCTCACC
5UTR-V1-69-2*01  GAGCATCACACACAACAGCCACATCCCTCCCTCACAGAAGCCCCC-AGAGAGCAGCACCTCACC
5UTR-V1-58*01    GAGCATCATCCAGAAACCACAACCACATCCCTCCGCTAGAGAAGCC--CTGACGGCACAGTTCCTCACT
5UTR-V1-58*02    GAGCATCATCCAGAAACCACAACCACATCCCTCCGCTAGAGAAGCC--CTGACGGCACAGTTCCTCACT
5UTR-V1-69*04    GAGCATCACATAACAACCAACCAGATTCCTCCTCTAAAGAAGCCCCTGGGAGCACAGCTCATCACC
5UTR-V1-69*06    GAGCATCACATAACAACCAACCACATTCCTCCTCTGAAGAAGCCCCTGGGAGCACAGCTCATCACC
5UTR-V1-69*14    GAGCATCACATAACAACCAACCACATTCCTCCTCTGAAGAAGCCCCTGGGAGCACAGCTCATCACC
5UTR-V1-69*01    GAGCATCACATAACAACCAACCACATTCCTCCTCTAAAGAAGCCCCTGGGAGCACAGCTCATCACC
5UTR-V1-69D*01   GAGCATCACATAACAACCAACCACATTCCTCCTCTAAAGAAGCCCCT-AGATCACAGCTCCTCACC
5UTR-V1-18*04    GAGCATCACCCAAAAACCACACCCCATCTGTCCTCTAGAGAATCCCCT-AGATCACAGCTCCTCACC
5UTR-V1-02*04    GAGCATCACCCAGCAACCACATCCCTCCTCTAGAGAATCCCCTGAGAGCTCCGTTCCTCACC
5UTR-V1-45*02    GAGCATCACCCAACAACCACATCCCTCCTCTAGAGAATCCCCTGAAAGCACAGCTCCTCACC
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VH2

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5UTR-V2-70*13    AGTGAATCCTGCTCTCCACC
5UTR-V2-70D*04   AGTGAATCCTGCTCTCCACC
5UTR-V2-70D*14   AGTGAATCCTGCTCTCCACC
5UTR-V2-05*01    AGTGACTCCTGTGCACCACC
5UTR-V2-05*02    AGTGACTCCTGTGCCCCACC
5UTR-V2-26*01    AGTGACTCCTGTGCCCCACC
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VH3

```
5UTR-V3-48*01    AGCTCTCAGAGAGGTGCCTTAGCCCTG-GATTCCAAGGCATTTCCACTTGGTGATC-AGCACTGAACACAGAGGACTCACC
5UTR-V3-48*02    AGCTCTCAGAGAGGTGCCTTAGCCCTG-GATTCCAAGGCATTTCCACTTGGTGATC-AGCACTGAACTGAACACAGAGGACTCACC
5UTR-V3-73*01    AGCTCTGGGAGAGAGCTCCAGCCTTGGGATTCCCAGCTGTCTCCACTCGGTGATC-GGCACTGAATACAGGAGAGAACTCACC
5UTR-V3-23*01    AGCTCTGAGAGAGGAGCCC-AGCCCTGGGATTTTCAGTGTGTTTCATTTGGTGATC-AGGACTGAACAGAGAGAACTCACC
5UTR-V3-23D*01   AGCTCTGAGAGAGGAGCCC-AGCCCTGGGATTTTCAGTGTGTTTCATTTGGTGATC-AGGACTGAACAGAGAGAACTCACC
5UTR-V3-23D*02   AGCTCTGAGAGAGGAGCCC-AGCCCTGGGATTTTCAGTGTGTTTCATTTGGTGATC-AGGACTGAACAGAGAGAGAACTCACC
5UTR-V3-64*01    AGCTCTGGGAGAGAGCCCCCCCCCANCCGTGAGATTCCCAGAGTTTCCACTTGGTGATC-AGCACTGAACCAGACACCAACC
5UTR-V3-49*01    AGCTCTGGGAGAGAGCCCCAGCCGTGAGATTCCCAGAGTTTCCACTTGGTGATC-AGCACTGAACCAGACCACCAACC
5UTR-V3-49*03    AGCTCTGGGAGAGAGCCCCAGCCGTGAGATTCCCAGAGTTTCCACTTGGTGATC-AGCACTGAACCAGACCACCAACC
```

5UTR-V3-13*02	AGCTCTGGGAGTGGAGCCCCCAGCCTTGGGATTCCCAAGTGTTTGTATTCAGTGATC-AGGACTGAACACACAGGACTCACC
5UTR-V3-13*05	AGCTCTGGGAGTGGAGCCCCCAGCCTTGGGATTCCCAAGTGTTTGTATTCAGTGATC-AGGACTGAACACACAGGACTCACC
5UTR-V3-73*02	AGCTCTGAGAGCGGAGCCCCCAGCCCCAGAATTCCCAGGTGTTTCATTTGGTGATC-AGCACTGAACACACAGAGGACTCACC
5UTR-V3-11*06	AGCTCTGGGAGAGAGCCCCCAGCCCCAGCCCCAGAGTTCCATTCGGTGATC-AGCACTGAACACACAGAGGACTCGCC
5UTR-V3-66*03	AGCTCTGGGAGAGAGCCCC-AGCACTGGGATTCCGAGTGTTTCCATTCAGTGATC-TGCACTGAACACACAGAGGACTCACC
5UTR-V3-53*02	AGCTCTGGGAGAGAGCCCC-AGCACTGGGATTCCGAGTGTTTCCATTCGGTGATC-AGCACTGAACACACAGAGGACTCACC
5UTR-V3-53*03	AGCTCTG-GAGAGAGCCCC-AGCACTGGA--TTCCGAGTGTTTCCATTCGGTGATC-AAGCACTGAACACACAGAGGACTCACC
5UTR-V3-30*01	AGCTCTGGGAGAGAGCCCC-AGCACTGGAAGTCGCCGGTGTTTCCATTCGGTGATC-AGCACTGAACACACAGAGGACTCACC
5UTR-V3-33*01	AGCTCTGGGAGAGAGCCCC-AGCACTAGAAGTCGGCGGTGTTTCCATTCGGTGATC-AGCACTGAACACACAGAGGACTCACC
5UTR-V3-64D*06	AGCTCTGGGAGAGAGCCCCCAGGCCCGGGATTCCCAGGTGTTTCCATTCAGTGATC-AGCACTGAAGACAGAAGACTCATC
5UTR-V3-43*01	AGCTCTGGGAGAGAGCCCCCAGCCCCAGCCCCTGAGATTCCCAGGTGTTTCCATTCGGTGATC-AGCACTGAACACACAGAGGACTCACC
5UTR-V3-09*01	AGCTCTGGGAGAGAGCCCCCAGCCCCAGCCCCTGAGATTCCCAGGTGTTTCCATTCGGTGATC-AGCACTGAACACACAGAGGACTCACC
5UTR-V3-09*03	AGCTCTGGGAGAGAGCCCCCAGCCCCAGCCCCTGAGATTCCCAGGTGTTTCCATTCAGTGATC-AGCACTGAACACACAGAGGACTCACC
5UTR-V3-20*01	AGCTCTGGGAGAGGACCCCCCAGCCCCAGCCCCTGAGATTCCCACGTGTTTCCATTCAGTGATC-AGCACTGAACACACAGAGGACTCGCC
5UTR-V3-20*03	AGCTCTGGGAGAGGACCCCCCAGCCCCAGCCCCTGAGATTCCCACGTGTTTCCATTCAGTGATC-AGCACTGAACACACAGAGGACTCGCC

VH4

5UTR-V4-39*01	ATGCTTTCTGAGAGTCA-TGGATCTCATGTGCAAGAAA
5UTR-V4-34*01	GTGCTTTCTGAGAGTCA-TGGACCTCCTGCACAAGAAC
5UTR-V4-34*02	GTGCTTTCTGAGAGTCA-TGGACCTCCTGCACAAGAAC
5UTR-V4-34*11	GTGCTTTCTGAGAGCTCATGGACCTCCTGCACAAGAAC
5UTR-V4-04*07	ATACTTTCTGAGACTCA-TGGACCTCCTGCACAAGAAC
5UTR-V4-38-2*02	ATGCTTTCTGAGAGTCA-TGGACCTCCTGTGCAAGAAC
5UTR-V4-04*01	ATACTTTCTGAGAGCTCATGGGCCTCCTGCACAAGAAC
5UTR-V4-04*08	ATACTTTCTGAGAGTCC-TGGACCTCCTGCACAAGAAC
5UTR-V4-28*01	ATACTTTCTGAGAGCTC-TGGACCTCCTGTGCAAGAAC
5UTR-V4-31*02	ATACTTTCTGAGAGTCC-TGGACCTCCTGTGCGAGAAC
5UTR-V4-59*02	ATACTTTCTGAGAGCC--TGGACCTCCTGTGCAAGAAC
5UTR-V4-61*01	ATACTTTCTGAGAGCC--TGGACCTCCTGTGCAAGAAC
5UTR-V4-28*07	ATACTTTCTGAGAGTCC-TGGACCTCCTGTGCAAGAAC
5UTR-V4-30-4*07	ATACTTTCTGAGAGTCC-TGGACCTCCTGTGCAAGAAC
5UTR-V4-59*01	ATACTTTCTGAGAGTCC-TGGACCTCCTGTGCAAGAAC
5UTR-V4-61*08	ATACTTTCTGAGAGTCC-TGGACCTCCTGTGCAAGAAC
5UTR-V4-28*02	ATACTTTCTGAGAGTCC-TGGACCTC-TGTGCAAGAAC

Table 7
(continued)

VH5

5UTR-V5-51*01	GAGTCTCCCTCACTGCCCAGCTGGGATCTCAGGGCTTCATTTTCTGTCCTCCACCATC
5UTR-V5-51*02	GAGTCTCCCTCACTGCCCAGCTGGGATCTCAGGGCTTCATTTTCTGTCCTCCACCATC

VH6

5UTR-V6-01*01	AGAGCCTGCTGAATTCTGGCTGACCAGGGCAGTCACCAGAGCTCCAGACA

VH7

5UTR-V7-4-1*02	Ccacagctccacacc

Alignment of genomic 5UTR of different VH families

5UTR-V1-46*01	AAGCATCATCCAACAACCA-CATCCCTTCT-----CTACAGAAGCCTCTGAGAGGAAAGTTC------------- ------TTCACC
5UTR-V1-46*03	AAGCATCATCCAACAACCA-CATCCCTTCT-----CTACAGAAGCCTCTGAGAGGAAAGTTC------------- -------TTCACC
5UTR-V1-24*01	GACCATCACACAACAGCCA-CATCCCTCCC-----CTACAGAAGCCCCCAG-------------AGCGCAGCAC-- ------CTCACC
5UTR-V1-69-2*01	GAGCATCACACAACAGCCA-CATCCCTCCC-----CTACAGAAGCCCCCAG-------------AGAGCAGCAC-- ------CTCACC
5UTR-V1-58*01	GAGCATCATCCAGAAAACCA-CATCCCTCCG-----CTAGAGAAG-CCCTGACGGCACAGTTC------------- ------CTCACT
5UTR-V1-58*02	GAGCATCATCCAGAAAACCA-CATCCCTCCG-----CTAGAGAAGCCCCTGACGGCACAGTTC------------- ------CTCACT
5UTR-V1-69*04	GAGCATCACATAACAACCA-GATTCCTCCT-----CTAAAGAAGCCCCTGGGAGCACAGCTC------------- ------ATCACC
5UTR-V1-69*06	GAGCATCACATAACAACCA-CATTCCTCCT-----CTGAAGAAGCCCCTGGGAGCACAGCTC------------- ------ATCACC
5UTR-V1-69*14	GAGCATCACATAACAACCA-CATTCCTCCT-----CTGAAGAAGCCCCTGGGAGCACAGCTC------------- ------ATCACC
5UTT-V1-69*01	GAGCATCACATAACAACCA-CATTCCTCCT-----CTAAAGAAGCCCCTGGGAGCACAGCTC------------- ------ATCACC
5UTR-V1-69D*01	GAGCATCACATAACAACCA-CATTCCTCCT-----CTAAAGAAGCCCCTGGGAGCACAGCTC------------- ------ATCACC

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5UTR-V1-18*04    GAGCATCACCCAAAAACCA-CACCCCTCCT------TGGGAGAATCCCCTAGATCACAGCTC---------------------------------------
                 -----CTCACC
5UTR-V1-02*04    GAGCATCACCCAGCAACCA-CATCTGTCCT------CTAGAGAATCCCCTGAGAGCTCCGTTC----------------------------
                 ------CTCACC
5UTR-V1-45*02    GAGCATCACCCAACAACCA-CATCCCTCCT------CTAGAGAATCCCCTGAAAGCACAGCTC------------------------
                 ------CTCACC
5UTR-V6-01*01    -------------AGAGCCTGCTGAATTCTGGCTGACCAGGGCAGTCACCAGAGCTCCAG-----------------------
                 --------ACA
5UTR-V5-51*01    -------------GAGTCTC-CCTCACTGCC------CAGCTGGGATCTCAGGGCTTCATTTTCTGTCCTCCA------------
                 ------CCATC
5UTR-V5-51*02    -------------GAGTCTC-CCTCACTGCC------CAGCTGGGATCTCAGGGCTTCATTTTCTGTCCTCCA------------
                 ------CCATC
5UTR-V3-48*01    AGCTCTCAGAGAGTGCCT-TAGCCCT---------------GGATTCCAAGGCATTTCCACTTGGTGATC-AGCACTGAACAC
                 AGAGGACTCACC
5UTR-V3-48*02    AGCTCTCAGAGAGTGCCT-TAGCCCT---------------GGATTCCAAGGCATTTCCACTTGGTGATC-AGCACTGAACAC
                 AGAGGACTCACC
5UTR-V3-73*01    AGCTCTGGGAGAGAGCTC-CAGCCTTG------------GGATTCCCAGCTGTCTCCACTCGGTGATC-GGCACTGAATAC
                 AGGAGACTCACC
5UTR-V3-23*01    AGCTCTGAGAGAGGAGCC---CAGCCCTG------------GGATTTTCAGGTGTTTTCATTGGTGATC-AGGACTGAACAG
                 AGAGAACTCACC
5UTR-V3-23D*01   AGCTCTGAGAGAGGAGCC---CAGCCCTG------------GGATTTTCAGGTGTTTTCATTGGTGATC-AGGACTGAACA
                 GAGAGAACTCACC
5UTR-V3-23D*02   AGCTCTGAGAGAGGAGCC---CAGCCCTG------------GGATTTTCAGGTGTTTTCATTGGTGATC-AGGACTGAACA
                 GAGAGAACTCACC
5UTR-V3-64*01    AGCTCTGGGAGAGAGCCC--CCCCCCTG------------GGATTCCCAGGTGTTTTCATTGGTGATC-AGCACTGAACAC
                 AGAAGAGTC----
5UTR-V3-49*01    AGCTCTGGGAGAGAGCCC--CANCCGTG------------AGATTCCCAGGAGTTCCACTTGGTGATC-AGCACTGAACAC
                 AGACCACCAACC
5UTR-V3-49*03    AGCTCTGGGAGAGAGCCC--CAGCCGTG------------AGATTCCCAGGAGTTCCACTTGGTGATC-AGCACTGAACAC
                 AGACCACCAACC
5UTR-V3-13*02    AGCTCTGGGAGTGGAGCCC--CAGCCTTG------------GGATTCCCAAGTGTTTGTATTCAGTGATC-AGGACTGAACAC
                 ACAGGACTCACC
5UTR-V3-13*05    AGCTCTGGGAGTGGAGCCC--CAGCCTTG------------GGATTCCCAAGTGTTTGTATTCAGTGATC-AGGACTGAACAC
                 ACAGGACTCACC
5UTR-V3-73*02    AGCTCTGAGAGCGGAGCCC--CAGCCCCA------------GAATTCCCAGGTGTTTTCATTGGTGATC-AGCACTGAACAC
                 AGAGGACTCACC
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Table 7
(continued)

5UTR-V3-11*06	AGCTCTGGGAGAGAGCCC--CAGCCCCA------------GAATTCCCAGGAGTTTCCATTCGGTGATC-AGCACTGAACAC AGAGGACTCACC
5UTR-V3-66*03	AGCTCTGGGAGAGAGCC---CAGCACTG------------GGATTCCGAGGTGTTTCCATTCAGTGATC-TGCACTGAACAC AGAGGACTCGCC
5UTR-V3-53*02	AGCTCTGGGAGAGAGCC---CAGCACTG------------GGATTCCGAGGTGTTTCCATTCGGTGATC-AGCACTGAACAC AGAGGACTCACC
5UTR-V3-53*03	-AGCTCTGGGAGAGAGCCC-AGACACTG------------GATTCCGAGTGTTTCCATTCGGTGATCAAGCACTGAACAC AGAGGACTCACC
5UTR-V3-30*01	AGCTCTGGGAGAGAGCC---CAGCACTG------------GAAGTCGCCGGTGTTTCCATTCGGTGATC-AGCACTGAACAC AGAGGACTCACC
5UTR-V3-33*01	AGCTCTGGGAGAGAGCC---CAGCACTA------------GAAGTCGGCGGTGTTTCCATTCGGTGATC-AGCACTGAACAC AGAGGACTCACC
5UTR-V3-64D*06	AGCTCTGGGAGAGAGCCC-CAGGCCCG------------GGATTCCCAGGTGTTTCCATTCAGTGATC-AGCACTGAAGA CAGAAGACTCATC
5UTR-V3-43*01	AGCTCTGGGAGAGAGCCC-CAGCCCTG------------AGATTCCCAGGTGTTTCCATTCGGTGATC-AGCACTGAACAC AGAGAACGCACC
5UTR-V3-09*01	AGCTCTGGGAGAGAGCCC-CAGCCCTG------------AGATTCCCAGGTGTTTCCATTCAGTGATC-AGCACTGAACAC AGAGGACTCACC
5UTR-V3-09*03	AGCTCTGGGAGAGAGCCC-CAGCCCTG------------AGATTCCCAGGTGTTTCCATTCAGTGATC-AGCACTGAACAC AGAGGACTCACC
5UTR-V3-20*01	AGCTCTGGGAGAGAGCCCCC-CAGCCCTG------------AGATTCCCACGTGTTTCCATTCAGTGATC-AGCACTGAACAC AGAGGACTCGCC
5UTR-V3-20*03	AGCTCTGGGAGAGAGCCC-CAGCCCTG------------AGATTCCCACGTGTTTCCATTCAGTGATC-AGCACTGAACAC AGAGGACTCGCC
5UTR-V2-05*01	-----------AGTGACTC-CTGTGCAC----------------------------- ---------CACC
5UTR-V2-05*02	------------AGTGACTC--CTGTGCCC----------------------------- --------CACC
5UTR-V2-26*01	---------AGTGACTC-CTGTGCCC----------------------------- --------CACC
5UTR-V2-70*13	----------AGTGAATC--CTGCTCTC----------------------------- --------CACC

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5UTR-V2-70D*04   -----------AGTGAATC-CTGCTCTC------------------------------
5UTR-V2-70D*14   --------CACC-----AGTGAATC-CTGCTCTC------------------------
5UTR-V4-39*01    ATGCTTTCT--GAGAGTCA-TGGATCTCAT-----GTGCAAGAAA-------------
5UTR-V4-34*01    GTGCTTTCT--GAGAGTCA-TGGACCTCCT-----GCACAAGAAC-------------
5UTR-V4-34*02    GTGCTTTCT--GAGAGTCA-TGGACCTCCT-----GCACAAGAAC-------------
5UTR-V4-34*11    GTGCTTTCT--GAGAGCTCATGGACCTCCT-----GCACAAGAAC-------------
5UTR-V4-04*07    ATACTTTCT--GAGACTCA-TGGACCTCCT-----GCACAAGAAC-------------
5UTR-V4-38-2*02  ATGCTTTCT--GAGAGTCA-TGGACCTCCT-----GTGCAAGAAC-------------
5UTR-V4-04*01    ATACTTTCT--GAGAGCTCATGGCCTCCT-----GCACAAGAAC-------------
5UTR-V4-04*08    ATACTTTCT--GAGAGTCC-TGGACCTCCT-----GCACAAGAAC-------------
5UTR-V4-28*01    ATACTTTCT--GAGAGCTC-TGGACCTCCT-----GTGCAAGAAC-------------
5UTR-V4-31*02    ATACTTTCT--GAGAGTCC-TGGACCTCCT-----GTGCGAGAAC-------------
5UTR-V4-59*02    ATACTTTCT--GAGAGCC--TGGACCTCCT-----GTGCAAGAAC-------------
5UTR-V4-61*01    ATACTTTCT--GAGAGCC--TGGACCTCCT-----GTGCAAGAAC-------------
5UTR-V4-28*07    ATACTTTCT--GAGAGTCC-TGGACCTCCT-----GTGCAAGAAC-------------
5UTR-V4-30-4*07  ATACTTTCT--GAGAGTCC-TGGACCTCCT-----GTGCAAGAAC-------------
5UTR-V4-59*01    ATACTTTCT--GAGAGTCC-TGGACCTCCT-----GTGCAAGAAC-------------
5UTR-V4-61*08    ATACTTTCT--GAGAGTCC-TGGACCTCCT-T-----GTGCAAGAAC-----------
5UTR-V4-28*02    ATACTTTCT--GAGAGTCC-TGGACCTC-T-----GTGCAAGAAC-------------
```

Table 8
Leader sequences of human VH genes

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VH1
L-V1-46*01    ATGGACTGGACCTGGAGGGTCTTCTGCTTGCTGCTGTAGCTCCAGGTGCTCACTCC
L-V1-46*03    ATGGACTGGACCTGGAGGGTCTTCTGCTTGCTGCTGTAGCTCCAGGTGCTCACTCC
L-V1-69*01    ATGGACTGGACCTGGAGGTTCCTCTTCTGCTGGTGGCAGCAGCTACAGGTGTCCAGTCC
L-V1-69*04    ATGGACTGGACCTGGAGGTTCCTCTTCTGCTGGTGGCAGCAGCTACAGGTGTCCAGTCC
L-V1-69*06    ATGGACTGGACCTGGAGGTTCCTCTTCTGCTGGTGGCAGCAGCTACAGGTGTCCAGTCC
L-V1-69*14    ATGGACTGGACCTGGAGGTTCCTCTTCTGCTGGTGGCAGCAGCTACAGGTGTCCAGTCC
L-V1-69D*01   ATGGACTGGACCTGGAGGTTCCTCTTCTGCTGGTGGCAGCAGCTACAGGTGTCCAGTCC
L-V1-24*01    ATGGACTGCACCTGGAGCTCCTCTTCCTCCTGCTGGTGGCAGCAGCTACAGGCACCGCC
L-V1-69-2*01  ATGGACTGCACCTGGAGGATCCTCCTCTTGGTGGCAGCAGCTACAGGCACCCACGCC
L-V1-58*01    ATGGACTGGATTTGGAGGATCCTCTTCTTGGTGGGAGCAGCGACAGGTGCCCACTCC
L-V1-58*02    ATGGACTGGATTTGGAGGATCCTCTTCTTGGTGGGAGCAGCGACAGGTGCCCACTCC
L-V1-45*01    ATGGACTGGACCTGGAGAATCCTCTTCTTGGTGGCAGCAGCCACAGNTGCCTACTCC
L-V1-18*04    ATGGACTGGACCTGGAGCATCCTTTTCTTGGTGGCAGCAGCAACAGGTGCCCACTCC
L-V1-2*04     ATGGACTGGACCTGGAGGATCCTCTTCTTGGTGGCAGCAGCCACAGGAGCCCACTCC
              ******** * ****** ***   ** **** * **** ***    * **

VH2
L-V2-70*13    ATGGACATACTTTGTTCCACGCTCCTGCTACTGACTGTCCCGTCCTGGGTCTTATCC
L-V2-70D*04   ATGGACATACTTTGTTCCACGCTCCTGCTACTGACTGTCCCGTCCTGGGTCTTATCC
L-V2-70D*14   ATGGACATACTTTGTTCCACGCTCCTGCTACTGACTGTCCCGTCCTGGGTCTTATCC
L-V2-05*01    ATGGACACACTTTGCTCCACGCTCCTGCTGCTGACCATCCCTTCATGGGTCTTGTCC
L-V2-05*02    ATGGACACACTTTGCTCCACGCTCCTGCTGCTGACCATCCCTTCATGGGTCTTGTCC
L-V2-26*01    ATGGACACACTTTGCTACACACTCCTGCTGCTGACCACCCCTTCCTGGGTCTTGTCC
              ******** * **** ******** *** ** ********* * ******** ***

VH3
L-V3-09*01    ---ATGGAGTTTGGGACTGAGCTGGATTTTCCTTTTGGCTATTTTAAAAGGTGTCCAGTGT
L-V3-09*03    ---ATGGAGTTTGGGACTGAGCTGGATTTTCCTTTTGGCTATTTTAAAAGGTGTCCAGTGT
L-V3-64D*06   ---ATGGAGTTTCTGGCTGAGCTGGGTTCTCCTTGTTGGCTATTTTAAAAGATGTCCAGTGT
L-V3-48*01    ---ATGGAGTTTGGGCTGTGCTGGGTTTTCCTTGTTGCTATTTTAGAAGGTGTCCAGTGT
L-V3-48*02    ---ATGGAGTTTGGGCTGTGCTGGGTTTTCCTTGTTGCTATTTTAGAAGGTGTCCAGTGT
L-V3-13*02    ---ATGGAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTTTAGAAGGTGTCCAGTGT
L-V3-13*05    ---ATGGAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATATTAGAAGGTGTCCAGTGT
L-V3-23*01    ---ATGGAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAGTGT
L-V3-23D*01   ---ATGGAGTTTGGGCTGAGCTGGCTTTTTCTTGTGGCTATTTTAAAAGGTGTCCAGTGT
L-V3-23D*02   ---ATGGAGTTTGGGCTGAGCTGGCTTTTTCTTGTGGCTATTTTAAAAGGTGTCCAGTGT
              *** ** ********* *** ******** ***
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L-V3-30*01	----ATGGAGTTTGGGCTGAGCTGGGTTTTCCTCGTTGCTCTTTTAAGAGGTGTCCAGTGT
L-V3-33*01	----ATGGAGTTTGGGCTGAGCTGGGTTTTCCTCGTTGCTCTTTTAAGAGGTGTCCAGTGT
L-V3-64*01	ATGACGGAGTTTGGGCTGAGCTGGGTTTTCCTCGTTGCTCTATTTTAAAAGGTGTCCAGTGT
L-V3-49*01	----ATGGAGTTTGGGCTTAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAATGT
L-V3-49*03	----ATGGAGTTTGGGCTTAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAATGT
L-V3-11*06	----ATGGAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTATAAAAGGTGTCCAGTGT
L-V3-43*01	----ATGGAGTTTGGACTGAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAGTGT
L-V3-53*02	----ATGGAGTTTGGCTGAGCTGGGTTTTCCTTGTTGCTATTTCAAAAGGTGTCCAGTGT
L-V3-20*01	----ATGGAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAGTGT
L-V3-20*03	----ATGGAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAGTGT
L-V3-66*03	----ATGGAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAGTGT
L-V3-73*01	----ATGGAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAGTGT
L-V3-73*02	----ATGGAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAGTGT
L-V3-53*03	----ATGGAGTTTGGCTGAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAGTGT
	* ******** * *** ****** ** ***** * ** ** ******* ***

VH4

L-V4-39*01	ATGAAGCACCTGTGGTTCTTCCTCCTGCTGGTGGCGGCTCCCAGATGGGTCCTGTCC
L-V4-38-2*02	ATGAAGCACCTGTGGTTTTCCTCCTGCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-59*01	ATGAAACATCTGTGGTTCTTCCTTCTCCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-59*02	ATGAAACATCTGTGGTTCTTCCTTCTCCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-31*02	ATGAAACACCTGTGGTTCTTCCTCCTCCTGGTGGCAGCTCCCAGATGGGTCCTGCCC
L-V4-4*07	ATGAAACACCTGTGGTTCTTCCTCCTCCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-28*01	ATGAAACACCTGTGGTTCTTCCTCCTCCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-28*02	ATGAAACACCTGTGGTTCTTCCTCCTCCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-28*07	ATGAAACACCTGTGGTTCTTCCTCCTCCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-30-4*07	ATGAAACACCTGTGTGGTTCTTCCTCCTCCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-4*08	ATGAAACACCTGTGGTTCTTCCTCCTCCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-34*01	ATGAAACACCTGTGGTTCTTCCTCCTCCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-34*02	ATGAAACACCTGTGGTTCTTCCTCCTCCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-34*11	ATGAAACACCTGTGGTTCTTCCTCCTCCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-61*01	ATGAAACACCTGTGGTTCTTCCTCCTCCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-61*08	ATGAAACACCTGTGGTTCTTCCTCCTCCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-4*01	ATGAAACACCTGTGGTTCTTCCTCCTCCTGGTGGCAGCTCCCAGATGGGTCCTGTCT
	* ******** * ****** * ***** ** ******************** *

Table 8
(continued)

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VH5
L-V5-51*01    ATGGGGTCAACCGCCATCCTCGCCCTCCTCCTGGCTGTTCTCCAAGGAGTCTGTTCC
L-V5-51*02    ATGGGGTCAACCGCCATCCTCGCCCTCCTCCTAGCTATTCTCCAAGGAGTCTGTGCC
              ************************** **** **** *****************  **

VH6
L-V6-01*01    ATGTCTGTCTCCTTCCTCATCTTCCTGCCCGTGCTGGGCCTCCCATGGGGTGTCCTGTCA
```

Alignment of genomic leader of different VH families

```
L-V5-51*01    ---GGGTCAACCGCCATCCTCGCCCTCCTCCTGGCTGTTCTCCAAGGAGTCTGTTCC
L-V5-51*02    ---GGGTCAACCGCCATCCTCGCCCTCCTCCTAGCTATTCTCCAAGGAGTCTGTGCC
L-V6-01*01    ---ATGTCTGTCTCCTTCCTCATCTTCCTGCCCGTGCTGGGCCTCCCATGGGGTGTCCTGTCA
L-V3-09*01    ---ATG----GAGTTGGGACTGAGCTGGATTTCCTTTTGGCTATTTTAAAAGGTGTCCAGTGT
L-V3-09*03    ---ATG----GAGTTGGGACTGAGCTGGATTTCCTTTTGGCTATTTTAAAAGGTGTCCAGTGT
L-V3-64D*06   ---ATG----GAGTTCTGGCTGAGCTGGGTTCTCCTTGTTGCCATTTTAAAAGATGTCCAGTGT
L-V3-48*01    ---ATG----GAGTTGGGGCTGTGCTGGGTTTTCCTTGTTGCTATTTTAGAAGGTGTCCAGTGT
L-V3-48*02    ---ATG----GAGTTGGGGCTGTGCTGGGTTTTCCTTGTTGCTATTTTAGAAGGTGTCCAGTGT
L-V3-13*02    ---ATG----GAGTTGGGGCTGAGCTGGGTTTTCCTTGTTGCTATATTAGAAGGTGTCCAGTGT
L-V3-13*05    ---ATG----GAGTTGGGGCTGAGCTGGGTTTTCCTTGTTGCTATATTAGAAGGTGTCCAGTGT
L-V3-23*01    ---ATG----GAGTTTGGGCTGAGCTGGCTTTTTCTTGTGGCTATTTTAAAAGGTGTCCAGTGT
L-V3-23D*01   ---ATG----GAGTTTGGGCTGAGCTGGCTTTTTCTTGTGGCTATTTTAAAAGGTGTCCAGTGT
L-V3-23D*02   ---ATG----GAGTTTGGGCTGAGCTGGCTTTTTCTTGTGGCTATTTTAAAAGGTGTCCAGTGT
L-V3-30*01    ---ATG----GAGTTTGGGCTGAGCTGGGTTTTTCTCGTTGCTCTTTTAAGAGGTGTCCAGTGT
L-V3-33*01    ---ATG----GAGTTTGGGCTGAGCTGGGTTTTTCTCGTTGCTCTTTTAAGAGGTGTCCAGTGT
L-V3-64*01    ATGACG----GAGTTTGGGCTGAGCTGGGTTTTTCTTGTTGCTATTTTAAAAGGTGTCCAATGT
L-V3-49*01    ---ATG----GAGTTTGGGCTTAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAATGT
L-V3-49*03    ---ATG----GAGTTTGGGCTTAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAATGT
L-V3-11*06    ---ATG----GAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTTTATAAAAGGTGTCCAGTGT
L-V3-43*01    ---ATG----GAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTTTATAAAAGGTGTCCAGTGT
L-V3-53*02    ---ATG----GAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTTCAAAAGGTGTCCAGTGT
L-V3-20*01    ---ATG----GAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAGTGT
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L-V3-20*03     ---ATG----GAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAGTGT
L-V3-66*03     ---ATG----GAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAGTGT
L-V3-73*01     ---ATG----GAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAGTGT
L-V3-73*02     ---ATG----GAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAGTGT
L-V3-53*03     ---ATG----GAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCTATTTTAAAAGGTGTCCAGTGT
L-V2-70*13     ---ATG----GACATACTTTGTTCCACGCTCCTGCTACTGACTGTCCCGTCCTGGGTCTTATCC
L-V2-70D*04    ---ATG----GACATACTTTGTTCCACGCTCCTGCTACTGACTGTCCCGTCCTGGGTCTTATCC
L-V2-70D*14    ---ATG----GACATACTTTGTTCCACGCTCCTGCTACTGACTGTCCCGTCCTGGGTCTTATCC
L-V2-05*01     ---ATG----GACACACTTTGCTCCACGCTCCTGCTGCTGACCATCCTTCATGGGTCTTGTCC
L-V2-05*02     ---ATG----GACACACTTTGCTCCACGCTCCTGCTGCTGACCATCCTTCATGGGTCTTGTCC
L-V2-26*01     ---ATG----GACACACTTTGCTACACACTCCTGCTGCTGACCACCCTTCCTGGGTCTTGTCC
L-V4-39*01     ---ATG----AAGCACCTGTGGTTCTTCCTCCTGCTGGTGGCGGCTCCCAGATGGGTCCTGTCC
L-V4-38-2*02   ----ATG---AAGCACCTGTGGTTTTCCTCCTGCTGGTGGCAGCTCCCAGATGGTCCTGTCC
L-V4-59*01     ---ATG----AAACATCTGTGGTTCTTCCTCCTGCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-59*02     ---ATG----AAACATCTGTGGTTCTTCCTCCTGCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-31*02     ---ATG----AAACACCTGTGGTTCTTCCTCCTGCTGGTGGCAGCTCCCAGATGGGTCCTGCCC
L-V4-4*07      ----ATG---AAACACCTGTGGTTCTTCCTCCTGCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-28*01     ---ATG----AAACACCTGTGGTTCTTCCTCCTGCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-28*02     ---ATG----AAACACCTGTGGTTCTTCCTCCTGCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-28*07     ---ATG----AAACACCTGTGGTTCTTCCTCCTGCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-30-4*07   ----ATG---AAACACCTGTGGTTCTTCCTCCTGCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-4*08      ---ATG----AAACACCTGTGGTTCTTCCTCCTGCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-34*01     ---ATG----AAACACCTGTGGTTCTTCCTCCTGCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-34*02     ---ATG----AAACACCTGTGGTTCTTCCTCCTGCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-34*11     ---ATG----AAACACCTGTGGTTCTTCCTCCTGCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-61*01     ---ATG----AAACACCTGTGGTTCTTCCTCCTGCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-61*08     ---ATG----AAACACCTGTGGTTCTTCCTCCTGCTGGTGGCAGCTCCCAGATGGGTCCTGTCC
L-V4-4*01      ---ATG----AAACACCTGTGGTTCTTCCTCCTGCTGGTGGCAGCTCCCAGATGGGTCCTGTCT
L-V1-46*01     ---ATG----GACTGGACCTGGAGGGTCTTCTGCTTGCTGGCTGTAGCTCCAGGTGCTCACTCC
L-V1-46*03     ---ATG----GACTGGACCTGGAGGGTCTTCTGCTTGCTGGCTGTAGCTCCAGGTGCTCACTCC
L-V1-69*01     ---ATG----GACTGGACCTGGAGGGTCTTCTGCTTGCTGGCTGTAGCTCCAGGTGTCCAGTCC
L-V1-69*04     ---ATG----GACTGGACCTGGAGGGTCTTCTGCTTGCTGGCTGTAGCTCCAGGTGTCCAGTCC
L-V1-69*06     ---ATG----GACTGGACCTGGAGGGTCTTCTGCTTGCTGGCTGTAGCTCCAGGTGTCCAGTCC
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(continued)

Table 8
(continued)

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L-V1-69*14     ----ATG----GACTGGACCTGGAGGTTCCTCTTTGTGGTGGCAGCAGCTACAGGTGTCCAGTCC
L-V1-69D*01    ----ATG----GACTGGACCTGGAGGTTCCTCTTTGTGGTGGCAGCAGCTACAGGTGTCCAGTCC
L-V1-24*01     ----ATG----GACTGCACCTGGAGGATCCTCTTCTTGGTGGCAGCAGCTACAGGCACCCACGCC
L-V1-69-2*01   ----ATG----GACTGCACCTGGAGGATCCTCCTCCTTGGTGGCAGCAGCTACAGCAGCCCACGCC
L-V1-58*01     ----ATG----GACTGGATTTGGAGGATCCTCTTCTTGGTGGGGAGCAGCAGGTGCCCACTCC

L-V1-58*02     ----ATG----GACTGGATTTGGAGGGTCCTCTTCTTGGTGGGGAGCAGCGACAGGTGCCCACTCC
L-V1-45*01     ----ATG----GACTGGACCTGGAGAATCCTCTTCTTGGTGGCAGCAGCCACAGNTGCCTACTCC
L-V1-18*04     ----ATG----GACTGGACCTGGAGCATCCTTTCTTGGTGGCAGCAGCAACAGGTGCCCACTCC
L-V1-2*04      ----ATG----GACTGGACCTGGAGGATCCTCTTCTTGGTGGCAGCAGCCACAGGAGCCCACTCC
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Chapter 6

Analysis of Signaling Events in B-1a Cells

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Abstract

B-1a cells are a unique subset of B cells that are phenotypically and functionally distinguishable from conventional (B-2) cells. Here we detail methods to analyze signaling events in B-1a cells. We demonstrate by flow cytometry and Western blot that mouse peritoneal B-1a but not splenic B-2 cells have constitutive activation of signal transducers and activators of transcription-3 (Stat3) and extracellular signal-regulated kinase (Erk).

Key words B-1a cells, B-2 cells, Peritoneum, Stat3, Erk

1 Introduction

B-1a and B-2 cells exist in humans and mice. They can be distinguished from each other by their anatomical localization, cell surface phenotype and self-renewal properties. B-1a cells are mainly found in the pleural and peritoneal cavities, and they express moderate levels of cell surface CD5. In contrast, B-2 cells are the predominant B cell population found in the spleen and lymph nodes, and they lack surface expression of CD5 [1–3].

Previous studies have shown that unstimulated ex vivo B1-a and B-2 B cells have distinct signal transduction events [4, 5]. Here, we describe the isolation of murine B-1a and B-2 cells from the peritoneal cavities and spleen, respectively and examine the activation status of signal transducer and activator of transcription-3 (STAT3) and the extracellular signal-regulated kinase (ERK) in these cells by both flow cytometry and Western blot analyses.

2 Materials

2.1 General Tools

1. Dissection board.
2. Dissection scissors.

3. Dissection forceps.

4. Needles (22G).

5. Glass pipette.

6. 15-mL polystyrene tubes (15 and 50 mL).

7. Nylon cell strainer.

8. Plastic syringe (5 mL).

9. X-ray film cassette.

10. MACS MS and LD columns (Miltenyi Biotec).

2.2   Reagents

1. 70% ethanol.

2. RPMI 1640 medium.

3. Complete RPMI 1640 medium: RPMI medium with 10% fetal calf serum (FCS).

4. Red blood cell lysis buffer: 155 mM NH_4Cl, 0.1 mM EDTA. Adjust to pH 7.2 with NaOH.

5. B-1a Cell Isolation Kit (Miltenyi Biotec).

6. CD43 (Ly-48) MicroBeads (Mouse, Miltenyi Biotec).

7. EDTA-free protease inhibitor cocktail tablets. One for 50 mL cell lysis buffer (Roche Diagnostics).

8. Cell lysis buffer: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, protease inhibitor.

9. Bio-Rad Bradford Protein Assay Kit (Bio-Rad Laboratories).

10. MACS buffer: PBS, 0.5% bovine serum albumin (BSA), 2 mM EDTA. Adjust to pH 7.2 with HCl.

11. Bio-Rad BSA protein standard (Bio-Rad Laboratories).

12. Loading buffer: 100 mM Tris–HCl, pH 6.8, 200 mM DTT, 20% glycerol, 4% SDS, 0.2% Bromophenol Blue.

13. Precision Plus Protein™ All Blue Prestained Protein Standards (Bio-Rad).

14. NuPAGE Novex 4–12% Bis-Tris Protein Gels (1.0 mm, 10-well, Thermo Fisher Scientific).

15. Bio-Rad Trans-Blot Turbo Midi PVDF Transfer Packs (Bio-Rad Laboratories).

16. TBST buffer: 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.2% Tween 20.

17. Blocking buffer: TBST, 5% BSA.

18. PE anti-phosphorylated Stat3 (pY705) (BD Phosflow).

19. Alexa Fluor 488 anti-phosphorylated p44/42 MAPK (D13.14.4E) (Rabbit mAb, Cell Signaling Technology).

20. Stat3 (124H6) mouse mAb (Cell Signaling Technology).

21. p42 MAP Kinase (Erk2) antibody (Cell Signaling Technology).

22. HRP-linked anti-rabbit IgG (Cell Signaling Technology).

23. HRP-linked anti-mouse IgG (Cell Signaling Technology).

24. SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

25. SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific).

26. BD Stain Buffer (BD Pharmingen).

27. BD Perm/Wash Buffer (BD Phosflow).

28. BD Lyse/Fix Buffer (BD Phosflow).

2.3 Equipment

1. Aspirator.

2. 37 °C water bath.

3. Centrifuge (*Sorvall ST16R*).

4. Eppendorf Centrifuge (5415R) (Eppendorf AG).

5. Microscope (CKX41 Inverted Microscopes, Olympus).

6. EVE Automated Cell Counter (NanoEnTek).

7. Infinite 200 PRO Microplate Reader (Tecan).

8. Misonix Sonicator 3000 (Misonix).

9. MiniMACS Separator (Miltenyi Biotec).

10. MACS MultiStand (Miltenyi Biotec).

11. Bio-Rad Power Pac 300 Electrophoresis Power Supply (Bio-Rad Laboratories).

12. Bio-Rad Trans-Blot Turbo Transfer System (Bio-Rad Laboratories).

13. Thermo Scientific Digital Tube Revolver—0.5–50 mL tubes (Thermo Scientific).

14. Grant Bio PSU-20i Orbital Multi-Platform Shaker (Grant Instruments).

15. Kodak Medical X-ray Processor (Carestream).

16. Canon CanoScan 8800F (Canon).

17. BD LSR II Flow Cytometer (BD Bioscience).

18. FlowJo version 7.6.5 software (FlowJo).

2.4 Mice

1. C57BL/6 mice.

2. Normal chow: 4% of crude fat, 11% calories from fat (maintenance diet for rats/mice, Altromin).

3   Methods

3.1   Mouse Maintenance and Experiments

All procedures and experiments with mice are performed according to guidelines from the National Advisory Committee on Laboratory Animal Research. C57BL/6 mice are housed under a 12-h light–dark cycle and given normal chow. Female mice are subjected to experiments. All mice are used between 4 and 6 months of age (*see* **Note 1**).

3.2   Isolation of B-1a Cells

This protocol describes a procedure to obtain viable immune cells from the peritoneal cavity of mice, which can then be used for phenotypic analysis by flow cytometry and Western blot.

1. Euthanize mouse, spray mouse body with 70% ethanol, and mount it on its back on the dissection board.

2. Cut the outer skin of the peritoneum and gently pull it apart to expose the peritoneal cavity.

3. Pick up inner skin of the peritoneal cavity with forceps and inject 5 mL of complete RPMI 1640 Medium into the peritoneal cavity using a 22G needle (*see* **Note 2**).

4. Remove the needle after injection and rotate the mouse's body gently and continuously for about 1 min to dislodge attached cells into the medium.

5. Pick and hold up a bit of the inner skin of peritoneal cavity with the forceps and cut a small incision with a pair of dissection scissors, and gently collect the fluid with a glass pipette (*see* **Note 3**).

6. Withdraw as much fluid as possible into a 15 mL polystyrene tube. Approximately 4–4.5 mL cell suspension can be collected (*see* **Note 4**).

7. Centrifuge the cell suspension at $300 \times g$ (4 °C) for 5 min, aspirate the supernatant, and resuspend the cell pellet in complete RPMI 1640 Medium for cell counting.

8. Centrifuge the cell suspension at $300 \times g$ (4 °C) for 5 min, aspirate the supernatant, and resuspend the cell pellet in 40 μL of MACS buffer per 10^7 cells.

9. Add 10 μL of B-1a Cell Biotin-Antibody cocktail (from B-1a Cell Isolation Kit) per 10^7 cells and incubate cells for 5 min at 4 °C. Top up 30 μL of MACS buffer and 20 μL of anti-Biotin Microbeads per 10^7 cells and incubate cells for an additional 10 min at 4 °C.

10. Place a LD Column in the MiniMACS Separator and equilibrate column with 5 mL of MACS buffer.

11. Apply cell suspension onto the column. Collect the unlabeled cells and wash the column with 2×1 mL of MACS buffer.

12. Centrifuge the cell suspension at $300 \times g$ (4 °C) for 5 min, aspirate the supernatant, and resuspend the cell pellet in 40 μL of MACS buffer and 10 μL of anti-APC Microbeads. Incubate the cells for 15 min at 4 °C.

13. Top up to 1 mL with MACS buffer and centrifuge the cell suspension at $300 \times g$ (4 °C) for 5 min. Resuspend the cell pellet in 500 μL of MACS buffer.

14. Place a MS Column in the MiniMACS Separator and equilibrate the column with 500 μL of MACS buffer.

15. Apply cell suspension onto the column. Collect the flow-through and wash column with 3×500 μL of MACS buffer.

16. Remove the column from the separator and place it on a 15 mL polystyrene tube.

17. Pipette 1 mL of RPMI 1640 Medium onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.

18. Quantify the cell by counting.

3.3 Isolation of B-2 Cells from Spleen

1. Remove the spleen, which is located to the left side of the abdomen and inferior to the stomach.

2. Slice the spleen into small pieces and place the fragments onto a strainer attached to a 15 mL polystyrene tube.

3. Press the excised spleen through the nylon cell strainer with the plunger end of a syringe and wash the cells through the strainer with complete RPMI 1640 Medium.

4. Centrifuge the cell suspension at $300 \times g$ (4 °C) for 5 min and aspirate the supernatant.

5. Gently resuspend the cell pellet in 2 mL of red blood cell lysis buffer and incubate cells for 5 min on ice.

6. Add 2 mL of complete RPMI 1640 Medium and centrifuge cells at $300 \times g$ (4 °C) for 5 min.

7. Aspirate the supernatant and resuspend the cell pellet in 10 mL of complete RPMI 1640 Medium for cell counting.

8. Centrifuge the cell suspension at $300 \times g$ (4 °C) for 10 min and aspirate the supernatant.

9. Resuspend cell pellet in 90 μL of MACS Buffer per 10^7 cells and add 10 μL of CD43 (Ly-48) MicroBeads per 10^7 cells.

10. Mix well and incubate cells for 15 min at 4 °C.

11. Place a MS Column in the MiniMACS Separator and equilibrate the column with 5 mL of MACS buffer.

12. Add 2 mL of MACS buffer to the cell suspension and centrifuge at $300 \times g$ (4 °C) for 10 min and aspirate the supernatant.

13. Resuspend the cell pellet in 500 μL of MACS buffer.

14. Apply cell suspension onto the column. Collect the flow-through unlabeled cells and wash the column with 2×1 mL of MACS buffer.

15. Quantify the cell by counting.

3.4 Intracellular Staining

1. Adjust cell concentration of the isolated B-1a and B-2 cells to 5×10^6/mL.

2. Add 10 volumes of prewarmed BD Lyse/Fix Buffer to $1–2 \times 10^6$ cell suspension and mix well.

3. Incubate cells for 10 min at 37 °C.

4. Centrifuge the cell suspensions at $300 \times g$ for 8 min and aspirate the supernatant.

5. Vortex briefly to loosen the cell pellet.

6. Add one volume of BD Stain Buffer equivalent to the volume of Lyse/Fix Buffer used and centrifuge the cell suspensions at $300 \times g$ for 8 min and aspirate the supernatant.

7. Add 1 mL of BD Perm/Wash Buffer and incubate the cells for 30 min at room temperature.

8. Centrifuge the cell suspensions at $300 \times g$ for 8 min and aspirate the supernatant.

9. Incubate the cells for 60 min at room temperature with 50 μL of Stain Buffer containing PE-conjugated anti-phosphorylated Stat3 (pY705) and Alexa Fluor 488-conjugated anti-phosphorylated p44/42 MAPK antibodies (*see* **Note 5**).

10. Add 3 mL of BD Perm/Wash Buffer. Centrifuge the cell suspensions at $300 \times g$ for 8 min and aspirate supernatant.

11. Resuspend the cell pellet in 500 μL of BD Perm/Wash Buffer and analyze cells on a LSR II flow cytometer.

12. Results can be analyzed with FlowJo version 7.6.5 software and are shown in Fig. 1.

3.5 Cell Lysis Preparation

1. Centrifuge isolated B-1a cells at $300 \times g$ (4 °C) for 5 min, aspirate the supernatant, and resuspend cell pellet in 0.1 mL cell lysis buffer.

2. Lyse the cells by sonication in Eppendorf tube placed in ice–water mix at output level, 1.0; total process time, 15 s; pulse-ON time, 5 s; pulse-OFF time, 3 s (*see* **Note 6**).

3. Centrifuge the cell lysate in Eppendorf tube at $16,000 \times g$ (4 °C) for 5 min and transfer the supernatant to a new Eppendorf tube for protein concentration assay.
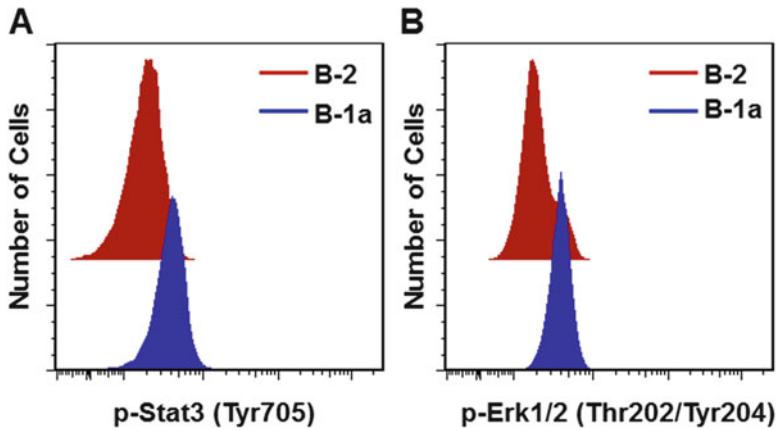
Fig. 1 Flow cytometry analyses of Stat3 and Erk activation in B-1a and B-2 B cells. The activation status of Stat3 (**a**) and Erk1/2 (**b**) in B-1a and B-2 cells ex vivo was examined by intracellular staining with phosphorylation-specific anti-Stat3 (Tyr705)-PE and anti-Erk1/2(Thr202/Tyr204)-AX488 (clone D13.14.4E) antibodies. Cells were analyzed followed by flow cytometry. Data shown are representative of three separate experiments

3.6 Protein Concentration Assay and Preparation of Samples for Western Blot

Cell lysate protein concentrations were measured by Bio-Rad Bradford Protein Assay kit based on the change of absorption of Coomassie Brilliant Blue G-250 dye (shifts from 465 nm to 595 nm when binding to protein) in response to various concentrations of protein.

1. Mix B-2 and B-1a cell lysate (5 µL of each sample) or 5 µL of BSA protein standard series (0, 31.25, 62.5, 125, 250, 500, 1000, and 2000 µg/mL) with 195 µL of Bio-Rad Bradford Protein Assay reagent in 96-well flat-bottom plates and incubate for 10 min at room temperature (*see* **Note 7**).

2. Measure the absorbance at 595 nm with a microplate reader.

3. Calculate the protein concentrations in samples by comparing the standard curve obtained from known concentrations of protein, which was examined in parallel.

4. Mix aliquots (~15–25 µL) of samples at protein amount of 20 µg/Eppendorf tube with equal volume of loading buffer, boil for 5 min at 100 °C, centrifuged at $16{,}000 \times g$ for 1 min and subject to Western blot analysis.

3.7 Western Blot Analysis

1. Load 10 µL of protein maker, B-2 and B-1 samples into gel and electrophorese gel at 120 V. Allow sample migration to continue until the blue dye front is at the end of the plate.

2. Remove gel from electrophoresis apparatus. Transfer protein onto PVDF membrane with Bio-Rad Trans-Blot Turbo Transfer System according to manufacturer's instructions.

3. After transfer, wash PVDF membrane twice with TBST buffer and block it with blocking buffer with constant rocking for 2 h at room temperature or overnight at 4 °C.

4. Dilute primary antibody (PE anti-phosphorylated Stat3 or Alexa Fluor 488 anti-phosphorylated p44/42 MAPK) in blocking solution with a dilution ratio of 1:1000.

5. Incubate membrane with the primary antibody with constant mixing for 2 h at room temperature or overnight at 4 °C on the revolver.

6. Wash membrane three times with TBST buffer for 10 min each on the shaker.

7. Incubate the membrane with a suitable HRP-conjugated secondary antibody (recognizing the host species of the primary antibody, HRP-linked anti-rabbit IgG, or HRP-linked anti-mouse IgG), diluted at 1:10,000 in blocking buffer. Incubate for 1 h with constant rotating on the revolver.

8. Wash membrane three times with TBST buffer for 15 min each on the shaker.

9. Prepare ECL substrate according to the manufacturer's instructions. Incubate the PVDF membrane with substrate for 1–5 min.

10. Place the PVDF membrane on a piece of transparent sheet, remove extra ECL substrate liquid and cover the membrane with another piece of transparent sheet. Load PVDF membrane onto a X-ray film cassette.

11. Expose the membrane to an autoradiography film in a dark room. Adjust the exposure time according to the image intensity found on the film.

12. Line up the developed film in the correct orientation, label the film border and mark the bands of the MW ladder directly onto the film. Add notes such as lane content, film exposure time, substrate used for ECL, date, and the batch of experiment.

13. Wash the PVDF membrane in TBST for 5 min and probe with antibody (Stat3 or p42 MAPK) respectively for loading control analyses.

14. Dilute primary antibody (Stat3 or p42 MAPK) in blocking buffer with a dilution ratio of 1:1000. Repeat **steps 7–12**.

15. Scan the film and crop the image properly for generating publication quality picture (Fig. 2).
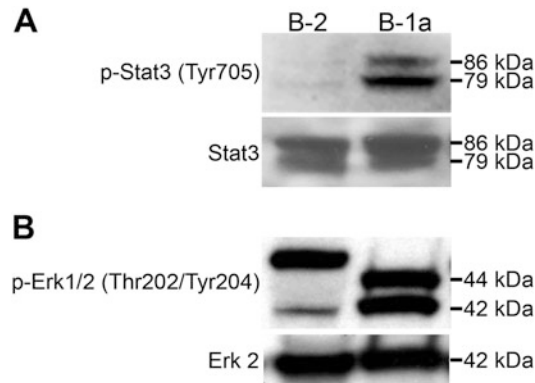
Fig. 2 Western blot analyses of Stat3 and Erk activation in B-1a and B-2 B cells. The activation of (**a**) Stat3 (Tyr705) and (**b**) Erk1/2 (Thr202/Tyr204) ex vivo in B-1a and B-2 whole cell lysates were examined by Western blot analyses using anti-phospho-Stat3 (pY705) and anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibodies. Subsequently, total amount of Stat-3 and Erk2 for loading controls were probed with anti-Stat3 and anti-Erk2 antibodies in second round Western blot analyses. Data shown are representative of three separate experiments

4 Notes

1. Gender and age of mice are critical for the isolation of B-1a cell population.

2. Inject slowly to avoid puncturing any organs.

3. Try to avoid clogging by fat or other organs.

4. If blood contamination is visible and can be readily detected, then the affected sample should be lysed again with red blood cell lysis buffer.

5. Protect from light.

6. Ensure that the micro-probe of the sonicator is at the bottom or near the bottom of the Eppendorf tube.

7. Ensure sufficient incubation time at room temperature as it is critical for the protein concentration assay.

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Chapter 7

Exploring the Role of Microbiota in the Limiting of B1 and MZ B-Cell Numbers by Naturally Secreted Immunoglobulins

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Abstract

Immunoglobulins (Igs)—or antibodies (Ab)—are important to combat foreign pathogens but also to the immune system homeostasis. We developed the $AID^{-/-}\mu S^{-/-}$ mouse model devoid of total soluble Igs and suitable to monitor the role of Igs on immune homeostasis. We used this experimental system to uncover a negative feedback control of marginal zone (MZ) and B1 B cells numbers by naturally secreted Igs. We raised $AID^{-/-}\mu S^{-/-}$ mice in germ-free conditions demonstrating that this effect of natural secreted Igs is independent of the microbiota. Herein, we provide a comprehensive description of the protocols to establish and use the $AID^{-/-}\mu S^{-/-}$ mice to study the role of total secreted Igs or of different Ig classes. This study involves Igs injections to $AID^{-/-}\mu S^{-/-}$ mice or establishment of $AID^{-/-}\mu S^{-/-}$ mixed bone marrow chimeras that provide a powerful system to study $AID^{-/-}\mu S^{-/-}$ B cells in the presence of stable concentrations of different Ig classes. While we describe flow cytometric and histological methods to analyze MZ and B1 B cell subsets, $AID^{-/-}\mu S^{-/-}$ mice can be used to study the effects of natural Igs on other B cell subsets or immune cells.

Key words B1 B cells, MZ B cells, Soluble immunoglobulins, $AID^{-/-}\mu S^{-/-}$ mice, Germ-free mice, Bone marrow chimeras

1 Introduction

Naturally secreted immunoglobulins (Igs) are mainly produced by marginal zone (MZ) [1] and B1 cells [2] and play a pleiotropic role in immune system and tissue homeostasis. Some of their functions result from the control they exert on microbiota. By preventing systemic invasion by symbiotic and pathogenic microorganisms, they limit persistent activation and proliferation of immune cells which can lead to chronic inflammation and tissue damages at mucosal barriers [3]. Independently of this role on the microbiota, natural Igs help clear metabolites and debris from cells dying during tissue regeneration, which could otherwise activate self-reactive lymphocytes and cause autoimmune disorders [4].

To investigate in vivo the direct implication of the microbiota on different aspects of homeostatic regulation by natural Igs, we developed a strategy where we compare the phenotype of genetically modified mice deficient in all or different classes of secreted Ig, bred in the presence (specific pathogen free (SPF)) or in the absence of microbiota (germ-free (GF)) [5]. Mice devoid of soluble Igs were generated by inter-crossing mice deficient for the activation-induced cytidine deaminase (named $AID^{-/-}$ mice thereafter) [6]—the key enzyme catalyzing class switch recombination and somatic hypermutations in Ig variable domains—with mice deficient in the secretory exon of the µ chain (µS) that allows IgM secretion by unswitched B cells (named $µS^{-/-}$ mice thereafter) [7]. The phenotype of $AID^{-/-}µS^{-/-}$ mice was compared to that of $AID^{-/-}$ and $µS^{-/-}$ single mutants, in order to correlate the different phenotypic traits observed in the double mutants to the lack of soluble IgM (in $µS^{-/-}$ mice), switched and hypermutated Igs (in $AID^{-/-}$ mice), or both types of Igs [5]. To verify the dependency of the phenotype observed on different Ig classes, we injected $AID^{-/-}µS^{-/-}$ mice with total serum Igs, polyclonal or monoclonal IgG.

We applied these methods to unveil a negative feedback control exerted by natural Igs on B1 and MZ B cells numbers, and its independency of the presence of microbiota. In this particular case, as AID and µS mutations were likely to cause intrinsic defects in B cell physiology [8–10], we sought to demonstrate that the phenotype of $AID^{-/-}µS^{-/-}$ mice regarding B1 and MZ B cell numbers was due to cell extrinsic lack of serum Igs. To this aim, we generated and analyzed bone marrow (BM) chimeras where $AID^{-/-}µS^{-/-}$ B cells were exposed to soluble Igs produced by wild-type (WT) B cells, soluble non-hypermutated IgM produced by $AID^{-/-}$ B cells, or soluble switched Ig produced by $µS^{-/-}$ B cells [5]. This strategy proved advantageous over Ig injections as it allowed stable exposure of $AID^{-/-}µS^{-/-}$ B cells to Igs, a condition that could not be met with Ig injections for Ig classes of short half-life times.

The general strategy as well as the analytical tools (flow cytometry and histology) we used are described in this chapter and can be applied to the study of other immune cell populations likely to be regulated by natural Igs, dependently or independently of their role on microbiota.

2   Materials

2.1 $AID^{-/-}µS^{-/-}$ Mouse Genotyping

1. Digesting buffer: 100 µg/mL proteinase K, 0.5% SDS, 100 mM NaCl, 50 mM Tris–HCl pH 8.0, 4 mM EDTA.

2. Thermomixer.

3. PCR cycler.

Table 1
PCR reaction for AID$^{cre/cre}$ genotyping

Reagent	Vol per reaction (μL)	Final concentration
Tissue DNA	1	
PCR buffer 5×[a]	5	1×
MgCl$_2$ 25 mM	2	2 mM
dNTP 10 mM	0.5	0.2 mM each
oIMR 7537 20 mM	0.625	0.5 mM
oIMR 7538 20 mM	0.625	0.5 mM
oIMR 7505 20 mM	0.625	0.5 mM
Taq	0.2	1 Unit
H$_2$O to 25 mL	14.425	
Total	25	

[a]This concentration may vary according to manufacturer

4. Microtubes (1.5 mL).

5. Microtubes adapted to PCR cycler's block.

6. Taq polymerase.

7. PCR buffer.

8. MgCl$_2$ (25 mM).

9. Mixed dNTPs (10 mM).

10. Primers (20 μM) (oIMR 7537 5′-GGA CCC AAC CCA GGA GGC AGA TGT-3′; oIMR 7505 5′-CAC TCG TTG CAT CGA CCG GTA ATG-3′; oIMR 7538 5′-CCT CTA AGG CTT CGC TGT TAT TAC CAC-3′) (*see* Table 1).

11. Agarose gel electrophoresis tank.

12. Power source.

13. Agarose.

14. Electrophoresis buffer: 1.1 M Tris–HCl, pH 8.3, 900 mM Borate, 25 mM EDTA. Dilute 5× buffer to 0.5×.

15. DNA dye (safeRed™).

16. 100 bp ladder DNA.

17. Gel imager.

18. 96-well ELISA plates.

19. Coating buffer: 0.05 M phosphate buffer, pH 8.0. If necessary, correct pH with phosphoric acid or sodium hydroxide.

20. Goat anti-mouse IgM.

21. Washing buffer: PBS, 0.1% Tween 20.

22. Blocking buffer: PBS, 1% gelatin.

23. PBS-gelatin-Tween buffer: PBS, 1% gelatin, 0.05–0.1% Tween 20. For the dilution of mouse sera.

24. Anti-mouse IgM-HRPO.

25. Substrate buffer: 150 mM citrate-phosphate, pH 5.6, 1 μL/mL of 30% H_2O_2.

26. Substrate solution: 0.5 mg/mL of orto-phenylenediamine (ODP) in substrate buffer.

27. Stopping buffer: 10% SDS in water.

28. Spectrophotometer measuring at 490 nm.

29. Multichannel pipette. Although it is not strictly necessary, it greatly improves the performance and reproducibility of ELISAs.

30. Microcentrifuge.

2.2 AID$^{-/-}$μS$^{-/-}$ BM Chimeras

1. Irradiator compatible with mouse irradiation.

2. Sterile PBS.

3. Dissection tools: scissors, forceps.

4. Needles (23 G) and syringes.

2.3 Ig Injections

1. Sterile tubes to collect mouse serum.

2. General-purpose centrifuge.

3. −20 or −80 °C freezer for storage.

4. Syringes and needles for injection.

2.4 Germ-free AID$^{-/-}$μS$^{-/-}$ Mice

1. Germ-free facility: sterile isolators, transfer chambers compatible with the isolators to introduce items into the isolator, autoclave able to contain the transfer chambers using validated autoclaving programs.

2. Promon vet. (medroxyprogesterone acetate).

3. Surgical equipment and dissecting tools, including surgical clamps.

4. VirkonS: 1% solution, room temperature, Antec Int. Ltd. (or other strong germicidal liquids).

2.5 Flow-Cytometric analysis of Mouse B1 and Marginal Zone B Cells

1. Refrigerated general-purpose centrifuge.

2. Flow cytometer and acquisition software.

3. Lavage buffer: PBS, 2% fetal calf serum (FCS).

4. Latex beads (10 μm) (Coulter Corp.)

5. Dissecting tools including curved forceps.

6. Needles: 26 and 18 G.

7. Syringes (12 mL).

8. Small plastic Petri dishes.

9. Plastic tubes.

10. Microtubes (1.5 mL)

11. FACS buffer: PBS, 2% FCS, 0.1% of NaN_3.

12. Fc block solution: FACS buffer, 30 μg/mL of anti-CD16/32 (clone 2.4G2).

13. Propidium iodide (PI) solution: FACS staining buffer, 200 ng/mL PI.

14. Peritoneal cavity antibody mix: anti- CD11b (clone M1/70), CD19 (clone 1D3) or B220, CD5 (clone 53-7.3) fluorescent labeled antibodies.

15. Spleen antibody mix: anti-CD19 (clone 1D3) or B220, CD21/35 (7G6) and CD23 (B3B4) fluorescent labeled antibodies or anti-CD19 or B220, IgM (clone R331.24.12) and IgD (clone 11-26 or 1.19).

16. U- or V-bottom plates (96-well).

2.6 Analysis of Marginal Zone B cells by Immunohistology and Confocal Microscopy

1. Cryostat.

2. Liquid nitrogen and tank.

3. Aluminum foil.

4. Tissue TEK® OCT compound.

5. ICE-IT spray.

6. Multispot glass slides (Hendley, Hessex, UK).

7. Glass coverslips.

8. Cold acetone.

9. Glass-staining jars.

10. Magnetic stirrer.

11. Confocal staining buffer: PBS, 10% FCS, 0.1% NaN_3.

12. Normal mouse serum for blocking.

13. Antibodies: rat anti-CD23 (clone B3B4), polyclonal goat Cy3-conjugated anti-rat IgG, FITC-conjugated rat anti-CD21/35 antibody (clone 7G6).

14. Antifading mounting medium.

15. Confocal microscope equipped with 488 and 543 nm lasers and 10× objectives.

16. Imaging software.

3 Methods

3.1 AID$^{-/-}$μS$^{-/-}$ Mouse Establishment

1. Intercross AID$^{-/-}$ mice (also known as *aicda*$^{-/-}$) with μS$^{-/-}$ mice of the same genetic background. Several AID$^{-/-}$ [6, 11] and μS$^{-/-}$ [7, 8] mice have been generated (*see* Table 2).

Table 2
List of secreted Igs-deficient mice

Strain	PubMed ID	Original Lab	Link strain	Link genotyping protocol
aicda$^{-/-}$	11007474	T. Honjo	Aicdatm1Hon: http://www.informatics.jax.org/allele/MGI:2156156	http://www2.brc.riken.jp/animal/pdf/00897_PCR.pdf
AID$^{Cre/Cre}$	19070574	M.C. Nussenzeig	Aicdatm1(cre)Mnz: http://www.informatics.jax.org/allele/MGI:3720568	https://www2.jax.org/protocolsdb/f?p=116:5:0::NO:5:P5_MASTER_PROTOCOL_ID,P5_JRS_CODE:13339,007770
µS$^{-/-}$	9590224	J. Chen	Ighmtm1Che: http://www.informatics.jax.org/allele/MGI:1934014	https://www2.jax.org/protocolsdb/f?p=116:5:0::NO:5:P5_MASTER_PROTOCOL_ID,P5_JRS_CODE:13473,003751
µS$^{-/-}$	9707605	M.S. Neuberger	Ighmtm1Msn: http://www.informatics.jax.org/allele/MGI:2447178	

2. Intercross F1 AID$^{+/-}$µS$^{+/-}$ progeny to obtain AID$^{-/-}$µS$^{-/-}$ mice that are devoid of secreted Igs, but are able to generate B cells.

3. Intercross of F1 AID$^{+/-}$µS$^{+/-}$ mice generates as well littermate controls that produce secreted switched Igs only (µS$^{-/-}$ mice), secrete IgM only (AID$^{-/-}$ mice), or all soluble Igs (WT mice) (*see* **Note 1**).

3.2 Genotyping AID$^{cre/cre}$ by PCR

The following genotyping protocols relate to AID$^{cre/cre}$, abbreviated AID$^{-/-}$ in all other sections of this chapter, generated in M.C. Nussensweig's laboratory [6].

1. Extract DNA from tissues sampled from ear marks or by cutting 1–2 mm from the distal part of the tail.

2. Digest the tissues in 1.5 mL microtubes containing 200 µL of digesting buffer and place in a thermomixer overnight (O/N) or for minimum 2–3 h at 56 °C under 800 rpm agitation in thermomixer.

3. Centrifuge the samples in a microcentrifuge for 10 min at 13000 × *g* and precipitate DNA by transferring the supernatant into a new 1.5 mL microtube containing 200 µL of isopropanol. Shake the tube until seeing the precipitate (do

not vortex) and centrifuge for 5 min at $13000 \times g$ in a microcentrifuge.

4. Discard the supernatant by inverting the opened tube and let the DNA pellet dry by maintaining the tube upside down on absorbing paper on the bench.

5. When any trace of isopropanol disappeared (10–15 min), resuspend the pellet in 80 μL of MilliQ water and proceed to PCR.

6. Use the following primers to detect WT and AID[cre] alleles by PCR. IMR 7537 5′-GGA CCC AAC CCA GGA GGC AGA TGT-3′; IMR 7505 5′-CAC TCG TTG CAT CGA CCG GTA ATG-3′; IMR 7538 5′-CCT CTA AGG CTT CGC TGT TAT TAC CAC-3′ with the following PCR program: 94 °C for 4 min, 36× (94 °C for 30 s, 59 °C for 45 s, 72 °C for 2 min), 72 °C for 10 min, keep at room temperature on hold. The reaction is performed in standard conditions for Taq polymerase in buffer from the supplier, supplemented with 2 mM of $MgCl_2$, 2 mM of dNTP, and 5 mM of each primer (*see* Table 1).

7. The amplification gives 283 bp products from AID[cre/cre] mice, 484 bp products from AID^{+/+}, and products of both sizes from heterozygous AID[+/cre] mice.

3.3 Screening of μS⁻/⁻ Mice Using ELISA to Detect IgM

The following genotyping protocols relate to $\mu S^{-/-}$ mice generated in M.S. Neuberger's laboratory [7]. The $\mu S^{-/-}$ mice are devoid of IgM, while quasi-normal amounts of IgM are detected in $\mu S^{+/-}$ mice. Use a plate sealer to avoid evaporation in all incubation steps.

1. Collect mouse blood using a non-terminal method and prepare sera as in Subheading 3.5.

2. Precoat ELISA plates with 50 μL/well of anti-mouse IgM (2 μg/mL) in coating buffer, incubate the plates overnight at 4 °C or for 2 h at 37 °C.

3. Wash three times by submerging the plate in a container with washing buffer, empting it over a sink and tapping it upside down against layers of paper towels to remove residual liquid.

4. Incubate the plate with 100 μL/well of blocking buffer for at least 60 min.

5. Wash the plate as in **step 3**.

6. Distribute 100 μL/well of PBS-gelatin-Tween, in all wells except in the first lane.

7. Incubate 150 μL of mouse sera diluted in PBS-gelatin-Tween (150× dilution of sera). Make 1/3 serial dilution from 50 μL of the sample in the first lane. Discard 50 μL of the last lane. Incubate for at least 1 h at 37 °C or overnight at 4 °C.

8. Wash as in **step 3**.

9. Incubate with 50 μL/well of anti-mouse IgM HRPO diluted in PBS-gelatin-Tween. Incubate for 1 h at 37 °C.

10. Wash as in **step 3**.

11. Incubate with 50 μL/well of substrate solution protected from the light during 2–15 min. The substrate solution is unstable and should be used at room temperature soon after preparation.

12. Stop the reaction with 50 μL/well of stopping buffer.

13. Read the plate at 490 nm in a spectrophotometer.

3.4 AID$^{-/-}$μS$^{-/-}$ in Mixed Bone Marrow (BM) Chimeras

To study the effect of different Igs classes on AID$^{-/-}$μS$^{-/-}$ B cell homeostasis in mixed BM chimeras, it is necessary to distinguish AID$^{-/-}$μS$^{-/-}$ B cells from WT or other mutant B cells by using the allotypic markers CD45.1 and CD45.2. Therefore, it is necessary to cross AID$^{-/-}$μS$^{-/-}$CD45.2 mice with CD45.1 mice (see **Note 2** and Table 3).

1. Generate CD45.1$^{+/+}$AID$^{-/-}$μS$^{-/-}$ mice.

2. Lethally irradiate 6- to 12-weeks-old RAG-deficient mice (900 rads, ^{137}Cs source for C57BL/6 background) 1 day prior to BM reconstitution (see **Note 3**).

3. The day after irradiation, prepare BM cells by flushing tibias and femurs of donor mice with sterile PBS through a 23 G needle mounted on a syringe (see **Note 4**).

4. Count the BM cells and mix CD45.1$^+$ AID$^{-/-}$μS$^{-/-}$ BM cells at a 1:1 ratio with CD45.2$^+$ BM cells from WT, μS$^{-/-}$, AID$^{-/-}$, or AID$^{-/-}$μS$^{-/-}$ mice (see **Note 5**, Table 3).

5. Reconstitute irradiated RAG-deficient recipient mice by intravenous injection of 3–10 × 10^5 total BM cells in sterile PBS.

6. Analyze BM chimeras 10–14 weeks after reconstitution (see **Note 6**).

Table 3
Description of AID$^{-/-}$mS$^{-/-}$ mixed BM chimeras

Recipients	CD45.1+ BM 50%	CD45.2+ BM 50%		Igs in serum of BM chimeras	CD45.1+ B cells	CD45.2+ B cells
RAG$^{-/-}$	WT AID$^{-/-}$ μS$^{-/-}$	WT AID$^{-/-}$ μS$^{-/-}$	8 weeks after	All Ig classes No Igs	WT AID$^{-/-}$μS$^{-/-}$	WT AID$^{-/-}$μS$^{-/-}$
		AID$^{-/-}$ μS$^{-/-}$		Unmutated IgM Switched Igs (IgA, IgG, IgE)	AID$^{-/-}$μS$^{-/-}$ AID$^{-/-}$μS$^{-/-}$	AID$^{-/-}$μS$^{-/-}$ μS$^{-/-}$
		WT		All Ig classes	AID$^{-/-}$μS$^{-/-}$	WT

3.5 Immunoglobulin Injections

1. To prepare sera containing total Igs, collect blood from WT mice into a tube (*see* **Note 7**).

2. Incubate the tube(s) with mouse blood overnight at 4 °C or for 30 min at room temperature for clotting.

3. Centrifuge the tube(s) at $1000–2000 \times g$ for 10 min at 4 °C.

4. Transfer the supernatants—sera—immediately into a new tube.

5. Inject mouse sera or purified Igs from mouse sera in $AID^{-/-}\mu S^{-/-}$ mice depending on the project aim (*see* **Note 8**).

3.6 Generation of Germ-Free (GF) $AID^{-/-}\mu S^{-/-}$ Mice by Caesarean Section Rederivation

The general principle is to transfer $AID^{-/-}\mu S^{-/-}$ fetuses extracted by caesarean section from the sterile uterus of their mothers day 19 *post coitum* (*p.c.*) to surrogate mothers bred in an isolator (*see* **Note 9**).

1. On day −5, mate C3H/HeN mice inside the incubator to ensure that a surrogate mother that gave birth less than 5 days before will be available at the time of the transfer of $AID^{-/-}\mu S^{-/-}$ pups (*see* **Note 10**).

2. On days −4 to −3, after ascertaining the existence of a pregnant C3H/HeN female inside the incubator, mate $AID^{-/-}\mu S^{-/-}$ breeders (*see* **Note 11**). Check for mating plugs the morning after to identify donor mother(s).

3. Day 17 *p.c.* give the donor mother a subcutaneous injection of Promon (5 mg/0.1 mL).

4. Day 18 *p.c.* prepare the hysterectomy. Transfer all necessary supplies and instruments (water, paper towels, and surgical instruments), sterilized according to the entry procedures, into the incubator housing the surrogate mother(s), fill up the reservoir of the surgical transfer chamber with 1% VirkonS, sterilize the surgical compartment, and ventilate it overnight (*see* **Note 12**).

5. Day 19 *p.c.*, transfer all the necessary supplies and instruments from the incubator to the sterile compartment of the transfer chamber. In the nonsterile compartment of the transfer chamber, sacrifice the pregnant $AID^{-/-}\mu S^{-/-}$ donor female and submerge it entirely in the germicidal liquid (1% VirkonS) for 1 min.

6. Proceed to the hysterectomy. Using sterile gloves and surgical tools, open the abdomen and clamp the base of the uterus and the uterine horns. Cut the closed uterus and transfer it into the sterile compartment by dipping it for 1 min in the germicidal liquid of the transfer chamber.

7. Once inside the sterile compartment, rinse out the germicidal liquid with sterile water. Open the uterus, stimulate the pups to

breathe at the same time and clean them with paper towels. Pups should gain a pink color and breathe normally.

8. Transfer the pups into the isolator. Take out the surrogate mother litter to another cage and substitute some pups from her litter with $AID^{-/-}\mu S^{-/-}$ pups, such that the final number of pups remains the same. Rub $AID^{-/-}\mu S^{-/-}$ pups with bedding material from the foster mother cage and put the litter back with the mother. Check later for successful adoption (*see* **Note 13**).

9. Monitor the incubator microbiological status every 3 weeks (*see* **Note 14**).

3.7 Flow Cytometric Analysis (FACS) of Mouse B1 and MZ B Cells

This section describes the basic procedure to identify and quantify B1 and MZ B cell subsets by flow cytometry. B1 B cells are predominantly found in the peritoneal cavity, with a fewer residing in the spleen, while MZ B cells are restricted to the spleen. MZ B cells can be analyzed and quantified by FACS as described in this section, but as indicated by their name, these cells are primarily defined by their anatomical location. Their analysis and quantification can be confirmed by immunohistology using a confocal (or fluorescence) microscope (*see* Subheading 3.8). The same spleen can be analyzed by both FACS and immunohistology (*see* **Note 15**).

In the following protocol for FACS analysis, all centrifugations are performed at $400 \times g$ for 10 min at 4 °C.

1. Euthanize mice (*see* **Note 16**) and harvest peritoneal cavity cells and spleen.

2. Start by harvesting cells from peritoneal cavity. Pin the mouse on its back and cut out the skin of the mouse exposing as much as possible of the muscular wall of the peritoneum, leaving it intact.

3. Without punching the intestine, inject 8–12 mL (depending on the size of the mouse) of cold lavage buffer using 12 mL syringe with a 26 G needle (*see* **Note 17**). Take out the needle and substitute it for 18 G needle to withdraw the liquid containing peritoneal cavity cells. Harvest the cell suspension in a tube kept on ice.

4. Collect the spleen in a small plastic petri dish containing 1 mL of FACS buffer and a piece of 50 μm pore size nylon mesh (approximately 150×150 mm).

5. Mash the spleen between the folded mesh using curved microdissecting forceps to obtain a cell suspension, and transfer into a tube.

6. Centrifuge tubes and remove the supernatant. Resuspend the splenocytes in red blood cells lysis buffer (*see* **Note 18**). Wash
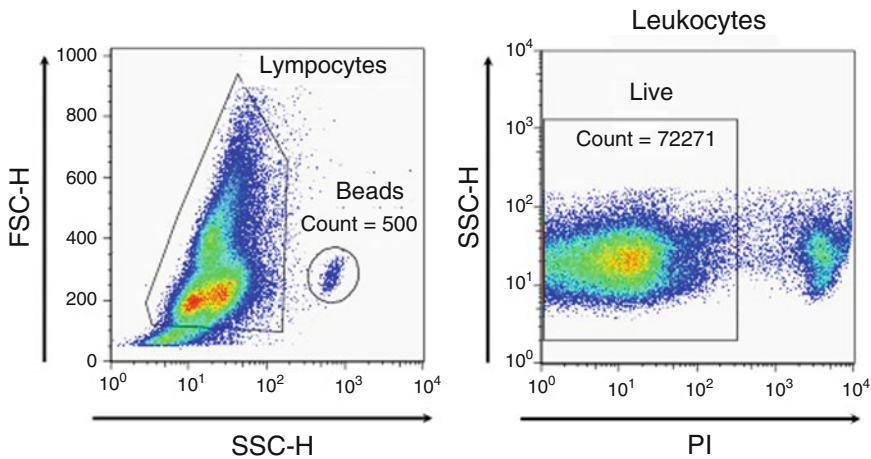
Fig. 1 Cell counting by FACS. Live lymphocyte number is determined using 10 μm latex beads (Coulter Corp.) and PI. Prepare a working solution of 10 μm latex beads at 5×10^5 beads/mL, by counting beads in the hemocytometer. Prepare a PI working solution by diluting PI in FACS buffer to a concentration of 200 ng/mL. For each cell suspension prepare a FACS sample by mixing 10 μL of beads working solution, 10 μL of cell suspension, and 80 μL of PI working solution. Set acquisition limit to 500 events in bead gate corresponding to 10 μL of sample. Read the corresponding number of live PI⁻negative lymphocytes (cells inside the lymphocyte gate) corresponding to the number of live lymphocytes contained in 10 μL of the sample, which is a ten times dilution of the cell suspension. The example of FACS plots shows the counts obtained for a cell suspension containing 7.2×10^7 mL^{-1} of live lymphocytes

samples with FACS buffer, centrifuge and resuspend in a defined volume of FACS buffer.

7. Count live lymphocytes using a flow cytometer, 10 μm latex beads and PI (Fig. 1) (*see* **Note 19**).

8. Plate $1–2.5 \times 10^6$ cells per well in 96-well round-bottom plates (*see* **Note 20**). Centrifuge and resuspend in 25 μL Fc block solution. Incubate for 10–15 min at 4 °C.

9. Wash with FACS buffer and centrifuge. Stain cells for 45 min at 4 °C in the dark with antibody staining mixes to detect B1 cells in peritoneal cavity and MZ B cells in the spleen. B1 cells in the peritoneal cavity can be identified as CD19⁺ or B220⁺and CD11b^{int} and can be subdivided into B1-a (CD5⁺) and B1-b (CD5⁻) cells. MZ in the spleen can be identified as CD19⁺B220⁺CD21/35^{hi}CD23^{low} or CD19⁺B220⁺IgM^{hi}Ig-D^{low}. In spleen, the CD19⁺B220⁺CD21/35⁻CD23⁻ subset contains B1 and activated B cells [5].

3.8 Detection and Quantification of MZ B Cells by Immunohistology

MZ B cells are defined anatomically as CD21/35⁺CD23⁻ cells residing outside of the follicles of the spleen. In this section, we describe how to quantify these cells by histology using confocal or fluorescence microscopy.
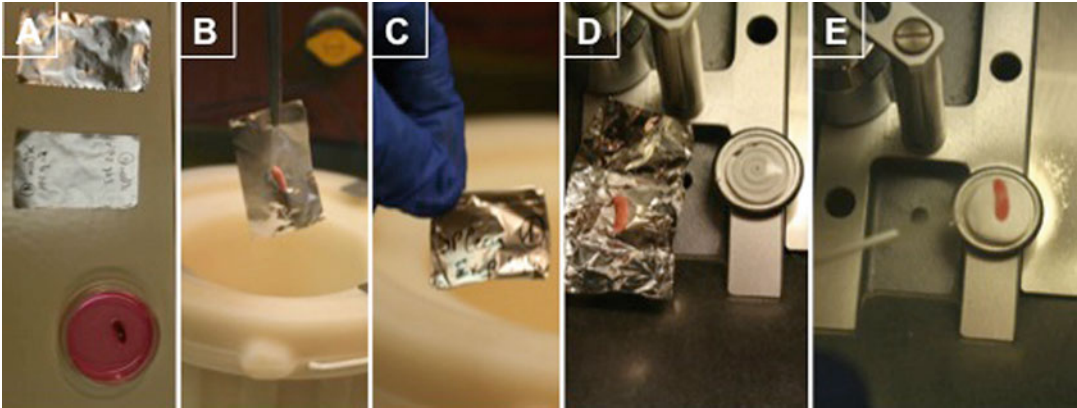
Fig. 2 Snap freezing and mounting method for mouse spleen cryo-sectioning. (**a**) Preparation of foil to store and identify harvested spleens. (**b**) Freezing of the spleen by successive plunging into liquid nitrogen until frozen. (**c**) Fold the foil around the spleen without touching the spleen, starting first by the bottom and top parts and then the sides for storage. (**d**) Mount the spleen for cutting; pour OCT compound onto prechilled object holder. (**e**) Place the spleen horizontally, its larger side up and use immediately freezing spray to avoid thawing

1. Snap freeze immediately the spleen or part of the spleen (*see* **Note 15**) harvested from the sacrificed mouse by placing the organ on its largest flat side onto the shiny side of a piece of foil with identification number written on the opposite side, and plunging it several times into liquid nitrogen until frozen (*see* Fig. 2a, b).

2. Fold rapidly the sides of the foil around the spleen without touching it (*see* Fig. 2c), and leave in liquid nitrogen until storage.

3. Put foiled organs in resealable polypropylene plastic bags to prevent dehydration and keep at −20 or −80 °C for short- or long-term storage, respectively.

4. In a cryostat chamber pour precooled OCT compound on object holder and place the frozen spleen, its largest flat side facing up.

5. Drown the spleen horizontally into the compound to embed the lower part, leaving 1–2 mm of the upper part out of the compound.

6. Spray immediately with freezing spray to prevent the spleen from thawing (*see* Fig. 2d, e, and **Note 21**).

7. Leave the mounted spleen to rest until complete freezing of the compound. For cutting, orientate the object holder such that the spleen is cut vertically.

8. Cut 5- to 7-μm-thick sections and collect onto multispot glass slides. Let it dry for 1 h at room temperature, fix for 20 min at 4 °C (in frigde) in a glass-staining jar containing cold acetone,

let it dry for 10 min at room temperature before placing in polypropylene bags to prevent air contact during storage at -20 or $-80\ ^\circ$C.

9. For immunofluorescent staining, take slides out of the freezer and let them warm up at room temperature for 10–15 min.

10. For rehydration and washing steps place the slides onto a glass or metal rack of a glass-staining jar, plunged into glass-staining jar containing PBS.

11. Put a magnetic stir bar between the rack and the bottom of the jar, place the jar on the top of a stirrer at a low speed and cover with foil to protect from the light. With this system, first rehydrate the slide for 10 min.

12. Proceed to immunostaining for CD23 and CD21/35 in a dark wet chamber (or covered with foil if the wet chamber is clear) (*see* **Note 22**) by incubating the sections for 1 h with rat anti-CD23 antibody (clone B3B4) at 7 μg/mL in staining buffer supplemented with 5% normal mouse serum.

13. Wash for 7 min with PBS in a glass jar under agitation.

14. Incubate for 30 min with polyclonal goat Cy3-conjugated anti-rat IgG antibody in staining buffer supplemented with 5% normal mouse serum, at a concentration to be determined extemporaneously (for indication, product A10522 from Invitrogen was used at 14 μg/mL).

15. Wash for 7 min with PBS in a glass-staining jar under agitation.

16. Incubate for 30 min in a dark wet chamber with FITC-conjugated rat anti-CD21/35 antibody (clone 7G6) at 10 μg/mL in confocal staining buffer.

17. Wash for 15 min with PBS in a glass-staining jar under agitation. Remove as much buffer as possible without letting the tissue dry, and mount in a antifading medium for confocal or fluorescence microscopy.

18. Acquire 1–5 images for each spleen in order to picture a representative area of the section (*see* Fig. 3). Using imaging software, delineate and measure the area covered by CD21/35+ CD23− MZ cells (Fig. 3).

19. Report each area in a counting table, as exemplified in Table 4.

20. Calculate the total area covered by MZ cells per total area of imaged spleen (Table 5).

4   Notes

1. AID$^{-/-}$μS$^{-/-}$ mice spontaneously develop enlarged MZ and B1 cells with age. At 3 weeks of age a slight but statistically different increase in MZ, B1 and activated B cell numbers
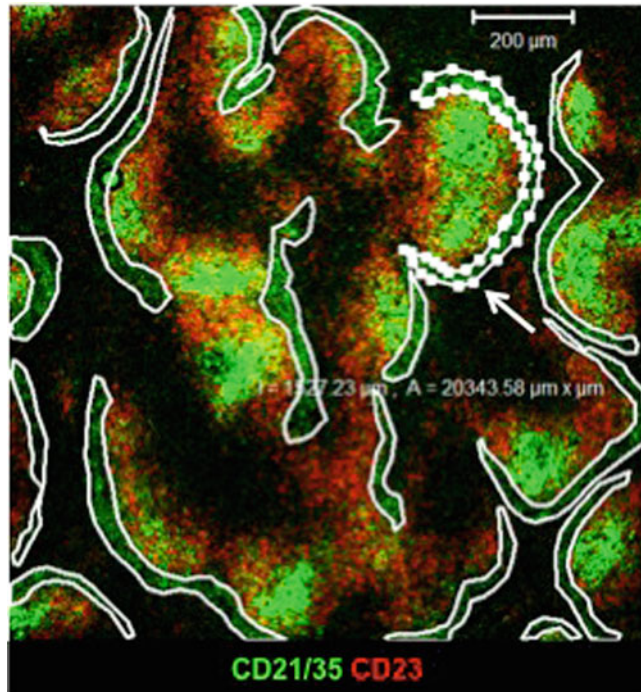
Fig. 3 Confocal image from the spleen of a wild type C57BL/6J mouse stained for CD21/35 (*green*), and CD23 (*red*). The follicles are delineated by CD23 staining and contain bright CD21/35 cells corresponding to follicular dendritic cells. MZ are CD21/35$^+$ CD23$^-$ cells outside of the follicles, and were delineated in *white*. The *arrow* shows a selected MZ area measured by the software. This area was reported in counting Table 4 (identified by a *star*). The total spleen area per image is 1,619,994.38 μm^2 Total imaged area per spleen: 8,099,971.9 μm^2, corresponding to five images/spleen (*see* Table 5)

    reflecting in increased total of B cells compared to WT mice can already be observed [5].

2. As μS$^-$ alleles are not readily detectable by PCR, the fastest and easiest way to produce CD45.1$^{+/+}$AID$^{-/-}$μS$^{-/-}$ is to fix first the μS deficiency by producing CD45.1$^{+/+}$μS$^{-/-}$ and AID$^{-/-}$μS$^{-/-}$ mice that are then inter-crossed. The progeny of this mating can be easily screened by PCR for AID$^{-/-}$ mutants. The presence of CD45.1 allotype can be screened by FACS staining a small sample of mouse blood with anti-CD45.1 (clone A20) and anti-CD45.2 (clone 104.2).

3. For optimal reconstitution and to avoid losing recipients due to the irradiation, recipient mice should be injected with donor BM between 12 and 24 h after irradiation.

4. BM cells are going to be injected into immune-compromised irradiated recipients; therefore, it is important to use sterile conditions to process the BM.

Table 4
Example of counting table to quantify MZ area by histology

Spleen nb 1	Image 1	Image 2	Image 3	Image 4	Image 5	Total CD21+CD23− area (μm^2)
CD21+CD23− areas (μm^2)	7475.67	23,464.91	10,584.09	25,320.12	20,085.56	
	19,436.99	9412.77	19,751.4	4587.71	9338.79	
	8474.32	36,488.88	10,769.24	19,177.48	29,104.51	
	15,215.48	17,694.98	16,816.68	10,597.81	5008.08	
	20,343.58[a]	62,900.7	8699.46	3567.72	9588.05	
	16,842.58	5117.19	7022.62	11,946.7	15,686.41	
	16,503.29		2554.14	4456.73	48,222.47	
	6630.35		4724.44	27,564.1	24,578.69	
	20,078.96		9754.06	5790.41		
	7119.86		10,796.8	17,623.41		
	11,499.97		14,833.03	30,973.85		
	8121.72		4428.31	1166.05		
	21,929.84		5457.51	2545.04		
	6093.29		13,192.05	12,484.42		
	7929.81		2772.2	4088.26		
	10,489.89					
	2296.49					
Total area/image	206,482.09	155,079.43	142,156.03	181,889.81	161,612.56	847,219.92

[*]correspond to the MZ area highlighted in Fig. 3

Table 5
Comparison of spleen area occupied by MZ B cells in WT and AIDμS mice

	Total CD21$^+$CD23$^-$ area (μm^2)	CD21$^+$CD23$^-$ area/μm^2	CD21$^+$CD23$^-$ area/μm^2
WT Spleen1	847,219.92	0.104595415	10.45954147
WT Spleen2	1,319,376.33	0.162886532	16.28865317
WT Spleen3	824,263.07	0.101761226	10.17612259
WT Spleen4	1,400,605.12	0.172914812	17.29148122
AIDμS Spleen4	2,910,292.13	0.359296571	35.92965711
AIDμS Spleen4	4,349,735.2	0.537006209	53.70062086
AIDμS Spleen4	5,917,101.92	0.730508944	73.05089441
AIDμS Spleen4	7,537,796.85	0.930595432	93.05954321

Note: Total area analyzed/spleen: 8099971.9 mm^2, corresponding to five pictures/spleen (*see* Fig. 3)

5. Other BM combinations can be done according to the project aim, e.g., the role of IgA on B cell subset homeostasis can be studied by constructing BM chimeras using CD45.1$^+$ AID$^{-/-}$μS$^{-/-}$ BM and CD45.2$^+$IgA$^{-/-}$ mice.

6. At 10 weeks after BM reconstitution the WT control BM chimeras have serum Ig titers comparable with those of WT mice. The number of total B cells in the WT control chimeras increases with time after reconstitution; therefore, it can be advantageous to analyze chimeras later in time if the number of B cells is an important factor for the project aims.

7. Several methods of mouse blood collection are available. The method of blood collection has to be determined according to the availability of WT mice. High amounts of WT blood are necessary for these experiments; therefore, cardiac puncture—a terminal procedure—can be used to higher yield. Cardiac puncture can be performed on terminally anesthetized mouse or in a recently euthanized mouse (do not use cervical dislocation to euthanize the mouse) by inserting a 1 mL syringe with a 22 G needle at a 45° angle at the center of the mouse thorax 1 cm deep. Blood should appear immediately when the plunger is pulled, if it does not, slightly withdraw the needle.

8. Sera Igs have different half-life times—varying from short half-life times for IgE and IgA to a week-long half-life time for IgGs [12]. Depending on the aim of the project this parameter has to be considered in the experimental design, for instances WT sera injections every other day are unable to generate detectable levels of IgA in sera of $AID^{-/-}\mu S^{-/-}$ mice even when sera are used for ELISA detections at dilutions allowing IgA detection in sera from GF WT animals (*see* Fig. 4). Igs can be purified from the sera of WT mice by affinity chromatography using protein G columns (IgGs). Hybridoma supernatants can be used to purify monoclonal IgGs that can serve as controls for the polyclonal IgG purified from sera of WT mice.
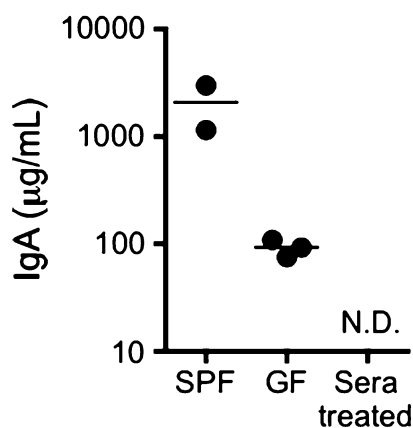
Fig. 4 Serum levels of IgA determined by ELISA. Serum IgA in sera from germ-free (GF) and specific pathogen-free (SPF) C57Bl/6 mice. Serum IgA is not detected (N.D.) in $AID^{-/-}\mu S^{-/-}$ that received WT sera injections every other day during 4 weeks ($n = 3$)

9. This section has been adapted from the description of generating germ-free mice on The European Mouse Mutant Archive (EMMA) [13]. A chapter in a previous series of Methods in Molecular Biology contains a detailed description as to how to breed and work with axenic mice [14]. Germ-free facilities usually have dedicated personal to operate the incubators; therefore, this brief description aims to give the researcher an idea of the procedure for a better experimental planning.

10. The space inside the incubator is limited, so everything should be optimized. Without a surrogate mother the transfer cannot be done; therefore, this is one of the most important steps of the procedure. C3H/HeN mice are good surrogate mothers, as they have big litters and easily adopt mice of C57BL/6 background. Check plugs in the morning after mating, as a confirmation that a surrogate mother will be available for transfer in 19 days. If space allows, more than one mating can be considered so as to ensure availability of surrogate mother.

11. It is advisable to set more $AID^{-/-}\mu S^{-/-}$ mating pairs outside the incubator to make sure that mice will available for transfer. If WT C57BL/6 controls are not available inside the GF incubators, a similar setup for C57BL/6 controls should be done.

12. The surgical transfer chamber is made of two compartments, one sterile and one non-sterile, separated by a reservoir that serves as a dipping tank. The uterus of the donor mother enters the isolator through this reservoir that contains a germicidal liquid.

13. $AID^{-/-}\mu S^{-/-}$ mice can be easily identified among the mixed litter by their black color, distinct from agouti C3H/HeN mice.

14. GF mice have enlarged cecum. Serum IgA levels are substantially reduced in WT GF compared to SPF mice (*see* Fig. 4). These two characteristics of GF mice are easy to check when analyzing the mice, although they do not substitute microbiological monitoring of the GF status.

15. Weigh the spleen. Cut in approximately half and weigh the piece to be used for FACS. Freeze the second half for immunohistology. Prepare cell suspension from the FACS piece and count the cells. Calculate the total numbers of cells in the whole spleen by multiplying the number found in the FACS piece by the ratio of total spleen weight over FACS piece weight.

16. If mice are euthanized by cervical dislocation be careful not to damage the abdomen to avoid blood in the peritoneal lavage.

17. The pressure made on the walls of the peritoneum by the liquid helps to extract cells that are attached; therefore, the more liquid injected the more cells can be collected. When extracting the injected liquid avoid aspirating fat as much as possible. Collecting from the side of the peritoneum may help to avoid the fat. Peritoneal lavage improves substantially with practice.

18. If peritoneal lavages contain RBC it is advisable to perform their lysis as for the spleen for an optimal FACS analysis.

19. Cells/mL function is available in some flow cytometers and can be used in conjunction to propidium iodide coloration as an alternative methodology to count live lymphocytes with beads.

20. V-bottom plates can be used for the peritoneal cavity samples as they help to reduce cell loss during centrifugation steps.

21. This way of freezing and mounting the spleen allows a perfect orientation of the tissue to obtain big sections. The fact that the upper part of the spleen is not embedded prevents folding of the sections caused by different resistances of the tissue and the compound to the blade of the cryostat. When the embedded part is reached, trim carefully the sides of the spleen to clear from the compound.

22. A dark wet chamber can be easily made out of the lid of a polystyrene box the size of 5 or 10 mL disposable plastic pipettes. Place two pipettes horizontally in the bottom of the lid spaced by a distance slightly smaller than the length of the slides. Between and around the pipettes put flatten paper towel and wet with distilled water. Place the slides across the pipettes, such that they lay horizontally above the wet paper without touching. Pour staining solutions onto the sections, and cover with foil to keep in the dark during the incubation.

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Assessment of Anti-Tumor Cytotoxic Activity of Naturally Occurring Antibodies in Human Serum or Plasma

Reinhard Schwartz-Albiez and Othmar Dill

Abstract

A small percentage of the Western population carries antibodies in the peripheral blood, which are able to kill human tumors such as neuroblastoma or melanoma. Several observations indicate that these antibodies, preferentially of IgM isotype, belong to the class of naturally occurring antibodies. Here, we describe two screening methods for the detection and quantification of such antibodies in human blood samples: a cellular ELISA technique and a flow cytometric assay, based on intercalation of fluorescent propidium iodide into the DNA of dying or dead cells.

Key words Cell death inducing serum search (CISS), Cellular cytotoxicity, Flow cytometry, Human tumors, Natural antibodies, Propidium iodide

1 Introduction

A small percentage of healthy blood donors carry antibodies in the blood with cytotoxic activity against distinct human tumors such as neuroblastoma or melanoma [1–3]. This cytotoxic activity is exerted in a complement-dependent way. These antibodies have been classified as so-called naturally occurring antibodies, present without stimulation by exogenous antigens [4]. The antibodies seem to detect primarily carbohydrate structures such as Lewis^y, Thomsen-Friedenreich antigen (CD176), or gangliosides, like GD2 and GD3, which have been described as tumor-associated antigens [2].

Screening of blood samples (sera or plasma) from larger groups of defined human populations for these cytotoxic antibodies can be advantageous for epidemiological studies in order to investigate their occurrence with regard to, for instance, sex, age, history of infectious diseases, life style, certain genetic traits, and more importantly susceptibility for distinct classes of tumors. The latter properties may help to clarify the role of natural antibodies as part of a humoral immune mechanism of tumor surveillance.

The here-presented screening methods may also be useful to investigate the function of therapeutic monoclonal antibodies in the physiological environment of blood components [5].

In order to measure cytotoxic activity in a high-throughput fashion, a cellular ELISA-type assay, called CISS (Cell Death Inducing Serum Search), was designed. Further, for closer examination, in particular to study the underlying mechanism, flow cytometric analysis of cytotoxicity can be analyzed. Both assay systems measure, as a basic principle, intercalation of propidium iodide into DNA of dying or dead cells.

2 Materials

2.1 General Equipment and Reagents

1. Flow cytometer.
2. Biological safety cabinet for cell culture.
3. Fluorometer: TECAN SpectraPlus.
4. PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4.
5. Culture medium: RPMI 1640 supplemented with 10% or less heat-inactivated fetal calf serum (FCS).
6. Flow cytometry buffer (FACS buffer): phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) (w/v) and 0.1% NaN_3 (w/v).
7. Veronal buffer (VBS): 5 mM sodium barbital, pH 7.4, 0.15 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl; 0.1% BSA.
8. GPBS: PBS containing 0.1% (v/v) gelatin.
9. Propidium iodide (PI): dissolved in PBS.
10. 20% (v/v) Triton-X 100: Prepared in PBS.
11. 96-flat-bottom well-microtiter plate.
12. FACS^R vials (Becton & Dickenson, Germany).

2.2 Blood Products

1. Serum of healthy donors. It can be supplied by blood banks in larger numbers or by individual sampling from voluntary donors (*see* **Note 1**).
2. Plasma from individual donors. They can be obtained from biobanks. The advantage of plasma is that it can be obtained in larger quantities in comparison to serum (*see* **Note 2**).

2.3 Cell Lines

1. Adherent (tumor) cell lines: human neuroblastoma cell line like Kelly and human melanoma cell lines such as MeWo, SK-Mel-28, SK-Mel-30, A-375, HT144, and MA-Mel-95 (*see* **Note 3**).
2. Non-adherent (tumor) cell lines: human B cell lymphoma cells like Raji, Molp-8, and Lp-1 (*see* **Note 3**).

3   Methods

3.1   Cell Death Inducing Serum Search Assay (CISS)

Cultivate the cells as a rule, in FCS-containing RPMI medium and keep in an incubator at 37 °C in an atmosphere with 5% CO_2. For the assay, cells should be incubated in a culture medium without a phenol red indicator.

For high-throughput screening of cytotoxic sera, a fluorescence microtiter ELISA-type assay is applied. Fluorescence is measured in a fluorometer.

1. Seed 5×10^4 adherent (tumor) cells/microtiter well in 200 μl of RPMI medium into a 96-flat-bottom well-microtiter plate and incubate overnight at 37 °C to let them become adherent again. For non-adherent tumor cells, such as leukemia or lymphoma cells, this step can be omitted and seed the cells into the wells shortly before or together with the respective serum without change of medium (omit **step 2**).

2. The following day, aspirate the medium and add 40 μl of the respective serum sample to each well.

3. For controls, add 40 ml of culture medium (without phenol red) instead of serum.

4. Incubate the plate for 1 h at 37 °C, 5% CO_2 atmosphere.

5. Measure the autofluorescence at 540 nm. This is defined as first measurement.

6. Subsequently, add 40 μl of 0.4 mg/ml PI per well and incubate for 1 h.

7. Measure PI fluorescence at 640 nm. This is defined as second measurement and should indicate the cytotoxicity of the individual serum sample.

8. Following, add 20 μl/well of 20% (v/v) Triton-X 100, gently mix the content, and incubate for an hour.

   Measure the fluorescence again for each well. This is defined as third measurement and should indicate the total, 100% cell lysis.

9. Calculate percentage of cytotoxicity using the formula:

$$x\% = \frac{(2\text{nd-measurement} - \text{autofluorescence})}{(3\text{rd-measurement} - \text{autofluorescence})} \times 100.$$

The threshold of cytotoxicity can be defined, e.g., as $3\times$ standard deviation (SD). Alternatively, in order to see at which values the data accumulate and which cut-off value would be feasible, cytotoxic activities can be plotted in a frequency histogram of percentage of cell death values with bins set up every 5% and the frequency of samples plotted against bins. Short graphical demonstration of CISS assay is shown in Fig. 1a.
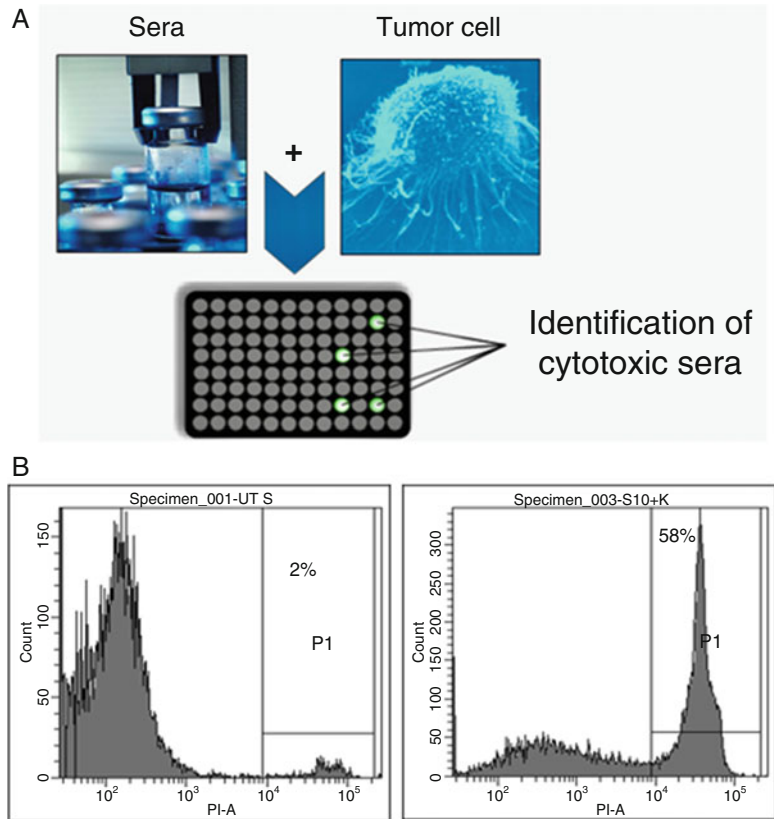
Fig. 1 CISS assay and flow cytometric analysis of cytotoxicity. (**a**) CISS assay. Sera are incubated together with tumor cell targets in a microtiter plate. Cytotoxic reaction can be optically observed by different grades of color reaction in the respective wells and the reaction quantified using a fluorometer. Also, the reaction can be qualitatively assessed by microscopic observation of cellular reaction. (**b**) Flow cytometric analysis of cytotoxicity. PI intercalation in DNA of dead or dying cells is quantitatively measured. Live cells are represented in the *left peak* of the *histograms* and dead cells with high PI incorporation in the *right peak*. By setting the gates with negative controls, percentage of PI intensity of positive cells can be assessed (*left histogram* = negative, *right histogram* = positive cytotoxic reaction). *x*-Axis: mean intensity of fluorescence, *y*-axis number of cells measured

3.2 Flow Cytometric Analysis of Cytotoxicity

Alternatively, immunoglobulins of serum or plasma samples can be screened for their cytotoxic potential against target cells by PI staining using flow cytometry. This assay is based on the same principle as the CISS fluorometric assay.

1. Add 5×10^5–1×10^6 target cells in FACS^R vials and incubate with 40–100 μl of serum/plasma for 1 h at 37 °C (this incubation time is sufficient for serum/plasma samples with cytotoxic potential to kill the target cell).

2. After the incubation, supplement additional 200 ml of FACS buffer to the cells and spin down the cells in a centrifuge by $300 \times g$ at 4 °C for 2 min and then wash two more times with 300 μl of FACS buffer by the same procedure.

3. Subsequently, add 300 ml of FACS buffer with 50 mg/ml of PI solution to the cells and analyze PI uptake by flow cytometry. This is named "1 step cytotox."

4. For positive control (100% cytotoxicity), incubate the cells with 20% (v/v) Triton-X-100 for 1 h at 37 °C.

5. Negative controls: (a) FACS buffer without addition of serum/plasma, (b) control without serum/plasma with subsequent addition of PI (spontaneous cytotox), and (c) incubation with a negative isotype immunoglobulin (human IgG or IgM) control (*see* **Note 4**).

6. Alternatively, cells can be incubated with serum/plasma or distinct monoclonal antibodies for 30 min on ice, then wash with pre-warmed GPBS, and incubate for 1 h at 37 °C with exogenous complement source (1:10 diluted normal human serum in 200 ml of VBS). Again, wash the cells with GPBS, stain with PI, and analyze by flow cytometry. This procedure is called "2 step cytotox." This variation may give information about the specific adherence of the immunoglobulins to the cell surface.

7. Perform quantitative analysis of flow cytometric data, as shown in Fig. 1b.

4   Notes

1. Please note that sera derived from blood donors of blood banks are, as a rule, anonymous samples and can neither be traced back to individual donors nor are these donors available for repetitive blood donations. For individual samples of healthy donors or patients one may need written, informed consent of the donor according to the ethical standards of the Helsinki Declaration.

2. For testing, cytotoxic plasma should be treated with sodium citrate to prevent clotting. For assay variations in which exogenous complement is needed, a human complement source can be prepared from pooled, non-cytotoxic sera of several healthy donors and stored frozen at −70 °C. Heat-inactivated serum (56 °C, 30 min) is used as negative control.

3. Established cell lines should be authenticated and determined to be negative for cross-contamination with other cells by cell line authentication tests (such as Multiplexion), and

    mycoplasma-free as verified by DAPI-staining of DNA and/or a PCR-based mycoplasma test.

4. The flow cytometric assay can be varied in order to investigate the cytotoxic mechanism or the possible molecular target of the cytototoxic attack:

(a) **Step 5** in Subheading 3.2 can be varied by incubation of test serum/plasma at 56 °C for 30 min to inactivate complement prior to cell incubation. Exogenous complement source can be added in the second incubation at 37 °C.

(b) Target cells can be treated with various enzymes, such as neuraminidase, prior to the assay as described elsewhere [2].

Acknowledgment

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Chapter 9

Hydrolysis and Dissolution of Amyloids by Catabodies

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Abstract

Catalytic antibodies (catabodies) hold potential for superior immunotherapy because of their turnover capability and no or minimal induction of inflammatory responses. Catabodies neutralize and remove target antigens more potently than conventional antibodies. Depending on the catalytic rate constant, a single catabody molecule degrades thousands to millions of target molecules over its useful lifespan, whereas conventional antibodies only form reversibly associated, stoichiometric complexes with the target. Thus, removal of the antibody-bound target requires accessory phagocytic cells that ingest the immune complexes, which is usually accompanied by release of inflammatory mediators. In comparison, catabodies bind the target only transiently, and the rapid and direct target destruction reduces the concentration of immune complexes that can activate inflammatory processes. These features are especially pertinent when large target amounts at anatomically vulnerable sites must be removed, e.g., amyloids. We reported specific catabodies to misfolded transthyretin (misTTR) amyloid and amyloid β peptide (Aβ). Accumulation of the oligomeric and fibrillized amyloid TTR forms causes diverse systemic pathologies, including cardiomyopathy, polyneuropathy, and skeletal diseases. Brain Aβ aggregates are thought to cause central nervous system degenerative disease, chiefly Alzheimer's disease. We describe methods for testing catabody-mediated degradation and dissolution of Aβ and TTR.

Key words Catalytic antibodies, Amyloids, Transthyretin, Amyloid beta, Hydrolysis assay, Dissolution assay

1 Introduction

Twenty seven proteins are known to form disease-associated, misfolded β-sheet aggregates. The soluble misfolded oligomers exert toxic effects via cell-surface receptors and membrane destabilization [1–3]. The particulate amyloids disrupt tissue anatomic integrity. No approved drugs capable of addressing the cause(s) of amyloidosis or reversing the course of amyloid disease are available. Accumulation of misfolded transthyretin (misTTR) aggregates with advancing age is thought to cause lumbar spinal stenosis [4, 5], carpal tunnel syndrome [6], cardiomyopathy [7], and polyneuropathy [8]. Renal, gastrointestinal, and lymphoid dysfunction is also common in patients with TTR amyloidosis [9, 10]. Along

with the tau protein aggregates, amyloid β (Aβ) peptide soluble oligomers and amyloid deposits in the brain play a central role in the pathogenesis of Alzheimer's disease (AD) [11, 12] and the more rare frontotemporal dementias. Mutations in the TTR, Aβ, and regulatory genes often cause increased TTR and Aβ misfolding, which results in early-onset hereditary amyloid disease [13–15].

A single catalytic antibody (catabody) molecule degrades multiple target antigen molecules. Moreover, the catabody-antigen complexes are transient, minimizing risk of immune complex-induced inflammation. The discovery of pathogenic autoantibodies with protease [16] and nuclease [17] activities generated excitement about their functional contributions to autoimmune disease [18–20]. Recent studies point to the novel role of naturally occurring catabodies as beneficial mediators [20–26]. We suggested that "innate" immunoglobulin (Ig) subsets with rapid and specific catabody activity constitute a natural defense system against misfolded amyloid proteins and microbial superantigens [21, 22, 27].

We describe here example methods useful in analyzing and validating catabody activities directed against misTTR and Aβ. Included are radioassays enabling sensitive and unambiguous detection of amyloid hydrolysis. Electrophoresis, analysis of β-sheet content, turbidity measurement, and microscopy methods allow prediction of catabody functional value, that is, direct catabody dissolution of particulate amyloid and removal of soluble oligomers. These assays do not rely on inflammatory cells needed for the Fc-receptor-dependent removal of amyloids complexed to conventional antibodies. Example methods for preparing antibodies suitable for screening activity are also described. Our hope is that these methods prove useful in identifying novel catabodies that are sufficiently rapid and specific to merit further development as immunotherapies for amyloid disease.

2 Materials

2.1 Buffers and Stock Solutions

All solutions should be prepared in ultrapure water using analytical grade reagents. pH should be verified with calibrated pH meter. Unless otherwise stated, all reagents are filtered with 0.22 μm membrane and stored at 4 °C.

2.1.1 IgM Catabody Purification

1. 1 M Tris–HCl, pH 7.5. Dissolve 31.7 g Tris–HCl, 5.9 g Tris Base in 100 mL of water. Adjust volume to 250 mL with water. Verify pH with pH meter.

2. Stock 100 mM 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS): 3 g CHAPS made up to 50 mL in water. Do not filter (*see* **Note 1**).

3. Buffer A: 50 mM Tris–HCl, pH 7.5, 0.1 mM CHAPS. Dilute 50 mL of 1 M Tris–HCl pH 7.5 in 300 mL water. Add 1 mL 100 mM CHAPS. Adjust volume to 1 L with water. Verify pH.

4. Buffer B: 50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 0.1 mM CHAPS. Dissolve 8.7 g NaCl in 100 mL of water. Add 50 mL of 1 M Tris–HCl pH 7.5 and 1 mL of 100 mM CHAPS. Adjust volume to 1 L with water. Verify pH with pH meter.

5. Buffer C: 0.1 M glycine–HCl, pH 2.7, 0.1 mM CHAPS. Dissolve 3.75 g glycine in 100 mL of water. Add 21.5 mL of 1 M HCl and 0.5 mL of 100 mM CHAPS. Adjust volume to 500 mL with water. Verify pH.

6. Buffer D: 1 M Tris–HCl, pH 9.0. Dissolve 1.6 g Tris–HCl and 11.5 g Tris base in 100 mL of water.

7. 10% NaN_3. Dissolve 10 g NaN_3 in 100 mL of water.

8. Column Storage Buffer: 50 mM Tris–HCl, pH 7.5, 0.1 mM CHAPS, 0.02% NaN_3. Dissolve 5 mL of 1 M Tris–HCl, pH 7.5. Add 0.2 mL of 10% NaN_3 and adjust the volume to 100 mL with water.

2.1.2 Substrate Preparation, Hydrolysis Assay, and Dissolution Assay

1. TTR Misfolding Buffer: 200 mM sodium acetate, 100 mM KCl, 1 mM EDTA, pH 4.2. Prepare 368 mL of 200 mM acetic acid by mixing 4.2 mL of 17.4 M acetic acid with 364 mL of water. Prepare 120 mL of 220 mM sodium acetate in water. Mix these solutions. Add 2 mL of 100 mM EDTA (in water, adjust to pH 8 with 1 N NaOH) and 1 M KCl (in water) each.

2. 1 M sodium phosphate, pH 7.4. Dissolve 11 g Na_2HPO_4 and 2.7 g NaH_2PO_4 in water and adjust the volume to 100 mL. Make 1 mL aliquots and store at $-20\ ^\circ C$.

3. 10× phosphate buffered saline (PBS): Prepare a 10× stock containing 100 mM sodium phosphate, 1.37 M NaCl, 27 mM KCl, pH 7.4 in water. Dilute tenfold to prepare 1× PBS.

4. PBS-C: 1× PBS with 0.1 mM CHAPS.

5. ^{125}I-Aβ40 hydrolysis buffer: PBS-C with 0.1% RIA grade bovine serum albumin (BSA).

6. ^{125}I-misTTR hydrolysis buffer: PBS-C with 0.003% RIA grade BSA.

7. Stock Thioflavin T (ThT). 5 mM ThT in DMSO. Dissolve 10 mg of ThT in 6.2 mL of DMSO. Make 100 μL aliquots, store the aliquots at $-20\ ^\circ C$ in an opaque bag to protect ThT from light.

8. Working ThT solution. 600 μM ThT in PBS-C. Mix 36 μL of 5 mM ThT with 288 μL of PBS-C.

9. 1% uranyl acetate. 0.1 g uranyl acetate in 10 mL of water. Filter (0.45 μm) and aliquot 0.1 mL. Store at $-80\ ^\circ C$ protected from light.

2.1.3 Electrophoresis, Blotting, and Immunostaining

1. 5× nonreducing electrophoresis loading buffer: 0.3 M Tris–HCl, pH 6.8, 10% SDS, 50% glycerol, 0.125 mg/mL bromophenol blue. Dissolve 1 g of SDS in 2 mL of water. Mix 5 mL of glycerol, 0.25 mL of 0.5% bromophenol blue, and 1.25 mL of 2.5 M Tris–HCL, pH 6.8. Adjust volume to 10 mL with water.

2. 5× reducing electrophoresis loading buffer: 300 mM Tris–HCl, pH 6.8, 10% SDS, 50% glycerol, 0.125 mg/mL bromophenol blue, 2.3 mM β-mercaptoethanol. Dissolve 1 g of SDS in 2 mL of water. Mix 5 mL of glycerol, 0.25 mL of 0.5% bromophenol blue, and 1.25 mL of 2.5 M Tris–HCl, pH 6.8. Adjust volume to 10 mL with water. Mix 1 mL of this solution with 0.2 mL of 14.4 M β-mercaptoethanol.

3. Electrophoresis running buffer (Biorad): 0.1 M Tris, pH 8.3, 0.1 M Tricine, 0.1% SDS in water.

4. Transfer Buffer: 92 mM glycine, 25 mM Tris base, 20% methanol. Dissolve 3.03 g of Tris-base, 14.4 g of glycine in 200 mL of water. Add 200 mL of methanol. Adjust volume to 1 L with water.

5. 10% Tween-20: Dilute 5 mL of Tween 20 with 45 mL of water.

6. PBS-T: PBS, 0.05% Tween-20. Add 5 mL of 10% Tween-20 to 995 mL of 1× PBS.

7. 1% skim milk PBST-T: Dissolve 1 g of skim milk powder in 100 mL of PBS-T.

8. Blot Blocking Buffer: 5% skim milk in PBS-T. Dissolve 2.5 g of skimmed milk powder in 50 mL of PBS-T.

9. Ponceau S: 0.1% Ponceau S in 5% (v/v) acetic acid. Dissolve 0.5 g of Ponceau in 100 mL of water. Add 25 mL of acetic acid and adjust volume to 500 mL with water.

10. Ponceau S destaining solution: Mix 225 mL of methanol, 50 mL of acetic acid, and 225 mL of water.

2.2 IgM Catabody Preparation

1. Anti-IgM-gel: Goat anti-human IgM antibody conjugated to agarose (Sigma). Use 1 mL of gel per 0.25 mL of human serum. Each mL gel binds 2–3 mg of human IgM. Human serum contains 1–2 mg/mL IgM. Before storage, wash gel in a column with 5 mL of Buffer C, followed by 5 mL of Column Storage Buffer. Store at 4 °C.

2. Poly-Prep chromatography columns (Biorad). These are 9 cm long columns with 2 mL bed volume capacity (0.8 × 4 cm) and ~10 mL reservoir.

3. Pooled serum from healthy human subjects (0.25 mL).

4. Micro bicinchoninic acid (microBCA) protein assay kit (Thermo Scientific).

5. Plate reader capable of reading absorbance at 562 nm (A562).

2.3 IgV Catabody
Preparation

1. Immunoglobulin variable domain (IgV) phagemid library (ref [28]) in HB2151 *E. coli* cells. Human IgVs amplified by the reverse transcriptase-polymerase chain reaction are cloned into pHEN2 expression vector containing LacZ operon inducible by isopropyl β-D-1-thiogalactopyranoside (IPTG).

2. 2YT medium: 31 g of 2YT broth in 1 L of water. Autoclave the medium.

3. 20% glucose: 200 g of glucose in 1 L of water. Sterilize by filtration using 0.22 μm filter.

4. 100 mg/mL ampicillin: 1 g of ampicillin in 10 mL of water. Sterilize by filtration using 0.22 μm filter. Make 1 mL aliquots and store at −20 °C.

5. Culture Medium: 2YT containing 2% glucose and 100 μg/mL ampicillin.

6. Induction Medium: 2YT containing 100 μg/mL ampicillin.

7. 100 mM IPTG. 5 mL/plate. Dilute 0.5 mL of 1 M IPTG with 4.5 mL of Induction Medium. The 1 M IPTG solution is prepared by dissolving 1 g in 4.1 mL of water. The 1 M IPTG solution is sterilized by filtration on a 0.22 μm filter. Make 1 mL aliquots of 1 M IPTG and store at −20 °C.

8. 2YT agar plates: 1.5% agar in 2YT medium containing 2% glucose and 100 μg/mL ampicillin. Add 7.5 g of agar to 0.5 L of 2YT medium and autoclave. Cool to about 60 °C. Add 55 mL of 20% glucose and 0.55 mL of 100 mg/mL ampicillin and dispense into a petri dish (100 mm × 10 mm). Let the agar solidify under the laminar flow hood.

9. Autoclaved toothpicks.

10. Inoculum Plate: Sterile 96-well polystyrene plates (Costar).

11. Induction Plate: 96-deep well plate (2 mL capacity) and lid for the deep well plate (Whatman). Wrap the plate and lid in foil and autoclave.

12. 96-well dot-blot apparatus (Biorad).

13. Nitrocellulose membrane (0.22 μm Trans-Blot sheets, Biorad).

14. 1 mg/mL human *c-myc* peptide standard in PBS (Genscript). Dissolve 1 mg powder in 1 mL of PBS. Make 0.01 mL aliquots and store −20 °C.

15. Anti-c-myc antibody solution. Murine IgG1/κ 9E10 (Sigma; directed to c-myc peptide EQKLISEEDL; 400 μg/mL in PBS). Dilute to 1:400 in 1% skim milk PBST-T.

16. Goat anti-mouse IgG (Fc-specific) conjugated to peroxidase (Sigma). Dilute to 1:1000 in PBS 1% BSA. The PBS 1% BSA is

prepared by dissolving 1 g of Fraction V BSA in 100 mL of PBS-T.

17. SuperSignal west pico chemiluminescent substrate (Thermo Scientific).

18. Fluoro S imager (Biorad).

19. QuantityOne software (Biorad) for densitometry analysis.

2.4 misTTR Preparation and Assays

2.4.1 Iodination

1. TTR purified from human plasma (Cell Science, >95% purity). Recombinant TTR is also suitable. Store TTR at 6 mg/mL of PBS-C buffer in 0.1 mL single-use aliquots at −20 °C.

2. Na[125]I in 0.01 N sodium hydroxide, 100 mCi/mL (MP Biomedicals) (*see* **Note 2**).

3. Thermo Scientific™ Pierce™ iodination tubes. Borosilicate test tubes (12 × 75 mm) precoated with 50 μg of tetra-chloro-3α, 6α-diphenylglycouril (*see* **Note 3**).

4. Bio-Spin 6 gel filtration columns (Biorad) for removing non-proteinaceous [125]I.

5. Slide-A-Lyzer™ dialysis cassettes (7 kDa cutoff, Thermo Scientific).

6. γ-counter for ^{125}I detection (CobraII, Hewlett Packard).

7. microBCA protein assay kit.

8. Plate reader capable of reading absorbance at 562 nm (A562).

9. Precast 16.5% Criterion™ Tris-Tricine SDS-PAGE electrophoresis gels (Biorad). Suitable for separating polypeptides ranging from 1.5 to 60 kDa.

10. Molecular weight standards. Low mass protein standards (GE Healthcare) and peptide standards (Biorad) to estimate nominal masses. The prestained molecular weight standards (Biorad) are useful to ensure proper blotting onto membranes.

11. Electrophoresis apparatus (Criterion, Biorad).

12. Power supply (Biorad).

13. Orbital shaker.

14. 37 °C incubator.

15. Phosphor Imager System for radiography (Cyclone, Packard).

16. Super Sensitive Storage Screens (Packard).

17. Autoradiography Development Cassettes (Thermo Scientific).

18. Portable fluorescent light table (Acculight™, Bretford) to inactivate Super Sensitive Storage Screen before exposure to radioactive gel.

19. ImageJ software (https://imagej.nih.gov/ij/download.html) for densitometry analysis.

2.4.2 Hydrolysis Assay	1. Purified human polyclonal IgM.

2. ^{125}I-misTTR in PBS-C.

3. Autoclaved 0.65 mL microcentrifuge tubes.

2.4.3 Dissolution Assays

1. Purified human polyclonal IgM.

2. misTTR prepared by treating non-radiolabeled TTR for 5 days at pH 4.2.

3. Autoclaved 0.65 mL microcentrifuge tubes.

4. Spectrophotometer (Cary Eclipse, Varian).

5. 0.1 mL cuvette with 1 cm path length (Varian) for turbidity measurement at 400 nm (quartz, plastic or glass).

6. Opaque white 96-well plates (Thermo Fisher).

7. Fluorescence plate reader (Cary Eclipse, Varian).

2.5 Aβ Preparation and Assays

2.5.1 Iodination

1. Synthetic Aβ40 (American Peptide; MW 4329.9 Da) pretreated with 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) (*see* **Note 4**). Dissolve peptide in HFIP (1 mg/mL), sonicate for 1 h in a water bath, filter through 0.02 μm syringe filter. Prepare 0.1 mg single-use aliquots in microcentrifuge tubes, remove HFIP under N2 stream. Store powder at −80 °C. For iodination, redissolve each aliquot in 4.6 μL of DMSO (yielding 5 mM peptide), vortex for 30 s, and sonicate for 10 min.

2. Na^{125}I in 0.01 N sodium hydroxide, 100 mCi/mL (MP Biomedicals) (*see* **Note 2**).

3. Thermo Scientific™ Pierce™ iodination tubes (*see* Subheading 2.4.1).

4. Phenol red-free Ham's F12 nutrient mixture.

5. Ultrasonication water bath.

6. Low protein binding 0.02 μm syringe filters (Anotop 10, GE Healthcare).

7. N$_2$ gas.

8. Reversed-phase HPLC (RP-HPLC) system equipped with a C18 column (Nova-Pak C18 Column, 60 Å, 4 μm, 3.9 mm × 150 mm; Waters) capable of generating binary gradients.

9. Solvent A: 0.1% trifluoroacetic acid (TFA) in water. Dilute 2 mL of TFA to 2 L with HPLC grade water and filter.

10. Solvent B: 80% acetonitrile, 0.1% TFA in water. Mix 800 mL of acetonitrile, 2 mL of TFA, and 198 mL of HPLC-grade water. Filter on a hydrophobic fluoropore polytetrafluoroethylene 0.22 μm membrane filter (*see* **Note 5**).

11. Sep-Pak® C18 Vac Cartridges 1 cc/100 mg, 55–105 µm particle size (Waters).

12. Vacuum manifold compatible with Sep-Pak cartridges.

13. Reacti-Vap™ evaporator (Thermo Scientific).

14. Borosilicate glass tubes (12 × 75 mm).

15. γ-counter for ^{125}I detection (CobraII, Hewlett Packard).

2.5.2 Hydrolysis Assay

1. Bacterial culture supernatants containing recombinant IgVs.

2. ^{125}I-Aβ40 peptide (*see* Subheading 2.5.1).

3. Repeat pipettor.

4. 50 mL Combitip (Eppendorf).

5. 5.5% TCA. Dilute 100% (w/v) trichloroacetic acid (TCA) stock to 5.5% (v/v) with water. Keep on ice.

6. 12 × 75 mm round-bottom polystyrene assay tubes and caps (Sarstedt).

2.5.3 Oligomer Disappearance Assay

1. Sonicator water bath.

2. Autoclaved 0.65 mL microcentrifuge tubes.

3. Synthetic Aβ42 (American peptide; MW 4514.04 Da). Prepare HFIP-pretreated 0.1 mg peptide aliquots as described for Aβ40 in Subheading 2.5.1. Redissolve each Aβ42 aliquot in 4.4 µL of DMSO (yielding 5 mM peptide), vortex for 30 s, and sonicate for 10 min.

4. Precast 16.5% Criterion™ Tris-Tricine SDS-PAGE electrophoresis gels.

5. Molecular weight standards. Low mass protein standards, peptide standards, and prestained protein standards.

6. Electrophoresis apparatus.

7. Electroblotting apparatus (Criterion).

8. Power supply.

9. Blotting cassettes, roller, filter pads, transfer filter paper (all from Biorad).

10. PVDF membrane (Biorad).

11. Mouse anti-human Aβ antibody mixture. Mixture of 1 µg/mL mouse monoclonal IgG 6D4 (MyBioSource; directed to Aβ C-terminus), 1 µg/mL mouse monoclonal 4G8 (Covance; to Aβ17–24), and 0.4 µg/mL mouse monoclonal 6E10 (Covance; epitope: to Aβ1–17) in PBS-T containing 1% of skim milk.

12. Peroxidase-conjugated goat anti-mouse IgG. 16 µL of peroxidase-conjugated goat anti-mouse IgG diluted in 40 mL of PBS-T containing 1% of skim milk.

13. SuperSignal west pico chemiluminescent substrate (Thermo Scientific).

14. Fluoro S imager (Biorad).

15. QuantityOne software (Biorad) for densitometry analysis.

2.5.4 Fibril Dissolution Assay

1. Aβ42 (5 mM in DMSO) (*see* Subheading 2.5.3).

2. Purified human polyclonal IgM.

3. Autoclaved 0.65 mL microcentrifuge tubes.

4. Opaque white 96-well plates.

5. Fluorescence plate reader (Cary Eclipse, Varian).

6. Transmission electron microscope (JEOL 1400 microscope).

7. Glow discharge unit (PELCO EasyGlow™).

8. 300-mesh Formvar carbon grids.

9. Dumont tweezer N5. Tweezers are used to hold the grid during the procedure.

10. Whatman paper grade 4. Whatman paper is used to wick excess solution.

3   Methods

3.1   Catabody Preparation

We present example catabody preparation methods that have been validated in studies that identified misTTR-directed [27] and Aβ-directed [21, 29, 30] catabodies. In the literature, antibodies from diverse biological sources and in distinct structural scaffolds are documented to express catalytic activity [20, 28, 31–36], e.g., polyclonal catabody preparations from serum, saliva, and milk; polyclonal and monoclonal catabodies from healthy humans and patients with various diseases in the IgM, IgA, and IgG scaffolds; polyclonal, monoclonal, and recombinant Ig light chain subunits or the Ig light chain variable domains from healthy humans, lupus patients, and multiple myeloma patients; and recombinant chains Fv constructs (light chain variable domain linked to heavy chain variable domain by a flexible peptide) from humans and mice.

3.1.1 IgM Catabodies

Polyclonal IgMs from healthy humans hydrolyze misTTR and Aβ selectively, prompting the suggestion of catabody defense against amyloidosis as an innate immune function [27, 37].

1. Pack Poly-Prep columns with 1 mL of anti-IgM-gel. Pre-equilibrate by washing with 5 mL of Buffer C and 5 mL of Buffer A, and then cap the column bottom.

2. To bind serum IgM to the column, layer 0.25 mL of pooled serum from healthy humans (pre-centrifuged at $21,000 \times g$ for

10 min to remove particulates) close to the bed surface. Add 0.75 mL of Buffer A and cap the column top. Place the column in 50 mL Falcon tubes and allow IgM binding to occur for 1 h at 37 °C in a rotary shaker (50–100 rpm; parafilm both column ends to avoid leakage).

3. Settle the gel by spinning the Falcon tube (1 min, $1000 \times g$), remove caps at column ends, recover flow-through fraction at unit gravity, wash with 3×9 mL of Buffer A (9 mL fills up the column completely), then with 2×9 mL of Buffer B (this salt wash improves IgM purity), and then with 1 mL of Buffer A (to eliminate salt contamination in the next step).

4. Elute bound IgM with 4×2 mL of acid Buffer C elutions into four individual tubes containing 0.11 mL of alkaline Buffer D (to neutralize the eluate). Generally, >90% of the serum IgM is recovered as an electrophoretically homogeneous preparation in the first two eluates (*see* **Note 6**).

5. Dialyze IgM against PBS-C prior to assay for catalytic activity.

6. Determine IgM concentration by the microBCA method or by ELISA using specific anti-IgM antibody for detection [38].

3.1.2 Recombinant Catabody Fragments

Human IgV fragments were documented to hydrolyze Aβ in the test-tube and clear brain Aβ deposits in transgenic mouse models [21, 29, 30]. If the catalytic rate is sufficiently rapid, the hydrolytic activity of the recombinant IgVs secreted by bacteria can be readily distinguished from the low-level Aβ hydrolytic activity of contaminant bacterial proteases (as validated from purified IgV studies). Thus, it is feasible to screen for IgV catabody fragments without purification of bacterial culture supernatants. We present an example screening protocol for an *E. coli* strain HB2151 library prepared by transfection with a vector expressing human IgVs as in ref. 28.

1. Isolate *E. coli* strain HB2151 clones expressing recombinant IgVs by growing the library as single colonies on a 2YT agar plate (0.05 mL of a 100,000-fold diluted bacterial suspension with A_{600} value 0.6–0.8; spread evenly on the agar surface with a disposable loop). Incubate for 16 h at 37 °C (*see* **Note 7**).

2. As controls for evaluating background protease contamination, isolate single colonies of the same *E. coli* strain HB2151 transfected with empty vector devoid of an IgV insert.

3. Pick individual bacterial colonies ($N = 50$) and inoculate into individual wells of the 96-well Inoculum Plate containing 0.1 mL of Culture Medium/well. Culture at 37 °C with shaking (50 rpm) until A_{600} reaches 0.5–0.7 (about 3–4 h). Remove 20 μL of the culture for **step 4**. Freeze the Inoculum Plate at −80 °C for later use after adding 0.08 mL of 50% glycerol.

4. Expand the bacterial clones (20 μL from **step 3**) by growth in a larger volume (0.5 mL/well of Induction Medium into 96-well Induction Plate). Culture at 37 °C with shaking (50 rpm) until A_{600} reaches 0.6–0.8 (about 3–4 h).

5. Induce IgV expression by adding 5 μL of 100 mM IPTG/well. Culture at 37 °C with shaking (50 rpm) for 24 h (*see* **Note 8**).

6. Centrifuge the plate (1800 × *g*, 30 min). Recover supernatants containing secreted IgVs. Also recover supernatants from control cultures of *E. coli* strain HB2151 transfected with empty vector devoid of an IgV insert.

7. Measure IgV expression by c-myc dot blotting (the IgV inserts contain a c-myc tag at the C-terminus). The method was described in detail previously [39]. Briefly, dispense 0.1 mL of culture supernatant or increasing amounts of reference *c-myc* peptide onto a 0.22 μm nitrocellulose membrane in a 96-well dot-blot apparatus. Block the membrane in Blot Blocking Buffer, wash with PBS-T, treat with anti-c-myc antibody, wash with PBS-T again, and then treat with peroxidase-conjugated goat anti-mouse IgG. Following washing with PBS-T, develop the membrane with SuperSignal west pico chemiluminescent substrate, and quantify the dot intensity using the Fluoro S imager and QuantityOne software. Interpolate IgV expression from the standard curve constructed using the c-myc peptide data.

3.2 misTTR Hydrolysis and Dissolution

3.2.1 ^{125}I–misTTR Substrate

^{125}I–misTTR is prepared by misfolding ^{125}I–TTR in a pH 4.2 buffer. The preparations contain both soluble misTTR oligomers and particulate misTTR fibrillary structures that can be separated by centrifugation and analyzed separately as substrates for catabodies (*see* **Note 9**). In addition to misTTR, the preparations contain minority amounts of TTR in its physiological tetramer state (54 kDa) that can be distinguished from misTTR by electrophoresis without prior heat treatment of the samples (ref. 27, under these conditions, the misTTR species dissociate into the 14 kDa monomer TTR band, whereas the physiologically folded TTR tetramers remain undissociated).

1. Equilibrate Bio-Spin 6 gel filtration columns. Centrifuge columns (1000 × *g*, 2 min) and remove storage buffer. Use the same centrifugation procedure to wash columns thrice with 0.5 mL of PBS-C.

2. Perform radioiodination by mixing TTR (3 mg/mL) and Na^{125}I (1 mCi) in a final volume of 0.2 mL of 0.1 M sodium phosphate, pH 7.4 (*see* **Note 2**).

3. Transfer the mixture into a Thermo Scientific™ Pierce™ iodination tube and allow reaction to proceed for 20 min.

Vortex briefly every 5 min. Then transfer the reaction mixture into a 1.7 mL microcentrifuge tube.

4. Recover any residual reaction mixture from the iodination tube by washing with 0.2 mL of PBS-C, pool, and remove free $Na^{125}I$ by separating the reaction mixture on a Bio-Spin 6 gel filtration column (0.05 mL/column, $1000 \times g$, 4 min).

5. Compute ^{125}I incorporation into TTR from measurements of protein and radioactivity content (cpm/µg protein units, *see* **Note 10**).

6. Measure TTR concentration by the microBCA protein assay kit in 96-well plates as described by Thermo Scientific. Use BSA as standard (diluted to 1, 2.5, 5, 10, 20, and 40 µg/mL in PBS-C). Dilute the samples to fall in the linear range of the standard curve (which is constructed by linear regression fitting).

7. Read absorbance 562 nm (A_{562}) and interpolate the sample protein concentrations from the standard curve. Count radio-activity in three 1 µL aliquots of ^{125}I-TTR from **step 4** using a γ-counter.

8. Check ^{125}I-TTR molecular integrity (about 200,000 cpm) by routine SDS-electrophoresis under reducing conditions using boiled samples (14 kDa monomer TTR band is anticipated unless fragmentation due to radiolysis has occurred, *see* **Note 11**).

9. Electrophorese 10 µL of sample or molecular mass protein standards per lane for 2 h at 150 mA/gel and 125 V. Apply routine methods for gel handling, fixation, silver nitrate treatment, and development (e.g., described in [40]). To facilitate diffusional entry and exit of reagents into gels, perform all steps at 37 °C with rotational shaking.

10. Visualize radioactive bands by Phosphor Imager System using polyvinyl chloride wrapped gels, Super Sensitive Storage Screens preexposed to fluorescent white light for 15 min, and exposure time sufficient to detect the radioactivity (we use exposure times of 5 min to 18 h).

11. Determine the band intensities by ImageJ software. Take care to use identical-sized rectangle to determine gel background (which is subtracted from the sample band intensities). Estimate molecular mass by reading off the standard curve constructed using proteins with known mass.

12. Prepare misTTR by mixing ^{125}I-TTR with an equal volume of TTR Misfolding Buffer in a clear plastic tube to a final concentration of 0.2 mg/mL, vortexing and incubation at 37 °C for 5 days.

13. For restoration to neutral pH, dialyze the reaction mixture (Slide-A-Lyzer cassette, three PBS-C changes × 2 h, 4 °C; *see*

Note 12). Misfolded fibrillar misTTR appears, evident from visible cloudiness and turbidity measurement (0.1 mL sample read in duplicate at A_{400} in a spectrophotometer pre-zeroed with the appropriate buffer; A_{400} immediately after misfolding is 0.7–1.1, and after dialysis, 0.4–0.6). A_{400} prior to misfolding is <0.05.

14. Perform SDS-electrophoresis using non-boiled samples under nonreducing conditions to enable distinction between the physiologically folded 4mer TTR band and the misTTR states (SDS treatment alone suffices to dissociate the particulate and soluble oligomer misTTR states into the 14 kDa monomer and minor 2mer bands). Fifty to eighty percent of the sample radioactivity is found as the 1mer band, whereas >95% of the sample radioactivity appears as the 4mer band without pre-aggregation reaction of the ^{125}I-TTR induced in the TTR Mis-folding Buffer.

15. Compute radioactivity incorporated into misTTR monomer band as in **steps 5–11** (*see* **Note 13**).

3.2.2 ^{125}I-misTTR Hydrolysis

This electrophoresis assay is useful for screening catabody activity of rigorously purified antibodies as well as unfractionated biological fluids (serum/plasma, cerebrospinal fluid) or culture supernatants containing monoclonal antibodies (or antibody fragments) secreted by B cells and bacteria transfected with antibody-expressing vectors. We recommend PBS-C as the base hydrolysis assay buffer, as this buffer simulates the ionic strength of biological fluids. Note that the ionic milieu can influence catabody conformation as well as substrate conformation. Both types of molecules must be maintained in their native conformation. The documented misTTR-directed catabodies use a serine protease-like mechanism [27], but metal-dependent catabodies to amyloids are also described, and we often include a divalent metal (1–50 μM) [30] in the assay buffer (*see* **Note 14**). Immune complexes formed by rapid catabodies are too short-lived to be detectable by conventional ELISA or immunoblotting procedures [21], but slow catabodies can form detectable complexes with antigens [41].

1. Treat ^{125}I-misTTR (100 nM, 21.6 μg/mL) in duplicate with the desired catabody concentration or diluent in a final volume of 0.02 mL of PBS-C containing 0.003% of BSA in microcentrifuge tubes. Vortex and spin briefly to draw the entire reaction mixture to the tube bottom. Oligomeric and particulate misTTR aggregates mediate distinct pathogenic effects. If desired, the hydrolysis reactions can be set up separately using the soluble ^{125}I-misTTR oligomers and particulate ^{125}I-misTTR fibrils as substrates fractionated by high-speed centrifugation (*see* **Note 9**).

2. Incubate 18 h at 37 °C with shaking.

3. Stop the reaction by adding 5 μL of 5× nonreducing electrophoresis buffer. Do not boil the samples (*see* **Note 15**). If necessary, samples can be stored at −20 °C for later electrophoresis analysis.

4. Conduct electrophoretic separation of reaction mixtures and radiography of the gels essentially as described under **steps 9–11** in Subheading 3.2.1. Nonreducing electrophoresis of non-boiled samples dissociates misTTR aggregates but not the physiologically folded tetramer TTR. Thus, selective hydrolysis of ^{125}I–misTTR but not physiologically folded ^{125}I–TTR is evident from the depletion of the major 1mer and minor 2mer bands but not the 4mer band. Appearance of product bands with mass smaller than the 1mer band provides definitive evidence of the hydrolytic reaction (Fig. 1a).
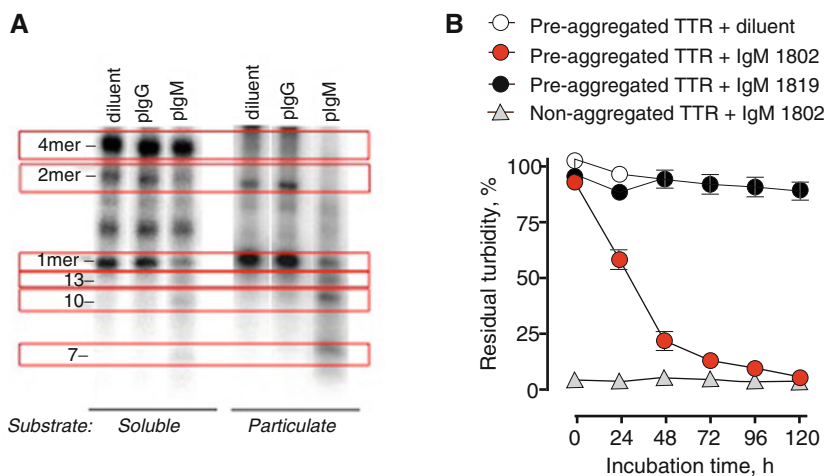
Fig. 1 misTTR hydrolysis and dissolution by polyclonal human IgMs. (**a**) Selective misTTR hydrolysis by polyclonal IgM. The soluble and particulate fractions of ^{125}I-misTTR (misTTR content, 37% and >95%, respectively) were separated by centrifugation (17,000 × *g*, 20 min) and treated for 18 h with diluent or polyclonal IgM (pIgM) and IgG (pIgG) purified from the same serum pool (130 μg/ mL) of healthy humans. Non-boiled reaction mixtures were analyzed by electrophoresis. The 1mer and 2mer bands derived from misTTR but not the physiologically folded TTR 4mer were selectively hydrolyzed upon IgM treatment, accompanied by appearance of polypeptide fragments with mass smaller than TTR 1mer. IgG did not express detectable hydrolytic activity. (**b**) Dissolution of misTTR by monoclonal IgM. A misTTR preparation (7.1 μM) was incubated with diluent, the hydrolytic human monoclonal IgM 1802 (120 μg/ mL, 0.67 μM), or an equivalent concentration of the non-hydrolytic monoclonal IgM 1819. IgM 1802 treatment reduced the turbidity compared to the basal value observed for control non-aggregated TTR treated with IgM 1802. Monoclonal IgMs were purified from the sera of Waldenström macroglobulinemia patients. *Error bars*, SD. Reproduced from J Biol Chem 2014 [27] with permission

5. We generally load reaction mixtures containing ~10,000 cpm/electrophoresis lane, and we conduct radiography with exposure of phosphor screens for 16 h.

6. Quantify % hydrolysis as follows: $[100 \times (\text{band intensity}_{\text{diluent}} - \text{band intensity}_{\text{catabody}})/\text{band Intensity}_{\text{diluent}}]$, wherein band intensities of the 1mer and 4mer bands are employed to determine hydrolysis, respectively, of the misTTR and physiologically folded TTR.

3.2.3   misTTR Dissolution   Misfolding reactions result in formation of β-sheet aggregates of TTR with progressively increasing in size ranging from soluble oligomers composed of a few monomers to well-organized particulate fibrils containing hundreds of monomers. Various environmental perturbations accelerate the aggregation reaction, including the pH 4.2 treatment employed for misTTR preparation in the present protocol. If the peptide bond hydrolytic reactions catalyzed by antibodies occur with sufficient rapidity, large amounts of amyloid aggregates are solubilized by small catabody amounts. The catabodies dissolve amyloids autonomously. There is no requirement for Fc receptor-mediated uptake of immune complexes by phagocytic cells to accomplish amyloid clearance.

1. Solubilization of a turbid misTTR suspension is measured as an index of amyloid dissolution capability of catabodies. Treat the misTTR suspension (7.5 μM) in duplicate with the desired catabody concentration or control diluent in a final volume of 0.2 mL of PBS-C in microfuge tubes (*see* **Note 16**). Also set up control reactions in which a non-turbid physiologically folded TTR solution (7.5 μM) is treated with diluent.

2. Vortex briefly, transfer the reaction mixture to a cuvette (path length 1 cm, 0.1 mL cuvette), and measure starting turbidity (A_{400}) at time 0. At the stated misTTR concentration, the starting A_{400} turbidity value is ~0.5.

3. Incubate the reaction mixture (37 °C) with shaking. Read A_{400} values at several time points until the misTTR suspension has clarified completely (A_{400} approximating the A_{400} value for physiologically folded TTR solution). Calculate % fibril dissolution as: $[100 \times (A_{400 \ \text{diluent}} - A_{400 \ \text{catabody}})/A_{400 \ \text{diluent}}]$ (Fig. 1b).

4. Reduction of amyloid binding to ThT, a reagent that selectively recognizes β-sheet structures, is measured as another index of amyloid dissolution. Treat the misTTR suspension (7.5 μM) in duplicate with the desired catabody concentration or control diluent in a final volume of 0.1 mL of PBS-C in an opaque 96-well plate (which affords superior fluorescence detection).

5. As before, also set up control reactions in which a non-turbid physiologically folded TTR solution (7.5 μM) is treated with diluent.

6. Incubate the plates for increasing time intervals (37 °C) on a plate shaker. Add 2.5 μL of 600 μM ThT/well (final ThT concentration 15 μM), incubate for 30 min, and measure fluorescence using a fluorescence plate reader (excitation wavelength 440 nm, emission wavelength 485 nm; excitation and emission slit widths, respectively, 5 and 10 nm; reading time/well 0.1 s).

7. Compute % fibril dissolution as: $[100 \times (FU_{diluent} - FU_{catabody})/FU_{diluent}]$, wherein $FU_{diluent}$ and $FU_{catabody}$ correspond, respectively, to the fluorescence in wells treated with diluent and catabody. All fluorescence values observed in catabody-containing wells are corrected for any low-level fluorescence attributable to the catabody itself (measured in wells containing the catabody alone; *see* **Note 17**).

3.3 Aβ Hydrolysis and Dissolution

The preponderance Aβ42 peptide with the additional C-terminal dipeptide unit is greater in brain parenchymal amyloid deposits, whereas the shorter Aβ40 peptide is somewhat more represented in amyloids surrounding small cerebral blood vessels. In the test tube, Aβ42 forms oligomeric and larger aggregates within minutes, and aggregation of Aβ40 occurs over hours to days. To minimize complications associated with a highly aggregated substrate, we conduct initial catabody screens at very small concentrations of the radiolabeled ^{125}I–Aβ40 peptide. Then, we test the oligomeric and higher order Aβ42 aggregates to confirm that catabodies recognize these pathogenic peptide states.

3.3.1 ^{125}I–Aβ40 Substrate

1. Using a vacuum manifold, pre-equilibrate a Sep-Pak C18 cartridge with 3×1 mL of Solvent B washes and 3×1 mL Solvent A washes (approximate flow rate 1 mL/min, *see* **Note 18**).

2. For radioiodination, mix 4.6 μL of stock Aβ40 (0.1 mg peptide) with 15 μL of 1 M sodium phosphate, pH 7.4, and 1 mCi $Na^{125}I$ (compute volume from initial concentration provided by manufacturer after correction for radioactivity decay) and bring the volume to 150 μL with water (*see* **Note 2**).

3. Transfer the reaction mixture into a Thermo Scientific™ Pierce™ iodination tube. Allow the reaction to proceed for 15 min.

4. Vortex briefly every 5 min. Then remove the reaction mixture, wash the iodination tube 2×0.2 mL with Solvent A, pool the original reaction mixture and washes, and pass the pooled mixture through the Sep-Pak cartridge.

5. Collect flow-through, wash the cartridge with Solvent A (5 × 1 mL, collect each wash in individual tubes; *see* **Note 19**).

6. Then elute radiolabeled peptide bound to the cartridge with Solvent B (5 × 0.6 mL, collect each eluate in individual tubes, and measure radioactivity in the washes and eluates recovered from the cartridge (1 μL aliquots). Usually, >50% of total radioactivity is recovered in the first Solvent B eluate, representing the crude ^{125}I–Aβ40 preparation. Bring this fraction to dryness under a stream of N_2 gas (about 20–30 min) in the Reacti-Vap™ Evaporator at low heat setting). Then dissolve the peptide in 0.02 mL of DMSO and add 0.48 mL of an 80% Solvent A-20% Solvent B mixture.

7. Fractionate the crude ^{125}I–Aβ on a Nova-Pak C-18 RP-HPLC column that has been pre-equilibrated with 20% Solvent B (in Solvent A). The column run protocol consists of washing with 20% Solvent B (0–5 min), a gradient of 20% to 30% Solvent B (5–10 min), and a gradient of 30–50% Solvent B (10–85 min; stated Solvent B concentrations obtained by mixing with Solvent A). Flow rate is 0.5 mL/min.

8. Monitor the absorbance using a flow-through detector (214 nm). Collect 0.5 mL fractions and monitor ^{125}I–Aβ elution by counting radioactivity in 1 μL aliquots.

9. Pool peak fractions (we typically pool the 45–50 min fractions). This yields purified ^{125}I–Aβ40 carrying the radiolabel at Tyr10 (*see* **Note 20**).

10. To improve ^{125}I–Aβ40 stability in storage, add the appropriate volumes of BSA (10%) and acetic acid (5 N) to bring these reagent concentrations to 0.2% and 0.1 N, respectively. Store at −80 °C in 0.1 mL aliquots.

3.3.2 Aβ42 Oligomer Substrate

1. Dilute stock Aβ42 to 0.1 mM in ice-cold Ham's F-12, immediately vortex for 30 s, and oligomerize by incubation at 4 °C for 24 h.

2. Verify oligomerization by reducing SDS-electrophoresis. Do not boil the samples (this reduces dissociation of native oligomers). Conduct electrophoretic separation of the reaction mixtures essentially as in Subheading 3.2.1, **step 9** (16.5% Criterion™ Tris-Tricine SDS-PAGE gels). Analyze protein standards (1–94 kDa) and peptide standards (1.4–26.6 kDa) with known mass in parallel. Including a lane with prestained protein standards is helpful to monitor the separations.

3. Oligomer bands are visualized by silver staining or anti-Aβ immunoblotting. Routine methods are applied to blot the electrophoresis gels onto PVDF membranes and stain with a mouse anti-human Aβ antibody mixture [21]. Care is taken to avoid trapping of air bubbles between the gel, filter pad, filter

paper, and PVDF membrane. An electro-blotting apparatus is used to ensure efficient blotting (60 min, 100 V, 400 mA). The blot segment containing protein/peptide standards is stained for 10 min with Ponceau S followed by destaining for 20 min. The blot segment containing test samples is blocked with the Blot Blocking Buffer (30 min, 37 °C on an orbital shaker), washed (3 × 5 min) with PBS-T. This blot segment is then treated with mouse anti-Aβ antibody mixture (40 mL, *see* Subheading 2.5.3) for 1 h with shaking. Following washing with PBS-T (3 × 5 min), the blot is stained with peroxidase-conjugated anti-mouse IgG (40 mL, 1 h with shaking), washed again (3 × 10 min), and then developed for 5 min with the peroxidase substrate (10 mL SuperSignal west pico chemiluminescent substrate) on a shaker. Visualize bands in the blot using the Fluoro S imager. We use exposure times varying from 10 s to 10 min, with photographs taken periodically to capture band intensities in the linear range of the instrument.

3.3.3   Aβ42 Fibrillar Substrate

1. Dilute stock Aβ42 to 0.05 mM in PBS-C, immediately vortex for 30 s, and fibrillize by incubation at 37 °C for 24 h.

2. Aβ42 fibrillization is verified at various time points by the ThT binding assay essentially as described in Subheading 3.2.3 (*see* **Note 21**). The fibrillization is rapid and increased ThT binding signals are evident within minutes of initiating the reaction (compared to ThT binding at time 0).

3.3.4 ^{125}I–Aβ40 Hydrolysis Assay

This method screens for catabody activity by means of selective precipitation of the intact ^{125}I–Aβ40 peptide using TCA, leaving the smaller hydrolytic products in the TCA-soluble fraction. The results correlate well with catabody hydrolytic activity measured by RP-HPLC separation of the reaction mixture [37], and catabodies identified by the TCA-precipitation screening method were further validated as peptide bond hydrolytic reagents using additional analytical techniques, e.g., mass spectrometry [37].

1. Treat ^{125}I–Aβ40 (~30,000 cpm) in duplicate with the desired catabody concentration or diluent buffer in a final volume of 0.1 mL PBS-C containing 0.1% BSA in 12 × 75 mm polystyrene tubes (*see* **Note 22**). Include tubes with ^{125}I–Aβ40 substrate alone as a measure of total available radioactivity. Cap tubes, vortex briefly, and spin to draw the reaction mixture to the tube bottom.

2. Incubate at 37 °C for 3–24 h.

3. Add 1 mL ice-cold 5.5% TCA v/v per tube using a repeat pipettor. Vortex briefly. Centrifuge (2000 × *g*, 4 °C, 30 min) to pellet intact ^{125}I–Aβ40. Aspirate supernatants into a radioactive waste bottle using a Pasteur pipette attached to a vacuum

line (*see* **Note 22**). Count radioactivity in pellets (1 min counting time/tube).

4. Generally >90% of the total radioactivity of the control ^{125}I–Aβ40 treated with diluent alone is TCA precipitable. Calculate % ^{125}I–Aβ40 hydrolysis as: $[100 \times (cpm_{diluent} - cpm_{catabody})/cpm_{diluent}]$, where $cpm_{diluent}$ and $cpm_{catabody}$ represent radioactivity in pellets recovered after diluent and catabody treatment, respectively.

3.3.5 Oligomer Aβ42 Disappearance Assay

Immunoblotting of electrophoretically separated Aβ42 oligomers is applied to determine their disappearance following catabody treatment. If the smaller mass product fragments contain the epitope recognized by the Aβ-directed antibody employed for immunoblotting, the product fragments can also be visualized. The method only reveals disposition of oligomers stable to SDS treatment, not the native Aβ oligomers. Alternative methods such as atomic force microscopy can be applied to determine disappearance of native oligomers by catabodies as in ref. 37.

1. Treat the Aβ42 oligomer preparation from Subheading 3.3.2 (corresponding to 40 μM monomer peptide concentration) in duplicate with the desired catabody concentration or control diluent in a final volume of 0.02 mL in PBS-C in microcentrifuge tubes.

2. Incubate for 24 h at 37 °C. Stop the reaction by adding 5 μL 5× reducing electrophoresis loading buffer. Do not boil the samples (this reduces dissociation of native oligomers). Conduct electrophoretic separation as in Subheading 3.2.1, **step 9**.

3. Immunoblot the gels with anti-Aβ antibody as in Subheading 3.3.2, **step 3**.

4. We generally observe bands corresponding to trimer (nominal mass ~13.5 kDa), tetramer (18 kDa), and higher-order oligomers (>45–84 kDa) in diluent that disappear following catabody treatment. A small mass product band at 2.5 kDa following catabody treatment is also evident, confirming the occurrence of a hydrolytic reaction. Band intensities are measured with QuantityOne software. Compute % hydrolysis as: $[100 \times (AVU_{diluent} - AVU_{catabody})/AVU_{diluent}]$, wherein $AVU_{diluent}$ and $AVU_{catabody}$ correspond to the intensities in Aβ42 treated with diluent and catabody, respectively, expressed in arbitrary volume units (Fig. 2).

3.3.6 Aβ42 Fibril Dissolution

We documented catabody-mediated dissolution of fibrillar Aβ42 from ThT binding and electron microscopy studies [21]. Additional techniques available for this purpose are turbidity and atomic force microscopy [27, 37]. Verification that fibrillar Aβ is
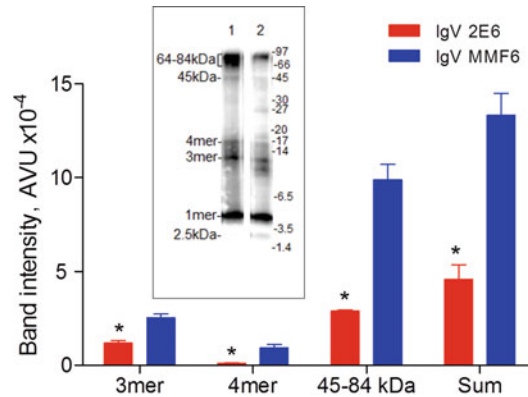
Fig. 2 Dissolution of preformed Aβ42 oligomers by recombinant immunoglobulin variable domain fragments (IgVs). Treatment of preoligomerized Aβ42 with catalytic IgV 2E6 but not control IgV MMF6 (3 μg/ mL, 24 h) depleted the SDS-stable Aβ trimers, tetramers, and high-mass oligomers (45 band, 64–84 kDa smear) evident in the electrophoretic gels. The catalytic and noncatalytic IgVs have an identical structural scaffold, two light chain variable domains linked by a flexible peptide. *$p < 0.005$. *Inset*, Aβ42 oligomers stained with a mixture of Aβ-binding monoclonal antibodies after treatment with IgV MMF6 (*lane 1*) or IgV 2E6 (*lane 2*). Migration of protein standards is shown by mass values on the right. *Error bars*, SD. Reproduced from J Biol Chem, 2015 [21] with permission

hydrolyzed by the catabody can be accomplished by RP-HPLC and mass spectrometric analysis of products [21, 37].

1. Perform tests of catabody-mediated Aβ42 fibril dissolution by the ThT binding method similar to that described for misTTR under Subheading 3.2.3. Treat the fibrillar Aβ42 suspension (20 μM monomer peptide equivalents) in duplicate with the desired catabody concentration or control diluent in a final volume of 0.1 mL PBS-C in a 96-well plate. In addition to the diluent control, a catabody inactivated with a specific inhibitor is a suitable negative control, e.g., the metal-dependent, Aβ-directed catabody clone 2E6 inactivated by the chelator EDTA [30]. Following incubation for increasing time intervals (37 °C), measure the ThT binding activity by fluorimetry, and compute % fibril dissolution as in Subheading 3.2.3.

2. Electron microscopy studies prove dissolution from disappearance of fibrillary Aβ disappearance. Perform electron microscopy by routine procedures as in [42]. Treat fibrillar Aβ42 (2.5 μM monomer peptide equivalents) in duplicate with the desired catabody concentration or control diluent in 50 μL of PBS-C. Place 5 μL sample (the reaction mixture) on a glow-discharged carbon grid for 1 min, remove excess sample by wicking the grid sides with Whatman filter paper, wash the grid with water (3×), remove excess water using filter paper as

before, and stain the grid with 1% uranyl acetate (5 μL, 1 min). Following three additional water washes, evaluate the size/number of fibrillary Aβ42 plaques on the grid with a transmission electron microscope. Catabody-induced changes in size and number of Aβ fibrillary aggregates >80% are discernible [21], providing additional confidence in the dissolution reaction measured by the ThT binding assay.

4   Notes

1. The critical micellar concentration (CMC) of CHAPS is 6–10 mM. Large micelles formed when the CHAPS concentrations exceed its CMC may be trapped by 0.22 μm filters. Therefore, filter CHAPS-containing buffers after CHAPS has been diluted to its working concentrations (generally 0.1–1 mM) that are below the CMC.

2. ^{125}I is a γ emitter with a half-life of 59.4 days. Store Na^{125}I in a lead container at room temperature. Follow guidelines in safe handling and disposal.

3. Oxidation with tetrachloro-3α,6α-diphenylglycouril generates iodine monochloride that reacts with the Tyr aromatic ring. TTR and Aβ have four and one Tyr residues, respectively, available for iodination.

4. HFIP treatment is thought to ensure that Aβ is converted into its monomer state, thereby providing a reproducible peptide preparation for preparing aggregates in aqueous buffers.

5. Polytetrafluoroethylene filters are compatible with most organic solvents.

6. The anti-IgM-gel is sufficient to recover >80% of IgM present in human serum. From 0.25 mL serum, we recover 0.2–0.4 mg IgM.

7. The concentration of the stock bacterial suspension is adjusted to yield about 100–500 single colonies per plate.

8. Medium should not contain glucose at the IgV expression step, as the LacZ operon is repressed by glucose.

9. Separate soluble and particulate ^{125}I–misTTR by centrifugation at centrifugation speeds >17,000 × g (20 min) as in ref. 27. Inclusion of BSA in buffers used for protein handling and hydrolysis assay minimizes protein loss due to adsorption on surfaces.

10. "cpm" is a measure of radioactivity. Under the stated conditions, we generally obtain ^{125}I–TTR preparations with specific activity of 0.8–1.7 × 10^6 cpm/μg.

11. Heavily iodinated polypeptides can undergo radiolytic fragmentation due to prolonged exposure to high-energy γ rays. Under the stated conditions, radiolytic fragmentation of ^{125}I–misTTR is <5%.

12. Repeatedly draw the misTTR suspension in the dialysis cassette into and out of the collection syringe to ensure collection of aggregates.

13. ^{125}I–misTTR may be used within 1.5 month after preparation.

14. Metal availability for antibodies is governed by several factors, including metal sequestration by non-antibody proteins present in the reaction mixture, presence of metal-bound fetal calf serum proteins included in the assay if direct screening of tissue culture supernatants containing such serum is performed, and low solubility of divalent metal salts obtained by reactions with buffer constituents.

15. Both physiologically folded and misfolded TTR are dissociated into the 14 kDa monomer species upon SDS treatment and boiling. misTTR aggregates but not the physiologically folded TTR tetramers are dissociable by SDS treatment alone without boiling [27].

16. Different misfolded amyloid proteins can revert to physiologically folded state to varying extents. Thus, dilution of misfolded proteins should be minimized.

17. The β-barrel structure of non-misfolded antibodies may bind ThT weakly. To correctly determine ThT binding to the amyloid, subtract the fluorescence due to the antibody alone from fluorescence values observed for the amyloid-antibody mixture.

18. Control the flow rate to ensure adequate time for peptide binding and elution.

19. Non-peptide ^{125}I is removed by Solvent A washing.

20. The pooled ^{125}I–Aβ typically contains ~0.25×10^6 cpm/μL. Specific activity is ~8×10^8 cpm/μg.

21. We observed increased ThT binding at this step in the 90–150 FU range (at 100 μM Aβ42).

22. Errors are minimized by obtaining hydrolysis in the linear range of the reaction (20–60% hydrolysis of available substrate). Incubation time and catabody concentrations are adjusted accordingly. Do not allow unnecessarily prolonged TCA contact with ^{125}I–Aβ during termination of the reaction, as excessive TCA treatment durations can fragment the peptide. Minimize disturbing the pellet while aspirating away the TCA from reaction tubes.

Acknowledgments

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Chapter 10

Methods for Posttranslational Induction of Polyreactivity of Antibodies

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Abstract

An antibody molecule that recognizes multiple unrelated antigens is defined as polyreactive. Polyreactivity is an intrinsic characteristic of immune repertoires. Degenerated antigen binding diversifies the repertoire of specificities, thus contributing to immune defense and immune regulation. Immune repertoire contains also a fraction of immunoglobulins, which acquire polyreactivity only following contact with various protein-destabilizing or pro-oxidative substances. Posttranslational induction of the antibody polyreactivity may have important repercussion for laboratory practice, as well as in cases of pathological conditions accompanied by liberation of large quantities of pro-oxidative substances such as heme, labile iron, or reactive oxygen species. Antibodies with induced polyreactivity have been demonstrated to exert pathogen neutralization and immune regulatory potential in inflammatory conditions, suggesting that this phenomenon may be exploited for design of therapeutic strategies. In this article, we provide description of the basic procedures for uncovering of the cryptic polyreactivity of antibodies by heme, ferrous ions, and acid pH solution.

Key words Immunoglobulins, Natural antibodies, IgG, Antibody polyreactivity, Cryptic antigenic specificities, Heme, Iron ions, Acidic pH

1 Introduction

Normal immune repertoires contain antibodies that bind to multiple self and foreign antigens. These antibodies are defined as polyreactive [1]. Polyreactive antibodies can be product both of thymus-independent and thymus-dependent immune responses. One of the characteristic features of the antibodies generated in the absence of stimulation by exogenous antigens, i.e. natural antibodies, is their prominent polyreactivity [2, 3]. Ability of antibodies to recognize many unrelated antigenic determinants contributes to the diversification of the repertoires of immune specificities. Hence, polyreactive antibodies facilitate early phases of the defense against

pathogens. These antibodies also participate in the regulation of the immune homeostasis [4].

Beside antibodies with genetically programed polyreactivity, a considerable fraction of antibodies in human immune repertoires can acquire ability to interact with multiple unrelated self and foreign antigens after transient exposure to certain agents that alter protein conformation. Thus, transient contact of polyclonal or monoclonal antibodies to chaotropic agents (urea, guanidine hydrochloride, thiocyanate), high salt concentrations, acid pH, or elevated temperatures have been demonstrated to result in drastic increase in the repertoire of recognized antigens [5–10]. This posttranslational acquisition of antibody polyreactivity can have important consequences in the laboratory practice and in industry. Purification of immunoglobulins from biological fluids is often accompanied by exposures to conditions that can induce polyreactivity of antibodies. For example, IgG antibodies are eluted from protein A or protein G columns by using acidic solutions (most commonly a solution of glycine with pH 2.6–2.8). Previous studies showed that transient exposure of human pooled IgG from healthy donors to pH below 4 is sufficient to uncover the cryptic polyreactivity of IgG [11, 12]. Comparison of the immunoreactivity of human IgG in whole sera or of the purified IgG (from the same sera) revealed dramatic enhancement of the reactivity to a large number of autoantigens following IgG purification by acidic pH elution [13–15]. Reactivity of IgG purified in mild conditions is similar to the antibody reactivity in whole normal sera [16].

More recent studies have revealed that contact of immunoglobulins with some pro-oxidative substances such as heme, iron ions, reactive oxygen species, and hypochlorous acid also results in induction of polyreactivity of immunoglobulins [17–22]. The pro-oxidative substances can be released in vivo in large quantities under certain pathological conditions, thus pointing the physiopathological importance of the posttranslational induction of antibody polyreactivity [23, 24]. Pro-oxidative substances possess considerably higher potency to induce polyreactivity in polyclonal immunoglobulin preparations as compared to protein-destabilizing agents such as urea or low pH [25]. The mechanism of the posttranslational acquisition of novel antigen binding characteristics of antibodies is not well understood. It is known that upon heme exposure certain antibodies start to recognize some proteinous antigens with high binding affinity (low nanomolar range of K_D value) [26, 27]. It was demonstrated that heme directly binds to variable region of the sensitive antibodies. This binding could result in reconfiguration of the polypeptide chain of the antigen binding sites and/or use of intrinsic binding promiscuity for recognition of versatile targets. We proposed a model for induction of binding polyreactivity by heme where heme serves as an interfacial cofactor for recognition of antigens [20, 26]. This model can partly also

explain the effect of ferrous ions but it is difficult to be used for explanation of the effect of low pH or chaotropic agents. The common feature of different substances that uncover polyreactivity of some antibodies is their ability to induce structural changes in the protein. It is noteworthy that not all antibodies acquire polyreactivity upon exposure to redox-active or protein-destabilizing agents. Thus, it was estimated that human IgG1 repertoire contain approximately 20% of antibodies that acquire polyreactivity upon exposure to heme [28].

Functions of antibodies with cryptic polyreactivity in immunity are not clear. In vivo induction of polyreactivity by heme or other pro-oxidative agents can contribute for localized recruitment of antigen binding specificities. Nonspecific antigen binding can serve as a first line for defense against pathogens, it may exert anti-inflammatory effect by quenching pro-inflammatory molecules or assisting in clearance of damaged cells and macromolecules. Indeed, administration of immunoglobulins with in vitro-induced polyreactivity in animal models of different inflammatory diseases results in amelioration of the inflammation and considerable improvement in survival or disease morbidity [18, 29, 30]. These data imply that cryptic polyreactive antibodies may represent a tool for the modulation of the pathologic immune response. Moreover, heme exposed antibodies acquire virus neutralizing activity [31].

In this article, we presented strategies for in vitro induction of polyreactivity of human IgG antibodies. Procedures for induction of polyreactivity by heme, ferrous ions, and low pH are described. Method of preparation of heme under different oxidized forms is provided, as well as the immunoblot technique that can be used to evaluate the level of immunoglobulins polyreactivity.

2 Materials

2.1 Immunoglobulins

1. Human pooled IgG preparation for therapeutic use (Endobulin S/D, Baxter).

2.2 Treatment of Immunoglobulins

1. Hemin (ferriprotoporphyrin IX) dry substance (Sigma-Aldrich) (*see* **Note 1**).

2. Black microcentrifuge tube (*see* **Note 2**).

3. 0.05 N sodium hydroxide in water.

4. Dimethyl sulfoxide (DMSO) suitable for cell culture.

5. Hydrogen peroxide—35% (w/v) solution (i.e., 10.29 M).

6. Ferrous (II) sulfate solution: 10 mM of $FeSO_4 \cdot 7H_2O$ in deionized water (*see* **Note 3**).

7. 0.2 M glycine-HCl, pH 4 or 2.6.

8. Sodium hydroxide 30% (m/v) solution.

9. 3 M solution of tris[hydroxymethyl]aminomethane, adjustment of pH to 10 by addition of 12 N HCl.

10. Solution of immunoglobulins (*see* **Note 4**).

11. Phosphate buffered saline (PBS).

12. Hepes buffered saline (HBS): 150 mM NaCl, 10 mM HEPES, adjustment to pH 7.4 by 7.5 N NaOH.

13. PD-10 desalting column (GE Healthcare) (optional).

14. Ultrapure EDTA 0.5 M (optional).

15. Dialysis bag (typically 12–14,000 Da pore size) or dialysis chambers (D-Tube Dialyzer Midi MWCO 6–8 kDa) (Calbiochem) (optional).

2.3 Analyses of IgG Immunoreactivity

1. Acrylamide gel electrophoresis (SDS-PAGE). We strongly recommend using precast gels. Typically, we use a 1-well mini gel (Novex NuPAGE 4–12% Bis-Tris gel, 1.0 mm, Life Technologies).

2. Running and loading buffers for SDS-PAGE. Commercially available (NuPAGE MES SDS Running Buffer Life Technologies).

3. Bacterial lysate in reducing sample buffer (1 mg/mL). Results shown here were obtained with lysate from *Bacillus anthracis*, but similar results were obtained with other bacterial strains (*Escherichia coli*, *Enterococcus faecalis*).

4. Transfer system. Results shown here were obtained using the iBlot dry transfer system (Life Technologies). Any other transfer method on a nitrocellulose membrane will be suitable.

5. Miniblot device, Miniblot System C-shell single, 28 channels (Immunetics).

6. Saran wrap.

7. Incubation vessel for membrane (Petri plate).

8. Rocking platform shaker.

9. PBS pH 7.2.

10. Tris buffered saline (TBS): 50 mM Tris, 150 mM NaCl, adjustment of pH to 7.2 with 12 N HCl.

11. TBS-T: 0.1% (v/v) Tween 20 in TBS.

12. Detection antibody. Goat anti-human IgG coupled to alkaline phosphatase (AP) (Southern Biotech).

13. Chromogenic detection reagents: nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3′-indolyphosphate (BCIP).

3   Methods

3.1   Preparation of Heme Solutions

3.1.1   Preparation of Hematin Solution

1. Weigh an appropriate quantity of dry substance of hemin (molecular weight 651.9 g/mol) in a black microcentrifuge tube.

2. Dissolve hemin in 0.05 N solution of sodium hydroxide. Calculate the volume of sodium hydroxide in order to obtain between 20 and 25 mM final hemin solution. Gently vortex the tube to help solubilizing. The Cl anion of hemin is displaced by an OH group leading to hematin formation (*see* Fig. 1).

3. Stock solution of hematin must be stored on ice and used on the day of preparation (*see* **Note 5**).

3.1.2   Preparation of Oxidized Hematin Solution

Hematin represents oxidized form (Fe^{3+}) of heme. Exposure of hematin to hydrogen peroxide results in further oxidation of hematin—formation of ferryl and perferryl oxidative forms of iron and modifications of the tetrapyrrole ring.

1. Dilute hematin solution to a final concentration of 20 mM in sodium hydroxide. Prepare this dilution in a large vessel, suitable for low-speed centrifugation. For example, prepare 1 mL of hematin solution in a 15 mL conical bottom tube.

2. Add hydrogen peroxide at a final concentration of 500 mM from the 35% stock solution: add 48.6 µL in 1 mL of hematin solution. Mix by inverting the tube. Oxygen bubbles will create foam.

Heme   Hemin   Hematin
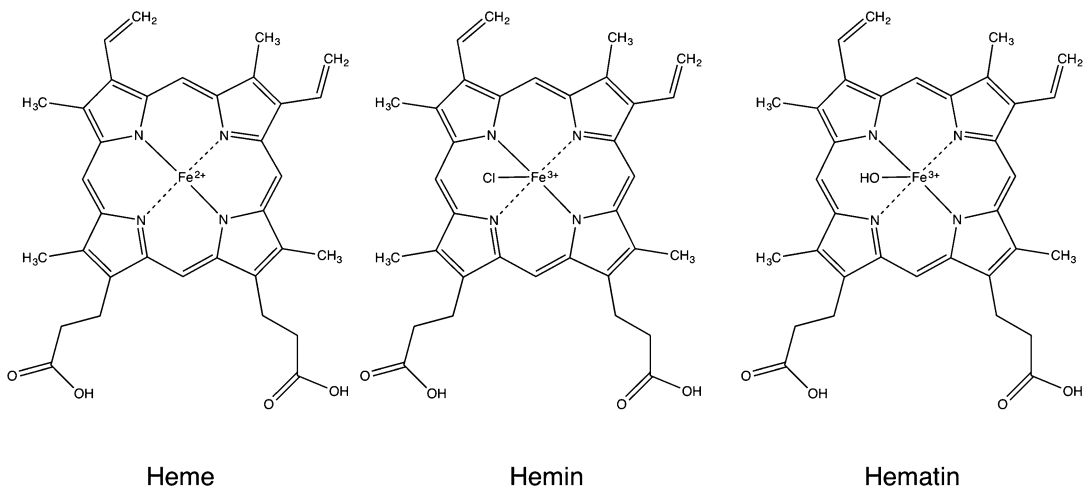
Fig. 1 Structural formulas of heme b (ferroprotoporphyrin IX) and oxidized forms (ferriprotoporphyrin IX) hemin and hematin

3. Centrifuge the tube for 1 min at $300 \times g$ to collapse the foam at the bottom of the tube.

4. The obtained solution should be stored on ice, protected from light and used promptly.

3.1.3 Preparation of Hemin Solution

1. Weigh an appropriate quantity of hemin in powder (molecular weight 651.9 g/mol) in a black microcentrifuge tube. The amount usually prepared is 30 mg.

2. Dissolve the powder in a volume of DMSO to obtain a 50 mM solution.

3. Stock solution of hemin must be stored on ice and used on the day of preparation (Please note that DMSO solution solidifies on ice).

3.2 Preparation of Immunoglobulin Solutions

1. Depending on the downstream applications, immunoglobulins should be dialyzed against an appropriate buffer.

2. For biochemical assays, dialyze against PBS; for cell culture, the immunoglobulin preparation can be diluted in or dialyzed against culture medium without serum (*see* **Note 6**).

3. Final IgG concentration should be determined according to requirements for the treatment. As a rule of thumb, monoclonal antibody preparation should be adjusted at a concentration ranging from 0.01 to 1 mg/ml ($0.067–6.67$ μM). Polyclonal preparations are usually used at higher concentrations to compensate for the heterogeneity of the preparation (1–100 mg/mL, i.e., $6.67–667$ μM).

3.3 Posttranslational Induction of Antibody Polyreactivity

3.3.1 Treatment of Immunoglobulins with Heme

1. Dilute heme solution (hematin, oxidized hematin, or hemin solution) prepared in Subheading 3.1 in order to follow these rules.

   (a) Heme treatment volume should not represent more than 1% of antibody volume.

   (b) Heme:IgG molar ratio should be determined according to the purpose of the experiment. The preferential treatment is exposure of 10 μM of IgG to 20 μM of heme. Although an IgG antibody molecule should bind two molecules of heme, in case of polyclonal preparations it is possible to choose an equimolar or subequimolar ratio in order to avoid the presence of unbound heme. Final heme concentration used for treatment of antibodies should not exceed 100 μM.

2. Pipette the right volume of heme solution into the diluted antibody. Incubate on ice for at least 5 min.

3. If required, free heme can be removed by using gel filtration column PD-10 (see manufacturer's instructions) equilibrated in PBS.

4. Heme-exposed IgG preparations should be stored at 4 °C and preferentially used within 24 h after preparation.

3.3.2 Treatment of Immunoglobulins with Iron Ions

1. Treat polyclonal preparation of immunoglobulins (10 mg/mL in PBS) by adding freshly prepared iron (II) sulfate solution to obtain a final concentration of Fe^{2+} of 1 mM. IgG preparations can be exposed to ferrous ions in other mediums—treatments of IgG in PBS, TBS, 0.25 M glycine (pH 6), or MES-buffered saline (pH 6) gave similar results (Fig. 2).

2. Iron ions exposed preparation can be used directly in immunological assays. Alternatively, iron sulfate can be removed by dialysis. Immunoglobulins preparation exposed to Fe^{2+} ions in different buffers and native immunoglobulins (10 mg/mL
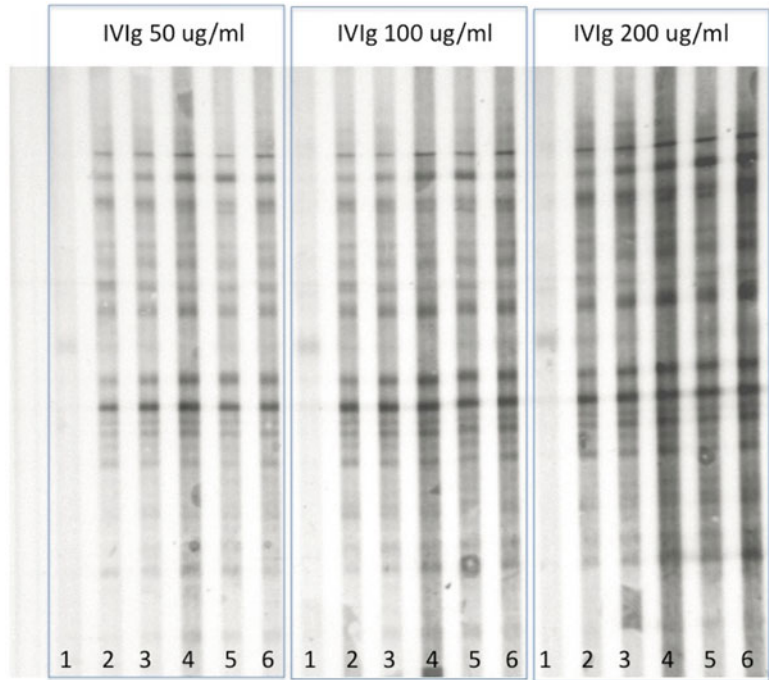
Fig. 2 Reactivity of native or Fe^{2+}-ions treated IVIg toward proteins present in endothelial cells lysate. The samples of IVIg were diluted in TBS-T to 50, 100, and 200 μg/mL. The *line numbers* correspond to following samples. (*1*) Native IVIg (dialyzed against EDTA). (*2*) Fe^{2+}−IVIg treated in *PBS* (dialyzed against EDTA). (*3*) Fe^{2+}−IVIg treated in *MES* (dialyzed against EDTA). (*4*) Fe^{2+}−IVIg treated in *TBS* (dialyzed against EDTA). (*5*) Fe^{2+}−IVIg treated in *Glycine 0.25 M* (dialyzed against EDTA). (*6*) Fe^{2+}−IVIg treated in *PBS* (NOT dialyzed against EDTA)

in PBS) should be transferred to dialysis chambers and dialyzed for at least 3 h at 4 °C against 400× excess of HBS containing 5 mM ultrapure EDTA. After, transfer the dialysis chambers to HBS buffer without EDTA and dialyze for 24 h at 4 °C with three changes of the buffer.

3. Store the immunoglobulins exposed to Fe^{2+} ions at 4 °C until analysis.

3.3.3 Treatment of Immunoglobulins with Acid pH Buffers

The simplest method for uncovering of cryptic IgG polyreactivity is to perform well-described purification of IgG by using protein A or protein G coupled sepharose and acidic buffer elution. Alternatively,

1. Dilute immunoglobulins at least tenfolds in the glycine buffer (either pH 2.6 or pH 4). Incubate for 5–10 min and then adjust pH to 7 with 3 M Tris pH 10.

2. Dialyze immunoglobulin preparation against PBS.

3.4 Evaluation of Induced Polyreactive Antibody Reactivity

3.4.1 SDS-PAGE and Transfer to Membrane

1. For electrophoretic separation of proteins, use gradient ready-gels. Load the single-well start gel with 100 μL of *B. anthracis* lysate in reducing sample buffer. Make sure that the volume is homogeneously spread across the well. The running buffer used was MES-SDS Running buffer. Run the electrophoresis at constant voltage of 200 V. Migrate until phenol red is running out of the gel (about 45 min).

2. Transfer the proteins from the gel on nitrocellulose membrane by using fast (7 min) electrotransfer system—iBlot and iBlot gel transfer stacks, nitrocellulose regular.

3. Block the membrane in TBS-T for 1 h at room temperature or overnight at 4 °C.

3.4.2 Immunoblot Procedure

1. Thoroughly clean the Miniblot apparatus with SDS and water.

2. Place the membrane in the apparatus. Make sure that the side of the membrane with bacterial lysate is correctly orientated.

3. Dilute treated antibody (or native control) tenfolds in TBS-T in order to reach 10–25 μg/mL for monoclonal antibodies or 50–100 μg/mL for polyclonal antibodies. Final volume should be at least 100 μL.

4. Load 60 μL of antibody per channel of Miniblot.

5. Incubate for 2 h at room temperature on a rocking platform shaker.

6. Aspirate the content of Miniblot channel. Open the Miniblot and transfer the membrane in a clean box.

7. Wash the membrane with PBS-T for 1 h on a rocking platform shaker. Change buffer every 10 min.

8. Prepare detection antibody by diluting 3000-folds in TBS-T. Prepare 15 mL for one membrane.

9. Remove PBS-T and incubate with detection antibody for 1 h at room temperature on a rocking platform shaker.

10. Wash the membrane with PBS-T for one hour on a rocking platform shaker. Change buffer every 10 min.

11. Prepare chromogenic BCIP/NBT mixture according to manufacturer's protocol.

12. Develop the membrane.

13. Wash and dry the membrane. Intensity of coloration reflects the quantity of immunoglobulin bound to the bacterial antigens (Fig. 2).

4   Notes

1. Different providers might offer different grades of purity in terms of chemical or biological contamination. One should choose the level of purity according to subsequent applications. It is advised to check the level of endotoxin in heme preparations.

2. Heme and its derivative are light-sensitive. We suggest preparing solutions in a black 1.5 mL microcentrifuge tube to protect solutions from direct light exposure. Alternatively, tubes can be covered with aluminum foil.

3. Store the solution at room temperature. Do not keep for more than one day, as ferrous ions (Fe^{2+}) will be oxidized into ferric ions (Fe^{3+}); the solution will turn brownish.

4. Immunoglobulin source can be either a monoclonal antibody or polyclonal solution, such as intravenous immunoglobulin (IVIg) therapeutic preparation.

5. Analysis carried out in our laboratory demonstrated a modification in the polyreactivity induction for hematin or hemin stored for more than 1 day.

6. Albumin contained in bovine or human serum can bind heme and quench its effect.

Acknowledgments

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Characterization of Natural IgM Antibodies Recognizing Oxidation-Specific Epitopes on Circulating Microvesicles

Florian Puhm and Christoph J. Binder

Abstract

Natural IgM antibodies specific for oxidation-specific epitopes (OSEs) have been found to bind to circulating microvesicles (MVs), also known as microparticles. This chapter describes how endogenous natural IgM antibodies bound to circulating MV subsets can be characterized on the one hand, and how monoclonal natural IgM antibodies can be used to characterize subsets of circulating MVs on the other hand.

Key words Natural IgM antibodies, Oxidation-specific epitopes, Microvesicles, Microparticles, Flow cytometry

1 Introduction

Natural IgM antibodies were found to recognize oxidation-specific epitopes (OSEs), which are particularly common on apoptotic cells [1] and a subset of circulating microvesicles (MVs) [2]. MVs, also known as microparticles or ectosomes, are 0.1–1µm large vesicles shed from the plasma membrane [3]. They are characterized by transmembrane proteins derived from their cell of origin and exposure of phosphatidylserine (PS) on their surface. In the circulation MVs can be derived from platelets, endothelial cells, leukocytes, and red blood cells [4, 5], but MVs of different cellular origin have been reported as well. MVs carry transmembrane proteins, as well as soluble proteins and nucleic acids. Therefore, they are capable of signaling by a variety of pathways. For example, information can be transferred by interaction with surface receptors or plasma membrane fusion and subsequent release of MV content [4]. Both MVs and OSEs have been implicated in the pathogenesis of inflammatory diseases, and binding of natural IgM antibodies to circulating MVs (e.g., via OSE) may represent a physiological clearance mechanism for cellular debris. In this regard, the characterization of the antigen specificity of natural IgM antibodies bound to circulating

MVs may provide important insights into immunological housekeeping mechanisms. In addition, natural IgM antibodies with defined antigen specificities represent a tool to quantify subsets of circulating MVs that may have different functional properties.

Because MVs expose phosphatidylserine (PS), they can typically be identified using Annexin-V staining for flow cytometric analysis. However, PS-negative MVs have been reported as well. As an alternative, Calcein-acetoxymethyl ester (Calcein-AM) staining of MVs can be performed. In this approach, the calcium binding domain of Calcein is blocked by the AM modification and Calcein-AM can permeate the plasma membrane. Inside the MV, esterases remove acetoxymethyl and expose the calcium binding site of Calcein and renders Calcein unable to permeate the membrane. Because Calcein forms fluorescent complexes (Excitation/Emission = 494/517) with calcium, it can be detected with flow cytometry to characterize labeled MVs.

Flow cytometric quantification of MVs bound by natural IgM antibodies primarily involves the characterization of the following parameters: (1) MV count (Annexin-V or Calcein positive events), (2) the frequency of MVs bound by natural IgM, and (3) the mean-fluorescence-intensity (MFI) of antibodies bound to MVs (= an estimate of the number of antibodies bound per MV). When purified natural IgM antibodies of defined specificity are used for labeling, the latter two will identify the frequency of MVs carrying unbound ("free") epitopes and density of these epitopes per MV. In addition, staining for transmembrane proteins present on the MVs can be used to determine the cellular origin of the MVs. For example, CD31 and CD41a positivity of circulating MVs indicate platelet origin. Finally, natural IgMs bound to circulating MVs can be characterized following lysis of the MVs and determination of binding specificities of eluted IgM by ELISA (*see* Sect. 3.5).

2   Materials

1. K$_2$EDTA-containing blood collection tubes.

2. Micro centrifuge tubes (1.5 mL).

3. 0.2 μm filters for filtration of buffers.

4. Phosphate buffered saline (PBS).

5. Fatty acid-free bovine serum albumin (BSA), for blocking.

6. Sample-buffer: PBS containing 0.5% fatty acid-free BSA. Filter with 0.2 μm filters (*see* **Note 1**).

7. Primary monoclonal natural IgM antibody (20 μg/mL final concentration). Prepare a 2× concentrated solution for staining (*see* **Note 2**).

8. Isotype control IgM (equivalent amount to natural IgM antibody).

9. Anti-IgM fluorophore-conjugated antibody (1 μg/mL final concentration) (*see* **Note 2**).

10. MV-Staining-Buffer: 10 mM HEPES, 2.5 mM $CaCl_2$, diluted in ddH_2O. Filter with 0.2 μm filters.

11. Fluorophore-conjugated Annexin-V. Dilute in MV-Staining-Buffer (*see* **Note 3**).

12. Calcein-AM staining solution: Filtered MV-Staining-Buffer containing Calcein-AM (5 μM final concentration [6]).

13. Latex beads (1.1 μm diameter). To determine upper size (side ward scatter, SSC and forward scatter, FSC) gate limit (*see* **Note 4**).

14. RIPA lysis buffer: 50 mM Tris–HCl, pH = 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, commercially available protease inhibitor cocktail.

15. Microdialysis cups. Molecular weight cutoff of 10 kDa.

16. Dialysis buffer: 0.27 mM EDTA in PBS.

3   Methods

3.1   Isolation of MVs from Whole-Blood

1. Collect blood samples into K_2EDTA-containing collection tubes (*see* **Note 5**).

2. Spin samples at room temperature for 30 min at $2000 \times g$ to obtain platelet-poor plasma.

3. The supernatant contains MVs and transfers them to fresh 1.5 mL micro centrifuge tubes.

4. Spin samples at $10,000–20,000 \times g$ [7] for 30 min at 4 °C to pellet MVs (*see* **Note 6**).

5. Resuspend MVs in filtered PBS and store at −20 °C or dilute pellet in filtered sample-buffer and proceed to staining for flow cytometric analysis.

3.2   Staining of MVs with Defined Monoclonal IgM Antibodies

An overview of all stainings required for flow cytometric analysis is presented in Table 1 (*see* **Note 7** for additional advice on setting up gating controls).

1. If samples are not already diluted in filtered sample-buffer (otherwise skip to step **2**), spin samples at $10,000–20,000 \times g$ for 30 min at 4 °C and resuspend pellets in filtered sample-buffer (*see* **Note 8**).

2. For primary monoclonal natural IgM antibody staining, mix 50 μL of the $2\times$ primary antibody solution with 50 μL sample and incubate for 30 min at 4 °C.

Table 1
The ideal amount of staining controls required for gating and analysis of MVs with IgM antibodies

Sample type	Sequence of the staining procedure		
	1	2	3
Unstained, gating	–	–	–
IgM, gating	IgM antibody	Anti-IgM antibody	–
Control IgM, gating	Control IgM antibody	Anti-IgM antibody	–
Annexin-V, gating	–	–	Annexin-V
Calcein-AM, gating	–	–	Calcein-AM
IgM, analysis	IgM antibody	Anti-IgM antibody	Annexin-V or Calcein-AM
Control IgM, analysis	Control IgM antibody	Anti-IgM antibody	Annexin-V or Calcein-AM

The three columns indicate the staining sequence, which is described in more detail in the protocol (*see* **Note 9**)

3. Spin sample for 15 min at $10,000–20,000 \times g$ at 4 °C and discard the supernatant.

4. Proceed to secondary antibody staining. Resuspend the pellet in 100 μL of the secondary antibody solution and incubate for 20 min at 4 °C in darkness.

5. Spin the sample for 15 min at $10,000–20,000 \times g$ at 4 °C and discard the supernatant.

6. Perform MVs Staining by Annexin-V or Calcein-AM staining. Resuspend the pellet in 100 μL of the Annexin -V or Calcein-AM staining solution and incubate for 20 min at room temperature in darkness (*see* **Note 9**).

7. Spin the sample for 15 min at $10,000–20,000 \times g$ at 4 °C and discard the supernatant.

8. Resuspend the pellet in 250 μL of filtered sample-buffer and proceed to measurement with the flow cytometer.

3.3 Flow Cytometry Analysis of the samples

1. For measurements, acquire the samples at low speed (12 μL/min) for 30 s (*see* **Note 10**).

2. Measure "filtered sample-buffer" to determine background signal and "size calibration beads (1.1 μm)" to determine the upper size limit for the analysis. The lower size limit depends on the machine. A cutoff is set according to the background signal, determined by measuring sample buffer, or the appropriate size calibration beads (*see* **Note 11**).

3. Main parameters obtained by the measurements are the following (*see* Fig. 1 for a graphical depiction of the analysis).
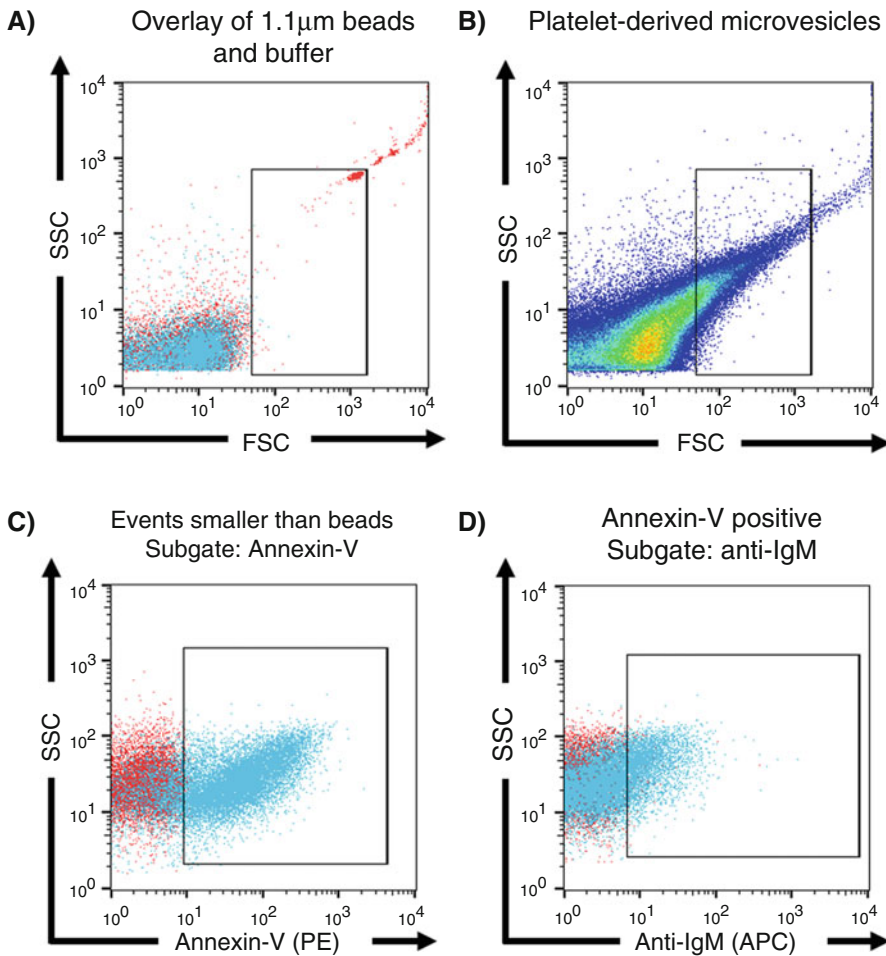
Fig. 1 Analysis of natural IgM-bound MVs by flow cytometry. (**a**) The minimal requirements to set up a size-gate (forward and side-scatter) for MV analysis using 1.1 μm beads and buffer as limitations. (**b**) An example of platelet-derived MVs (PMVs) that were generated by treating platelet-concentrate with ionomycin (10 μM) in the presence of $CaCl_2$ (1 mM) for 30 min at 37 °C. (**c**) An overlay of PMVs stained with Annexin-V and unstained PMVs. The main parameter for quantification is the number of Annexin-V-positive events. (**d**) Annexin-V-positive PMVs and an overlay of PMVs that were stained with natural IgM and anti-IgM (APC-conjugated) and PMVs stained with isotype control IgM and anti-IgM. The main parameters are, the number and frequency of anti-IgM-positive events, as well as the mean-fluorescence-intensity (MFI) of the anti-IgM signal

(a) Absolute number of MVs, quantified by the number of Annexin-V or Calcein-positive events.

(b) Absolute number of MVs bound by anti-IgM antibody, quantified by the number of Annexin-V/IgM or Calcein/IgM double-positive events.

(c) Frequency of IgM-positive events within all Annexin-V or Calcein-positive events.

(d) Mean fluorescent intensity (MFI) of the anti-IgM signal of Annexin-V/IgM or Calcein/IgM double-positive events.

(e) Total amount of anti-IgM bound to MVs is calculated by multiplication of MFI and number of IgM-positive MVs. The resulting number is the area-under-the-curve (AUC).

3.4 Characterization of MVs Bound by Endogenous Natural IgM Antibodies by Flow Cytometry

1. Quantify circulating MVs carrying endogenous natural IgM antibodies by flow cytometry using fluorophore-conjugated anti-IgM antibodies.

2. Perform the staining and quantification similar to the protocol described under Subheadings 3.2 and 3.3, except the staining step with primary monoclonal IgM antibodies. The advantage of this method is that the characterization of IgM-carrying circulating MVs can be combined with a more detailed characterization of these MVs with respect to cellular origin and other surface markers. For example, by costaining for CD31 and CD41a or CD45, platelet - or leukocyte-derived MVs can be determined, respectively.

3.5 Elution of Bound Endogenous Natural IgM Antibodies From Circulating MVs

1. Isolate MVs from the blood as described under Subheading 3.1.

2. Resuspend the MV pellet obtained after the final centrifugation step at $10,000–20,000 \times g$ in RIPA lysis buffer.

3. For complete lysis, keep the suspension overnight at 4 °C.

4. Transfer the lysates to microdialysis cups (molecular weight cutoff of 10 kDa) and dialyze against dialysis buffer overnight at 4 °C [2].

5. The concentration and antigen specificity of eluted endogenous natural IgM can then be determined by ELISA and compared to soluble plasma IgM, for example.

4   Notes

1. Alternatively to filtering, particle-free buffers can be prepared by centrifugation at $10,000–20,000 \times g$ for 30 min.

2. Optimal concentrations of antibodies have to be determined by titration. The primary antibody solution may also contain fluorophore-conjugated antibodies for surface marker proteins (e.g., anti-CD31 and anti-CD41a to determine platelet origin of MVs). Pay attention to the fluorescence emission wavelength. It is not possible to combine a FITC-conjugated antibody with Calcein-AM staining as the emission peak of Calcein overlaps with that of FITC.

3. Optimal concentration of conjugated Annexin-V should be determined by titration.

4. To control for changes in flow cytometer flow speed when quantifying MVs, counting beads can be added to the samples.

5. The anticoagulant used when collecting blood has been reported to affect MV quantity [8]. Therefore, only MVs from plasma samples collected with the same type of anticoagulant should be compared.

6. Smaller vesicles, such as exosomes, remain in the supernatant after $10,000$–$20,000 \times g$ centrifugation. Supernatants can be kept for analysis of released proteins such as cytokines.

7. To prepare gating controls it is advised to make a pool of all samples that are to be analyzed and to split it into the necessary number of aliquots.

8. As oxidation correlates with increasing temperature, samples should be prepared at 4 °C or even stored in the presence of anti-oxidants, such as butylated hydroxytoluene (BHT) [2], when testing natural IgMs against oxidation-specific epitopes. However, after staining with IgM antibodies, it is no longer necessary to keep the samples at 4 °C. Furthermore, staining with Annexin-V or Calcein-AM should be performed at room temperature to achieve optimal results.

9. Both Annexin-V and Calcein-AM staining depend on the availability of calcium, therefore these dyes may compete for calcium binding.

10. If speed and sample concentration are too high multiple particles will be simultaneously illuminated by the laser and the flow cytometer cannot distinguish between individual particles [9]. This is called swarm detection and causes underestimation of the total number of MVs, as well as the detection of numerous very small MVs as a single large event [9]. However, the resolution of flow cytometers differs. Especially more recent flow cytometers can tolerate higher concentrations of MVs in the sample.

11. Alternatively, mixtures of size calibration beads ranging from 0.1 to 1 μm can be employed. However, most flow cytometers cannot accurately detect particles below 0.5 μm size. Actually, smaller particles might be detected at high concentrations due to swarm detection [9].

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Chapter 12

Natural Monoclonal Antibody to Oxidized Low-Density Lipoprotein and *Aggregatibacter actinomycetemcomitans*

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Abstract

Natural antibodies are produced by B lymphocytes without exogenous antigenic exposure and are present at the time of birth. They usually bind to conserved epitopes on antigens of different chemical compositions. We cloned and characterized a natural mouse monoclonal IgM antibody (Aa_Mab) by selecting the binding to malondialdehyde acetaldehyde (MAA) adducts on low-density lipoprotein (LDL). The data showed that the Aa_Mab cross-reacted with *Aggregatibacter actinomycetemcomitans* (*Aa*) bacteria, an important oral pathogen in periodontitis associated with atherosclerosis. Surprisingly, the binding molecule of *Aa* bacteria to the Aa_Mab was *Aa* chaperonin 60 or HSP60, a protein that is not only responsible for maintaining cellular proteins conformation, but also functions as a potent virulence factor prompting bone resorption in periodontitis and as a putative pathogenic factor in atherosclerosis.

Key words Natural antibody, Oxidized low-density lipoprotein, Malondialdehyde acetaldehyde, *Aggregatibacter actinomycetemcomitans*, Chaperonin 60

1 Introduction

The innate immune response is important for the host's first-line defence against various pathogens and tissue injury. Natural antibodies are an essential part of innate immune system, they arise without exogenous antigenic stimulation and are present at birth [1]. Natural antibodies are produced T cell independently by B1 B-lymphocytes, marginal zone B cells and other B cell types, and include all immunoglobulin isotypes [1–3]. The role of natural antibodies is to protect against microbial antigens and to serve as innate recognition receptors for homeostatic housekeeping functions and removal of altered self-antigens [1, 4, 5]. Approximately one third of natural IgM isotype of antibodies in mice have been proposed to bind to oxidation-specific epitopes found in atherosclerotic plaques, such as malondialdehyde adducts (MDA-adducts), and therefore natural IgM antibodies have been focus of studies in atherosclerotic cardiovascular diseases. Mice lacking both

secretory IgM (sIgM$^{-/-}$) and low-density lipoprotein receptor (LDLR$^{-/-}$) have been shown to have more progressive atherosclerosis [6], suggesting a protective role of IgM isotype of antibodies. Also, mice lacking apolipoprotein E (ApoE$^{-/-}$) and spleen (splenectomized) showed reduced atherosclerosis and increased plasma IgM levels when adoptively transferred with B1a cells [7], suggesting that IgM secreted by B1a lymphocytes is atheroprotective. In humans, plasma levels of IgM binding to MDA modified low-density lipoprotein, MDA-LDL, are inversely associated with surrogate markers of atherosclerosis [8, 9], and new born babies have monoclonal IgM to malondialdehyde acetaldehyde modified low-density lipoprotein (MAA-LDL) displaying similar properties with the monoclonal mouse natural IgM linked to atheroprotection [10]. Malondialdehyde acetaldehyde is the immunodominant epitope in MDA-LDL and natural IgM to MAA-adducts in mice have been documented to cross-react with bacterial epitopes in *Porphyromonas gingivalis* [11] and *Aggregatibacter actinomycetemcomitans* [12].

P. gingivalis (Pg) and *A. actinomycetemcomitans (Aa)* are gram-negative rod bacteria considered the most important pathogens causing periodontitis, an inflammatory disease leading to loss of tooth-supportive tissue. Periodontitis is well established to be associated with atherosclerotic cardiovascular disease [13, 14], and it has been proposed that activation of the immune system by *P. gingivalis* and *A.actinomycetemcomitans* bacteria may be contributing factors [14–18]. Gingipain, a protease secreted by *P. gingivalis*, has been shown to share molecular identity with epitopes on MDA-LDL, and immunization with MDA-LDL moderates atherosclerosis in LDLR$^{-/-}$ mice challenged with live *P. gingivalis* [11, 19]. Yet, the causal relationship and pathological mechanism have remained elusive.

Here, a natural mouse monoclonal IgM (Aa_Mab) was selected by screening the IgM binding of hybridoma cell culture media to MAA-epitope on LDL. The hybridoma cell line had been generated from splenocytes of a non-immunized nrf2$^{-/-}$ mouse by fusion with mouse myeloma cell line. The binding of the purified Aa_Mab was screened to models of oxidized LDL and different bacteria including several serotypes of *A. acinomycetemcomitans* using chemiluminescence immunoassay, Dot blot, and Western blot. The monoclonal IgM antibody (Aa_Mab) was produced by growing the cell line in serum-free HyClone™ SFM4Mab-Utility media and purifying the culture media with Superose 6 10/30 GL high-performance size exclusion chromatography. The sequence analyses of the variable heavy (V$_H$) and variable light kappa (V$_k$) chains were attained by amplifying cDNAs reversely transcribed from hybridoma total RNA. Mass spectrometry analysis revealed that the epitope in *A. acinomycetemcomitans* recognized by the monoclonal Aa_Mab IgM was chaperonin 60, i.e., heat shock protein 60

(HSP60), a powerful virulence factor of the bacteria and also a known risk factor associated with atherosclerosis. The binding characteristics of the Aa_Mab IgM to *A. acinomycetemcomitans* chaperonin 60 was further demonstrated by using purified recombinant *Aa* chaperonin 60 expressed in *BL21(DE3) Escherichia coli* cells.

2 Materials

2.1 Bacteria and Hybridoma Cells

1. *A.actinomycetemcomitans* strains (ATCC 29523, ATCC 43718, ATCC 33384, IDH 781, IDH 1705, CU1000, C59A, representing six serotypes *a*, *b*, *c*, *d*, *e*, *f* and one non-serotypeable strain *x*) [20], *E. coli and Streptococcus pyogenes* (control strains).

2. Fastidious anaerobic agar (FAA) plates.

3. Dulbecco's phosphate buffered saline ($10\times$ PBS): Make the working solution by 1:10 dilution in water ($1\times$ PBS).

4. *DC™* (detergent compatible) protein assay kit.

5. CO_2 incubator.

6. Splenocytes from a non-immunized nrf2$^{-/-}$ mouse and P3$\times$63Ag8.653.1 myeloma cells.

7. Serum-free Dulbecco's Modified Eagle's Medium (DMEM).

8. 20% FBS-DMEM: DMEM, 20% fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.1 mM nonessential amino acids (NEAA), 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM β-mercaptoethanol.

9. $50\times$ hypoxanthine-aminopterin-thymidine (HAT)-media supplement.

10. 50% polyethylene glycol (PEG).

11. Cell storage solution: 85% FBS, 15% dimethyl sulfoxide (DMSO).

2.2 Chemiluminescence Immunoassay

1. Antigens.

    (a) Malondialdehyde acetaldehyde modified low-density lipoprotein (MAA-LDL).

    (b) Malondialdehyde modified low-density lipoprotein (MDA-LDL).

    (c) Copper oxidized LDL (CuOx-LDL).

    (d) Native LDL.

    (e) Bovine serum albumin (BSA).

    (f) Malondialdehyde acetaldehyde modified bovine serum albumin (MAA-BSA).

(g) Malondialdehyde modified bovine serum albumin (MDA-BSA).

(h) Oxidized phosphatidylcholine bovine serum albumin (PC-BSA).

(i) Cell wall polysaccharide (CWPS).

(j) 0.5% fish gelatin-PBS (FG-PBS).

(k) *A. actinomycetemcomitans* serotypes *a*, *b*, *c*, *d*, *e*, *f*, *x*.

(l) *E. coli* and *S. pyogenes*.

(m) Recombinant *Aa* chaperonin 60.

2. PBS-EDTA buffer: 1× PBS with 0.27 mM EDTA.

3. FG-PBS-EDTA buffer: 0.5% FG in 1× PBS with 0.27 mM EDTA.

4. Alkaline phosphatase-conjugated anti-mouse IgM.

5. Nunc Microfluor2 96-well plates.

6. An automated plate washer.

7. LumiPhos 530 (33%) (Lumigen).

8. A Wallac Victor3 multilabel counter.

2.3 Production and Purification of Antibody

1. Serum-free HyClone™ SFM4MAb-Utility Medium.

2. Amicon Ultra-15 Centrifugal Filter Units (MWCO 100 kDa).

3. Superose 6 10/30 GL high-performance size exclusion chromatography columns and ÄKTA explorer.

4. 10% sodium dodecyl sulfate (SDS) polyacrylamide gel.

5. Precision Plus Protein™ All Blue Prestained Protein Standards.

6. PageBlue™ Protein Staining Solution.

2.4 Total RNA Isolation, cDNA Amplification, Sequence Analysis

1. RNeasy Mini Kit.

2. RevertAid First Strand cDNA Synthesis Kit.

3. IgM heavy pair primers: forward (MH1) 5′-SARGTN-MAGCTGSAGSAGTC-3′, reverse (IgM) 5′-GACATTTGG-GAAGGACTGACTCTC-3′.

4. IgM light pair primers: forward (5MK) 5′-GAYATTGTGMT-SACMCARWCTMCA-3′, reverse (3KC) 5′-GGATACAGTT-GGTGCAGCATC-3′.

5. dNTPs.

6. AmpliTaq polymerase.

7. Nuclease-free H_2O.

8. Thermo cycler PCR equipment.

9. 1.2% agarose gel.

10. ChemiDoc XRS Gel Imaging System.

11. Quantity One software.

12. GeneJET PCR purification kit.

13. ABI3500xL Genetic Analyzer.

2.5 Mass Spectrometry

1. 10% SDS polyacrylamide gel.

2. Precision Plus Protein™ All Blue Prestained Protein Standards.

3. PageBlue™ Protein Staining Solution.

4. SDS-PAGE washing buffer: 40% acetonitrile, 60% water, 50 mM ammonium bicarbonate.

5. Dithiothreitol (DTT) buffer: SDS-PAGE washing buffer, 20 mM DTT.

6. Iodoacetamide.

7. Trypsin buffer: 9% acetonitrile, 91% water, 40 mM ammonium bicarbonate.

8. Trypsin solution: 20 ng/μL trypsin in trypsin buffer.

9. 800-384 anchor chip plate.

10. α-Cyano-4-hydroxycinnamic acid (MALDI matrix).

11. UltrafleXtreme MALDI Tof Tof instrument.

12. MASCOT software from Matrix science.

2.6 Expression and Purification of Aa Chaperonin 60

1. *Aa* chaperonin 60 cDNA in pET28a(+) expression vector (GenScript).

2. Competent *BL21(DE3) E. coli* cells.

3. SOB broth: 2% tryptone, 0.5% yeast extract, 8.56 mM NaCl, 2.5 mM KCl, 20 mM MgCl.

4. SOC broth: SOB broth, 20 mM glucose.

5. LB broth: 1% tryptone, 0.5% yeast extract, 1% NaCl.

6. LB kanamycin (LBK) plate: LB containing 30 μg/mL of kanamycin.

7. 0.5 M isopropyl β-D-1-thiogalactopyranoside (IPTG).

8. Lysozyme (50 mg/mL).

9. 1 M $MgCl_2$.

10. 1 M $CaCl_2$.

11. SIGMAFAST™ Protease Inhibitor Tablets, EDTA-Free.

12. Recombinant DNase I (RNase-free, 10 U/μL).

13. Digital Sonifier® Cell Disruptor.

14. Ultracentrifuge Beckman Optima L-100K.

15. HisPur™ Cobalt resin.

16. 3 M imidazole.

17. 50 mM sodium phosphate buffer, pH 7.4: 4.23 mL of 1 M NaH_2PO_4 (pH 7.4 adjusted by 10 M NaOH), 5.77 mL of 1 M Na_2HPO_4 (pH 7.4 adjusted by 37% HCl), 190 mL of H_2O.

18. Binding buffer: 300 mM NaCl, 50 mM sodium phosphate buffer, pH 7.4.

19. Washing buffer: 20 mM imidazole in binding buffer.

20. Elution buffer: 150 mM imidazole in binding buffer.

21. Amicon Ultra-15 Centrifugal Filter Units (MWCO 10 kDa).

22. 7 kDa MWCO Zeba Desalt Spin Column.

2.7 Dot Blot and Western Blot

1. Bio-Dot® microfiltration apparatus.

2. Nitrocellulose membrane.

3. Tris buffered saline (TBS): 20 mM Tris–HCl, pH 7.5, 150 mM NaCl.

4. Blocking buffer: TBS, 5% BSA.

5. Antibody buffer: TBS, 5% BSA, 0.05%Tween 20.

6. Washing buffer: TBS, 0.05% Tween 20.

7. Goat-anti-mouse IgM IRDye800CW (0.5 μg/mL).

8. Alexa Fluor 680 goat-anti-mouse IgM (0.5 μg/mL).

9. Odyssey Infrared imager and Image Studio™ Software.

3 Methods

3.1 Bacterial Cultivation

1. Grow *A. actinomycetemcomitans* serotypes (*a*, *b*, *c*, *d*, *e*, *f*, *x*), *E. coli and S. pyogenes* on FAA plates at 37 °C with 5% CO_2 for 2 days (*see* **Note 1**).

2. Pick bacterial cells from the plates and suspend in 1× PBS without heat inactivation.

3. Measure protein concentrations by *DC*™ (detergent compatible) protein assay kit.

3.2 Cloning of Mouse Monoclonal IgM

1. Generate hybridoma cells by fusion of splenocytes from a non-immunized nrf2$^{-/-}$ mouse with P3×63Ag8.653.1 myeloma cells using standard polyethylene glycol method [21] followed by selection in 1× HAT medium for a following 2 week period (*see* **Note 2**).

2. Grow the cells at 37 °C with 5% CO_2 in 20% FBS-DMEM. Collect and screen the culture medium (*see* **Note 3**).

3. Apply chemiluminescence immunoassay [10] to select IgM secreting hybridoma cells to MAA-LDL (*see* **Note 4**). Immobilize MAA-LDL (5 μg/mL) overnight at 4 °C to Nunc Microfluor2 96-well plates in 1× PBS. Block nonspecific binding

sites with 50 μL of FG-PBS-EDTA buffer. Add hybridoma culture medium (25–50 μL, *see* **Note 5**) and incubate at room temperature for 1 h. Use the same volume of alkaline phosphatase-conjugated anti-mouse IgM (1:30,000) as a secondary antibody and 25 μL of LumiPhos 530 (33%) as a substrate in the assay. Measure the chemiluminescence as relative light units (RLU) with a Wallac Victor3 multilabel counter.

4. Establish monoclonal hybridoma cell lines by limiting dilution from IgM producing cells. Propagate the selected clones and store in a cell storage solution in liquid nitrogen.

3.3 Production and Purification of Mouse Monoclonal Antibody to MAA-LDL

1. Aa_Mab (clone HMN-08_34) is selected to grow and expand it in DMEM containing 10% FBS at 37 °C with 5% CO_2 (*see* **Note 6**).

2. Following wash with PBS, transfer the cells to serum-free HyClone™ SFM4MAb-Utility medium and let it grow for 2 weeks.

3. Collect the medium and concentrate by Amicon Ultra-15 Centrifugal Filter Units (MWCO 100 kDa) (*see* **Note 7**).

4. Carry out the antibody purification using Superose 6 10/30 GL high-performance size exclusion chromatography columns with ÄKTAexplorer.

5. Verify purity of the antibody by running 10% SDS-polyacrylamide gel electrophoresis (PAGE) (*see* **Note 8**).

3.4 Total RNA Isolation, cDNA Amplification, Sequence Analysis

1. Grow the Aa_Mab (clone HMN-08_34) hybridoma cells in 35 mm plates in DMEM containing 10% FBS at 37 °C with 5% CO_2. Collect the cells following wash with PBS (*see* **Note 9**).

2. Isolate total RNA with RNeasy Mini Kit (*see* **Note 10**) and use this RNA to synthesize cDNA with RevertAid Reverse Transcriptase and oligo (dT) 18 primers included in the RevertAid First Strand cDNA Synthesis Kit (*see* **Note 11**).

3. Amplify the cDNA from reverse transcription by PCR using IgM heavy pair and IgM light pair primers [22]. The PCR reaction (100 μL) contains 2 μL of cDNA, 4 μL each of IgM primer (5 μM), 4 μL of dNTPs (2.5 mM each), 10 μL of $10\times$ PCR reaction buffer with 15 mM $MgCl_2$, 0.5 μL of AmpliTaq polymerase (5 U/mL), and 75.5 μL of nuclease-free H_2O. Run the PCR by using following program: 95 °C $\times$ 5 min; 95 °C $\times$ 1 min, 45 °C $\times$ 1 min, 72 °C $\times$ 2 min for 30 cycles; 72 °C $\times$ 10 min; 10 °C $\times$ 1 min; 4 °C $\times$ overnight.

4. Check the amplified PCR products by running 1.2% agarose gel electrophoresis and obtain the images by using ChemiDoc XRS Gel Imaging System and Quantity One software. Further

purify the PCR products with GeneJET PCR purification kit and analyze the nucleotide sequences by ABI3500xL Genetic Analyzer (*see* **Note 12**).

5. Align the antibody sequences to the germ-line genes with IMGT/V-QUEST sequence alignment tool (http://www.imgt.org).

3.5 Mass Spectrometry Analysis

1. Separate the bacterial protein recognized by Aa_Mab IgM by running 10% SDS-PAGE.

2. After being stained by Coomassie Brilliant Blue, cut the bands with correct size, destain with SDS-PAGE washing buffer, and reduce with DTT buffer for 30 min at room temperature.

3. Carry out alkylation by adding a stoichiometric amount of iodoacetamide. Incubate for 30 min, wash for 5 min once with SDS-PAGE washing buffer and twice with trypsin buffer.

4. Perform the sample digestion by first adding 5 μL of trypsin solution to the gel pieces for 20 min. Then add 15 μL of trypsin buffer and keep incubated at 35 °C overnight.

5. Dry 0.5 μL of the supernatant on an 800-384 anchor chip plate and dry a solution of α-Cyano-4-hydroxycinnamic acid as MALDI matrix on the top of the sample.

6. Measure the mass spectra on an UltrafleXtreme MALDI Tof Tof instrument with an automated method by first acquiring peptide fingerprint data in reflectron mode between m/z 700 and 4000, after which automatically select up to ten ions for MS/MS interrogation. Externally calibrate the spectra with peptide calibration mixture.

7. Combine the fingerprint and MS/MS data and subject to search in the NCBI nonredundant database with MASCOT using the following parameters: No species restriction, carbamidomethylation of cysteins as global modification, optional methionine oxidation, 20 ppm mass tolerance for peptide masses and 0.7 Da for MS/MS fragments.

3.6 Expression and Purification of Recombinant Aa Chaperonin 60

1. Thaw on ice competent *BL21(DE3) E. coli* cells from −80 °C and add 100 μL into a prechilled 14 mL polypropylene tube. Add β-mercaptoethanol to a final concentration of 25 mM. Incubate the reactions on ice for 10 min, swirling gently every 2 min.

2. Add 1 ng of the *Aa* chaperonin 60 cDNA in pET28a(+) expression vector to the transformation tube and incubate on ice for 30 min.

3. Then, heat in a 42 °C water bath for 45 s and incubate on ice for 2 min.

4. Add 0.9 mL of preheated (42 °C) SOC medium to the transformation reaction and incubate at 37 °C for 1 h with shaking at 250 rpm.

5. Spread 100 μL of the cells onto LBK agar plates and grow at 37 °C overnight (*see* **Note 13**).

6. Pick up a single colony and grow in 50 mL of LB broth containing kanamycin (30 μg/mL) at 37 °C with vigorous shaking (220 rpm) for 16–20 h.

7. Dilute the bacterial culture to 2 L by LB broth containing kanamycin.

8. Induce the protein expression at 30 °C for 18 h by adding 1 mM of IPTG when OD_{600} reaches 0.6–0.8.

9. Pellet the bacterial cells ($3000 \times g$ for 30 min at 4 °C), resuspend and lyse in binding buffer, supplement with proteinase inhibitor and lysozyme (1 mg/mL) at 37 °C for 1 h.

10. Add DNase I (4 U/mL), $MgCl_2$ (2.5 mM), and $CaCl_2$ (0.1 mM) and incubate for further 1 h.

11. Finally, lyse the cells with sonication by Digital Sonifier® Cell Disruptor. Pellet the cells debris by ultracentrifugation at $48,000 \times g$ for 30 min at 4 °C.

12. Collect the crude cell lysates and save at 4 °C.

13. Carry out the protein purification by using HisPur™ Cobalt resin. The binding is done by rotating the mix of cell lysates and resin at 4 °C overnight in binding buffer. Add imidazole to the buffer for washing (20 mM) and for elution (150 mM) respectively.

14. Desalt the eluted *Aa* chaperonin 60 by 7 kDa MWCO Zeba Desalt Spin Column with PBS (*see* **Note 14**).

3.7 Chemiluminescence Immunoassay With Aa_Mab Antibody

1. To investigate the direct binding characteristics of the monoclonal IgM antibody (Aa_Mab), immobilize various amounts (0–100 μg/mL) of antigens overnight at 4 °C to Nunc Microfluor2 96-well plates in PBS. The antigens are MAA-LDL, MDA-LDL, CuOx-LDL, native LDL, MAA-BSA, MDA-BSA, PC-BSA, BSA, CWPS, FG-PBS, seven serotypes (*a*, *b*, *c*, *d*, *e*, *f*, *x*) of *A. actinomycetemcomitans*, *E. coli*, *S. pyogenes*, and recombinant *Aa* chaperonin 60.

2. Wash the wells three times with PBS-EDTA buffer by an automated plate washer between each step of the immunoassays. Block the nonspecific binding sites with FG-PBS- EDTA buffer for 1 h at room temperature.

3. Add Aa_Mab (1–2.5 μg/mL) to incubate at room temperature for 1 h. Use 50 μL of alkaline phosphatase-conjugated anti-mouse IgM (1:30,000) as a secondary antibody and 25 μL of LumiPhos 530 (33%) as a substrate in the assay.

4. Measure the chemiluminescence as RLU with a Wallac Victor3 multilabel counter (*see* **Note 15**).

5. In competitive immunoassay, incubate Aa_Mab antibody (0.125–0.25 μg/mL) with either soluble antigens (MAA-LDL, MDA-LDL, CuOx-LDL, Native LDL, MAA-BSA, MDA-BSA, PC-BSA, BSA, CWPS, FG-PBS) or bacteria (seven serotypes of *A. actinomycetemcomitans, E. coli* and *S. pyogenes*) as competitors with concentrations at 0–100 μg/mL overnight at 4 °C. Centrifuge the samples at 16,000 × *g* for 30 min at 4 °C before adding to microtiter plates with immobilized MAA-LDL (5 μg/mL) or *Aa* chaperonin 60 (5 μg/mL). Complete the rest of the assay as in direct binding analysis (**steps 1–4**).

3.8 Dot Blot and Western Blot Analysis

1. For dot blot, load bacterial suspension (10 μg protein/strain/well) in 200 μL of TBS, by vacuum for 1 h at room temperature onto prewet nitrocellulose membrane using Bio-Dot® microfiltration apparatus (*see* **Note 16**).

2. For western blot, first separate the bacterial suspension (17 μg protein/strain/lane) by running 10% SDS-PAGE and then blot onto nitrocellulose membranes under 30 V at 4 °C overnight (*see* **Note 17**).

3. Block all blots in blocking buffer for 1 h at room temperature, incubate with Aa_Mab antibody (2.5 μg/mL for dot blot and 1 μg/mL for western blot) in antibody buffer for 1 h at room temperature.

4. Wash the blots by washing buffer for 5–10 min at room temperature for three times (*see* **Note 18**).

5. Visualize the binding of Aa_Mab to bacterial proteins by incubating further with goat-anti-mouse IgM IRDye800CW (0.5 μg/mL) for 1 h at room temperature (*see* **Note 18**).

6. Measure the fluorescent signals with an Odyssey IR imager and Image Studio™ Software.

7. For western blot analysis of recombinant *Aa* chaperonin 60, load 2 μg of proteins (*Aa* chaperonin 60, MAA-BSA, and BSA, respectively) per lane. Use Aa_Mab (0.1 μg/mL) as primary antibody, and Alexa Fluor 680 goat-anti-mouse IgM (0.5 μg/mL) as secondary antibody (*see* **Note 19**).

4   Notes

1. Purity of the bacterial cultures can be checked by colony morphology and gram-staining.

2. The 1× HAT selection medium is diluted from 50× HAT by 20% FBS-DMEM. The HAT selection medium needs to be

made freshly and changed every day for about 2 weeks until hybridoma clones are clearly visible.

3. The hybridoma culture media are screened freshly or stored at −20 °C temporarily for later use.

4. Otherwise specified, all procedures are carried out at room temperature for 1 h. All wells should be washed with an automated plate washer with PBS-EDTA buffer three times between each step of the immunoassays.

5. Either 25 or 50 μL of hybridoma culture media are used for chemiluminescence immunoassay depending on the volume of culture media in selection wells.

6. We allow the hybridoma cells to grow to reach a very high density because the more cells there are, the more antibodies they will produce.

7. The media are concentrated for about 200 times by centrifugation at $3200 \times g$, 4 °C.

8. SDS gels are usually run at 200 V for 1 h. The gels are washed by water for 3×10 min, stained in PageBlue™ Protein Staining Solution for 1 h, and destained with several changes of water.

9. The cells should be frozen at −80 °C if they are not used straight for total RNA isolation. However, the RNA yield from fresh cells is much higher than the one from frozen cells.

10. Total RNA is extracted according to the manufacturer's instruction from 1×10^5 to 1×10^7 cells with $A_{260/280}$ ratios of 1.9–2.1 and stored at −80 °C.

11. The cDNA amplification is carried out according to the manufacturer's instruction and stored at −20 °C.

12. The total volume of each PCR reaction is 100 μL and only 10 μL is used for agarose gel running (80 V for 1 h). The rest samples (90 μL) with successfully amplified PCR products are purified further for sequencing. The sequencing primers are MH1 or IgM for heavy chain and 5MK or 3KC for light chain.

13. The transformation is carried out according to the instructions from the competent cells supplier.

14. The binding of His-tagged proteins to Cobalt resin can also be done at room temperature for 1 h. The washing steps are usually repeated three times by spinning down the bounded resin at $3200 \times g$ for 2 min and discarding the unbounded supernatant. The elution is repeated three times, all eluates are combined and concentrated by Amicon Ultra-15 Centrifugal Filter Units (MWCO 10 kDa). The desalting procedures are carried out according to the manufacturer's instruction.

15. The chemiluminescence immunoassay is basically carried out as the same as in Subheading 3.2 except different antigens is immobilized overnight at 4 °C to Nunc Microfluor2 96-well plates in PBS.

16. MAA-BSA and PC-BSA (2 μg/well) are loaded as controls for dot blot assay.

17. MAA-BSA and BSA (2 μg/lane) are taken as controls for western blot analysis.

18. All procedures are carried out with shaking.

19. The proteins are separated by 10% SDS-PAGE, blotted to nitrocellulose membrane, incubated with Aa_Mab and secondary antibody, and visualized under the same conditions as used in bacterial dot blot and western blot.

Acknowledgment

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Chapter 13

Detection of Natural Antibodies and Serological Diagnosis of Pneumococcal Pneumonia Using a Bead-Based High-Throughput Assay

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Abstract

Surface-exposed proteins of pathogenic bacteria play a critical role during infections. The vast majority of these molecules are able to trigger strong immune responses. Measuring the humoral immune response against pathogenic bacteria through less-time consuming tests is necessary to reduce the window time for the diagnosis of diseases that may be associated with high morbidity and mortality rates. Due to the multiplex setup, Luminex xMAP® technology allows analysis of immune responses against many antigens in a single assay. Therefore, less volumes of sera samples are needed and inter assay coefficient of variation is much lower in comparison with other immunoassays. With this methodology, the carboxyl groups on the surface of the polystyrene microspheres must first be activated with a carbodiimide derivative prior to coupling antigens. After the antigen is coupled to a microsphere, different microspheres (all having a unique color) can be combined whereafter the presence of specific antibodies directed against the different antigens in sera can be determined simultaneously. The platform here described can also be useful for epidemiological surveillance programs and vaccine studies.

Key words Surface proteins, Proteomics, Multiplex assay, IgG antibodies

1 Introduction

Pathogenic bacteria display various proteins on their surface, which have key roles in many biological processes. Surface proteins are potential antigens and able to raise immune responses by infected hosts. During infectious processes, humoral immunity plays an important role in the bacterial clearance. Natural antibodies are among the first-line defense against invading pathogens. The majority of natural antibodies consist of IgM with smaller proportion of IgG and IgA [1]. The role of natural antibodies in human infections remains unclear because longitudinal studies in children have only demonstrated limited evidence of protection rendered by naturally induced anti-protein antibodies and no clear protective

effect against infections and colonization (e.g., upper respiratory tract) has been found [2, 3]. Conventional methods for detecting antibodies have been widely replaced by immunosorbent assays (e.g., ELISA). ELISA has proven to be highly sensitive and specific. Moreover, it has been shown less subjective and less time-consuming than classic methods such as double diffusion in agar, latex agglutination, and neutralization assay. Recently, flow cytometric bead-based technologies have been used for the development of assays for the simultaneous detection of multiple antibodies. The emerging multiplexed technology allows multiple analytes to be assessed simultaneously in a single sample [4]. The use of multiplexed bead-based immunoassays to a limited number of surface-exposed proteins has recently been reported to evaluate their immunogenicity in the pediatric population which is usually challenging due to limited sensitivity of current diagnostic tests and the interference with colonization. Carriage status has been also evaluated through this platform [5, 6]. Moreover, this methodology could be useful for investigating etiological diagnosis. The multiplex bead-based platform has proved to be comparable to ELISA and detects with high sensitivity a wide range of antibody concentrations in children sera from patients suffering infections (e.g., pneumonia) [7]. In the Luminex-based methodology, the 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) reacts with the carboxyl groups on the surface of the microspheres to form an active intermediate ester (e.g., O-acylisourea) which is more stable in the presence of N-hydroxysulfosuccinimide (Sulfo-NHS). This ester compound reacts with the primary amines (NH2 groups) of for instance proteins to form an amide linkage; in this way, the coupled proteins remain covalently attached on the surface of the microsphere. Although the multiplexed immunoassay here described was applied to assess seroresponses in children with pneumonia due to *Streptococcus pneumoniae,* this methodology could be extrapolated to assess seroresponses of an endlessness number of diseases caused by several microorganisms.

The multiplex bead-based assays have significant role in public health because sero-epidemiological surveys, which are important in estimating disease burden and evaluating the efficacy of interventions, could be carried out with the simultaneous detection of multiple antigens for a given disease and the simultaneous detection of multiple diseases [8]. The development of protein-based vaccines has also renewed the interest in the serological immunoassays using surface protein-based tests. Additionally, the capacity of anti-protein responses to identify children with bacterial diseases is insufficiently validated [7]. Therefore, the use of multiple antigens could markedly improve the performance of serological assays not only in the pediatric population.

Applying this methodology, it is possible to measure the immunoreactivity of sera antibodies against a collection of surface-exposed

proteins and discriminate sero-responses between patients and healthy children for a given disease. As a result, the developed platform is an ideal alternative to determine antibody titers in a less time-consuming workflow. Moreover, it is significantly sensitive, thus allowing the detection of very low antibody titers.

2 Materials

Prepare all solutions using filtered deionized water and analytical grade reagents. Prepare all reagents at room temperature and store them at 4 °C (unless indicated otherwise) (*see* **Note 1**). Two buffer solutions contain sodium azide. Because of its high toxicity, take extra caution while handling this compound.

1. Vortex.
2. Water bath sonicator.
3. Microcentrifuge.
4. Plate and tube incubator with shaker (Eppendorf).
5. Microscope.
6. Pipettes (10 μL, 20 μL, 200 μL, 1 mL).
7. Multi-channel pipettor.
8. Pipette tips.
9. 96-well filter plates (Millipore).
10. Vacuum manifold.
11. Millipore filter plate.
12. Luminex® analyzer.
13. SeroMAP™ microspheres (Luminex® technology).
14. Tubes (1.5 mL).
15. Low protein binding tubes (1.5 mL) (USA scientific microcentrifuge tubes).
16. Cell counting grid (Kova glasstic slide).
17. Activation Buffer: 0.1 M NaH_2PO_4, pH 6.2. For 1 L solution, add 12 g of NaH_2PO_4 in a 1 L graduated glass beaker or glass flask. Dissolve in 800 mL of distilled water, adjust the pH to 6.2 with NaOH, and then fill to 1 L. Eliminate any particle in suspension by filtering the solution with sterile vacuum bottles (Millipore).
18. Filtered distilled water.
19. Sulfo-NHS. Weigh 10 mg of *N*-hydroxylsulfosuccinimide and dissolve in 200 μL of distilled water (*see* **Note 2**).
20. 2-(*N*-morpholino)ethanesulfonic acid (EDC). Hydrate 1 vial of EDC by adding 200 μL of distilled water. Take out from the refrigerator prior to hydration (*see* **Note 3**).

21. Coupling Buffer: 0.05 M MES, pH 5.0. Dissolve 9.76 g of MES in 800 mL of distilled water, adjust the pH to 5.0 with NaOH, and then fill to 1 L. Eliminate any particle in suspension by filtering the solution with sterile vacuum bottles.

22. PBS-TBN Buffer: PBS, pH 7.4, 0.1% BSA, 0.05% sodium azide, 0.02% Tween 20. Dissolve 10 tablets of PBS (Genaxxon) in 800 mL of distilled water. Adjust pH with HCl. Add and dissolve 1 g of BSA and 0.5 g of sodium azide (*see* **Note 4**). Finally, add 200 μL of Tween 20 and then fill to 1 L. Eliminate any particle in suspension by filtering the solution with sterile vacuum bottles.

23. PBS-BN Buffer: PBS, pH 7.4, 1% BSA, 0.05% sodium azide. Dissolve 10 tablets of PBS in 800 mL distilled water. Adjust pH with HCl. Add and dissolve 10 g of BSA and 0.5 g of sodium azide and then fill to 1 L. Eliminate any particle in suspension by filtering the solution with sterile vacuum bottles.

24. R-Phycoerythrin (Sigma-Aldrich) (*see* **Note 5**).

25. Hycor glasstic slide.

3 Methods

We suggest designing the analysis in the 96-well plate before measuring the antibody levels against antigens under study. Protect photosensitive microspheres from light whenever possible throughout this entire procedure.

3.1 Coupling

This procedure is carried out at room temperature with accession to a vortex and water-bath sonicator. These two steps are the key to a successful microspheres homogenate (*see* **Note 6**). First steps are carried out to activate the microsphere surface. The second part refers to the coupling antigen.

3.1.1 Microsphere Activation

1. Remove kit and all reagents from the refrigerator and allow them to equilibrate at room temperature for 20–30 min.

2. Resuspend the stock microspheres (uncoupled microspheres) by vortexing the stock microsphere vial for 20 s and then sonicate for 20 s.

3. Transfer 2.0×10^6 microspheres (160 μL) from the stock into a 1.5 mL low protein binding tube (*see* **Notes 7** and **8**).

4. Pellet the microspheres by centrifugation at $15,000 \times g$ for 5 min (*see* **Notes 2** and **9**).

5. Wash the pelleted microspheres. First, remove the supernatant with pipette and then add 100 μL of distilled water.

6. Resuspend the microspheres by vortexing 20 s and sonicating 20 s.

7. Repeat **step 4** (*see* **Notes 3** and **9**).

8. Remove the supernatant, add 80 μL of activation buffer and resuspend by vortexing 20 s and sonicating 20 s (*see* **Note 4**).

9. Add 10 μL of 50 mg/mL Sulfo-NHS to the microspheres and mix gently by vortex.

10. Add 10 μL of 50 mg/mL EDC to the microspheres and mix gently by vortex.

11. Protect microspheres from light and incubate for 20 min at room temperature with gentle mixing at 500 rpm in the dark.

12. Pellet the activated microspheres by centrifugation at $15,000 \times g$ for 2 min.

13. Perform washing of microspheres. Remove the supernatant and resuspend the microspheres in 250 μL of coupling buffer by vortexing 20 s and sonicating 20 s.

14. Pellet the microspheres by centrifugation at $15,000 \times g$ for 2 min.

15. Repeat the wash step (two total washes with coupling buffer).

16. Remove the supernatant and resuspend the activated and washed microspheres in 100 μL of coupling buffer by vortex and sonication for approximately 20 s.

3.1.2 Antigen Coupling

1. Add 10 μg of protein to 2.0×10^6 resuspended microspheres (*see* **Note 10**). Fill up to a total volume of 500 μL with coupling buffer.

2. Mix coupling reaction by vortexing 20 s.

3. Protect microspheres from light and incubate for 2 h with gentle mixing (500 rpm) at room temperature in the dark.

4. Pellet the coupled microspheres by centrifugation at $15,000 \times g$ for 2 min.

5. Remove the supernatant and resuspend the pelleted microspheres in 500 μL of PBS-TBN buffer by vortexing 20 s and sonicating 20 s.

6. Incubate for 30 min with mixing (500 rpm) at room temperature in the dark.

7. Repeat **step 4**.

8. Perform washing of microspheres. Remove the supernatant and resuspend the pelleted microspheres in 1 mL of PBS-TBN buffer by vortexing 20 s and sonicating 20 s.

9. Repeat **step 4**.

10. Repeat the wash step once more (two total washes with PBS-TBN buffer). Do not forget vortexing the microspheres 20 s and sonicating for 20 s.

11. Remove the supernatant and resuspend the coupled and washed microspheres in 400 μL of PBS-BN buffer by vortexing 20 s and sonicating 20 s.

12. Count the microsphere suspension and store coupled microspheres at 2–8 °C in the dark.

3.2 Counting

Considering that coupled microspheres could be cleared during the washing steps, concentration of coupled microspheres must be adjusted to 3000 beads/μL. Counting is carried out using a plastic chamber divided in nine big quadrants which are subdivided into nine small squares each on the microscope (3 rows × 3 columns).

1. Add 5 μL of coupled microspheres to 45 μL of PBS-BN buffer. Mix by up and down pipetting.

2. Pipette 10 μL of diluted coupled microspheres into a chamber of a Kova glasstic slide.

3. Place the chamber on the microscope and focus until you see the sharp image. Look for the first counting grid square and start to count.

4. Count the beads of each quadrant. Beads on lines between quadrants are not taken into account.

5. Total amount of counted beads is divided by 3 that is the average amount of beads in one big row. This number is the amount of beads $\times 10^4$ in beads/mL.

6. Divide by 1000 to get the amount of beads in beads/μL.

3.3 Multiplex Bead-Based Assay

This describes the semiquantitative determination of antibody levels in serum using the Luminex® technology. Coupled antigens specifically bind to the natural antibodies in the serum sample. Antigen-antibody complexes are recognized with another fluorescently labeled antibody. The Luminex® engine identifies up to 99 microspheres colors and detects the fluorescence emitted by the second antibody.

1. Select the appropriate antigen-coupled microsphere sets.

2. Resuspend the microspheres by vortex and sonication for approximately 20 s.

3. Prepare a working microsphere mixture by diluting the coupled microsphere stocks to a final concentration of 3000 microspheres of each set/well in PBS-BN buffer (*see* **Notes 11** and **12**).

4. Prewet a 1.2 μm Millipore filter plate with 100 μL/well of PBS-BN buffer and aspirate by vacuum manifold.

5. Aliquot 50 μL of each working microsphere mixture into the wells of the filter plate (*see* **Note 13**).

6. Add 50 µL of PBS-BN buffer as blank sample into two wells.

7. Add 50 µL of standard or sample to the appropriate wells. Mix the reactions gently by pipetting up and down several times with a pipettor (*see* **Notes 14–16**).

8. Cover the filter plate and incubate for 35 min at room temperature on a plate shaker (800 rpm).

9. Aspirate the supernatant by vacuum manifold.

10. Wash each well twice with 200 µL of PBS-BN buffer by gently pipetting up and down several times with a pipettor and aspirate by vacuum manifold.

11. Resuspend the microspheres in 50 µL by gently pipetting up and down five times with a multi-channel pipettor.

12. Dilute phycoerythrin-labeled anti-species detection antibody in PBS-BN buffer (1:200). Protect the phycoerythrin from light (*see* **Note 16**).

13. Add 50 µL of the diluted detection antibody into the appropriate wells of the filter plate.

14. Cover the filter plate and incubate for 35 min at room temperature on a plate shaker (800 rpm).

15. Aspirate the supernatant by vacuum manifold.

16. Wash each well once with 200 µL of PBS-BN buffer by pipetting up and down and aspirate by vacuum manifold.

17. Resuspend the microspheres in 100 µL of PBS-BN buffer by gently pipetting up and down five times with a multi-channel pipette.

18. Analyze 100 µL on the Luminex® analyzer according to the system manual (*see* **Note 17**).

19. Perform statistical analysis (*see* **Note 13**).

4   Notes

1. All the solutions should be prepared at room temperature and stored (unless indicated otherwise) at 4 °C. Before use, check for precipitation.

2. We suggest preparing the Sulfo-NHS reagent, as indicated in Subheading 2, just prior to be used. In the text, this step is also indicated.

3. Hydrate the EDC reagent as indicated in Subheading 2. Due to the unstability of the EDC reagent in the solution, it should always be stored in its original sealed packaging until needed and discarded after one use. Reconstituted EDC should never be stored and reused. Once opened, the EDC solution must be

prepared and used quickly. If performing multiple reactions, be sure to prepare all of them prior to the preparation of the EDC solution, which can be quickly added to all of the reaction tubes immediately after dissolution. In the text we suggest at which moment this reagent could be prepared.

4. Sodium azide may be fatal in contact with skin or if swallowed. This compound inhibits cytochrome oxidase. Thus extreme caution must be necessary during the process.

5. This procedure describes the detection of natural human IgG antibodies by phycoerythrinilated secondary antibodies. Of course, immunoglobulins of other subclasses can be detected as well, just by changing the specificity of the secondary antibodies.

6. If using a 1 mL stock microsphere vial, vortex the stock vial for 20 s and then sonicate for 20 s to disperse the microspheres. Alternatively, the microsphere vial can be rotated on a rotator for 15 min. However, in this procedure, we successfully used the first option.

7. The total set of microspheres distributed by Luminex Corp. is 99 different colors according to the different ratios of fluorophores. Therefore, the 2×10^6 microspheres refer to microspheres of each one of the different colors.

8. Use low-binding tubes. It has been observed that, when using normal tubes, the amount of different coupled microspheres dramatically decreases after time.

9. The procedure here described was performed using a centrifuge what is useful when multiple reactions are prepared simultaneously instead of a magnetic separator, to pellet the microspheres during wash steps. As suggested by Luminex Corp, beads can be pelleted by microcentrifugation at $\geq 8000 \times g$ for 1–2 min. In this procedure, conditions were set up as $15,000 \times g$ for 5 min.

10. The protein to be coupled must be free of sodium azide, bovine serum albumin (BSA), glycine, tris(hydroxymethyl)aminomethane, glycerol, or amine-containing additives and should be suspended in PBS, pH 7.4.

11. We suggest preparing 200 μL as an extra volume of microsphere mixture to distribute in the 96-well plate.

12. One microsphere color, with no antigen coupled to its surface, is added in each set of beads to be analyzed to measure the nonspecific binding of sample antibodies to the beads themselves.

13. Duplicate measurements are required. Quality control is carried out by calculating the coefficient of variation (CV). Measurements with CV >25% are considered invalid.

14. Special care should be taken to avoid cross contamination, when performing multiple reactions simultaneously.

15. A positive control can be prepared by pooling a group of positive human sera.

16. Dilution of sample and detection antibody must be previously optimized according to the coupled protein to the microspheres. In this procedure, sera samples were diluted 1:100 and detection antibody was diluted 1:200.

17. It is recommended to make replicate assays in different days.

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Chapter 14

Detection of Naturally Occurring Human Antibodies Against Gangliosides by ELISA

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Abstract

Gangliosides are sialic acid-containing glycolipids that have been considered attractive targets for cancer immunotherapy, based on the qualitative and quantitative changes they suffer during malignant transformation and due to their importance for tumor biology. Natural antibodies against gangliosides have been detected not only in cancer patients but also in healthy donors. The presence of these antibodies can be used as diagnostic or prognostic factor. However, these responses are difficult to detect because anti-ganglioside antibodies are usually of IgM isotype and low affinity. Enzyme Linked Immunosorbent Assay (ELISA) is an immunoassay based on the specific binding of antibodies to antigens bound to a solid phase. These antigens can be glycolipids like gangliosides. An enzyme linked to the last reactant allows the detection of specific binding through the development of color after the addition of a suitable substrate. ELISA combines the specificity of antibodies with the sensitivity of enzyme reactions. The ELISA method described herein can be used to detect antibody responses against gangliosides not only related to cancer but also to autoimmune diseases and infections, both in healthy donors, and patients, untreated or receiving specific immunotherapy.

Key words ELISA, Naturally occurring antibodies, Gangliosides

1 Introduction

Gangliosides are sialic acid-containing glycosphingolipids commonly found in cell membranes of most vertebrates [1]. These glycolipids are involved in important cellular functions, including signal transduction, regulation of cell proliferation, differentiation, and death [2–5]. The expression patterns of membrane gangliosides suffer quantitative and qualitative changes during neoplastic transformations [6–9], overexpressing gangliosides that potentiate tumor intrinsic behavior, like metastatic and immunosuppressive capacities [10–15].

In particular, *N*-glycolylated gangliosides are very attractive tumor antigens because they are not normally synthesized in human tissues due to a 92 bp deletion in the gene that encodes

the cytidine-monophosphate-*N*-acetylneuraminic acid hydroxylase (CMAH), enzyme that catalyzes the conversion of *N*-acetyl to *N*-glycolyl sialic acid (NeuGc) [16]. However, several studies have reported the presence of NeuGc in human tumors [17], probably due to the preferential incorporation of NeuGc from dietary sources in tumor tissues, with a higher rate of division in comparison with normal cells [18] and the expression of a hypoxia-induced sialic acid transporter that enhances the incorporation from the external milieu [19].

Natural antibodies have been considered to be important in the primary defense against invading pathogens, the clearance of damaged structures, dying cells and oxidized epitopes, and the modulation of cell functions [20, 21]. Also, naturally occurring antibodies could play a role in the protection against neoplastic transformation [22–24]. Despite the fact that gangliosides are poorly immunogenic, due to their self and glycolipidic nature, several reports show the presence of naturally occurring antibodies against GM2, GD2, GM1, GD1a, GD1b, GT1b, GD3, GM3, and NeuGcGM3 among others, not only in cancer patients, but also in healthy individuals [8, 25–29]. Several of the anti-ganglioside antibodies detected in cancer patients and healthy donors were able not only to recognize, but also to kill tumor cells by different mechanisms [24, 27].

Our group reported the existence of naturally occurring IgM antibodies against NeuGcGM3 in healthy donors that diminishes with increasing age [27]. To detect these antibodies we used enzyme-linked immunosorbent assay (ELISA), an immunoassay that involves the stepwise addition and reaction of reagents to a solid phase-bound substance, through incubation and separation of bound and free reagents using washing steps. The specific binding is detected using enzymes attached to the last reactants which will transform a suitable substrate generating a colored reaction product. ELISA combines the specificity of antibodies with the sensitivity of enzyme reactions [30].

ELISA was first developed in 1971 and since then has become one of the most widely used techniques in clinical and research laboratories. This immunoassay was chosen for being a specific and highly sensitive method for detection and quantification of molecules of different nature, which allows testing many samples at the same time with multiple replicates. The microtiter plate format allows testing and optimizing multiple assay conditions simultaneously.

There are several kinds of ELISA. In the Direct ELISA the antigen is attached to the solid phase and directly recognized by enzyme-labeled antibodies. In the Indirect ELISA antibodies from a particular species react with the antigen attached to the solid phase and bound antibodies are detected by the addition of an anti-species antiserum labeled with enzyme. This system offers the

advantage that any number of antisera can be examined for binding to a given antigen using a single anti-species conjugate. The assay can also be performed as a Sandwich ELISA, where an antibody previously attached to the plate captures the antigen. The antigen is then detected using antibodies from another species directly conjugated or not to an enzyme, as previously described for Direct and Indirect ELISAs [30].

The main problem associated with ELISAs for studying the antibody response against gangliosides is the high unspecific binding in the control wells where the sample is added without ganglioside coating (background), especially when the sample is serum. This problem is even more evident in the detection of natural responses against these antigens, since the serum dilutions usually used are lower than when studying responses induced by vaccination. Also, natural responses against these glycolipids are of IgM isotype and low affinity; therefore, there has to be a compromise between the conditions to achieve a clean assay without losing the signal. Several studies report efforts to standardize efficient ELISAs to detect anti-ganglioside responses [31, 32]. The method ultimately followed in our laboratory to test natural responses against gangliosides is an indirect ELISA that follows recommendations reported in the protocol proposed by Dr. Ravindranath and colleagues [31], but with several modifications detailed bellow. With this protocol we did not face background problems and the results for the independent samples were consistent when the ELISA was repeated several times.

Although we used this ELISA to detect antibodies against NeuGcGM3 and NeuAcGM3, the same protocol can be used to study the response against any ganglioside, both to monitor endogenous immune responses to early or localized disease, and the induced responses against specific therapies. The detection of these responses could be used to reinforce earlier diagnostics or as prognostic factors. Furthermore, the scope of detecting anti-ganglioside responses goes well beyond studying anti-cancer responses, since these antibodies have been associated with several diseases like celiac disease, Guillain-Barre syndrome, amyotrophic lateral sclerosis, multiple sclerosis, rheumatoid arthritis, among others [33–38]. Finally, this ELISA can also be used to determine the specificity of human antiganglioside monoclonal antibodies.

2   Materials

2.1   Reagents
1. 15 mL conical tubes.
2. 1.5 mL vials.
3. Glass containers.

4. 5 mL glass pipets.

5. Catheter vein set for blood collection.

6. ELISA plates: 96-well polystyrene microtiter plates PolySorp (Nunc, Denmark).

7. Organic solvents: Chloroform and methanol, both-HPLC gradient grade.

8. Gangliosides: Lyophilized gangliosides NeuAcGM3 and NeuGcGM3 (at least 98% purity), purified from dog and horse erythrocytes respectively, by a modification of Folch extraction method [39] (Laboratory of Vaccines from the Center of Molecular Immunology, Havana, Cuba). Both gangliosides are also commercially available.

9. Biotin-conjugated goat antihuman IgG or IgM (Jackson ImmunoResearch Laboratories, Inc., USA).

10. Alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories).

11. *p*-nitrophenylphosphate.

12. Phosphate Buffered Saline (PBS): 0.27 mol/L NaCl, 0.005 mol/L KCl, 0.016 mol/L Na_2HPO_4, 0.003 mol/L KH_2PO_4, pH 7.4.

13. Human Serum Albumin (HSA).

14. Sample buffer and blocking buffer: 0.4% HSA in PBS (v:v) (PBS-HSA 0.4%).

15. Washing buffer: 0.1% Tween 20 in PBS (v:v).

16. Diethanolamine solution: 9.7% diethanolamine (v:v), 0.185 mL of saturated $MgCl_2$, in distillated water, adjust pH to 9.8 with 5 M HCl.

17. Substrate solution: 1 mg/mL of p-nitrofenilfosfato in diethanolamine solution, pH 9.8.

Prepare fresh blocking, sample, washing buffers, and substrate solution for the assay.

2.2 Equipment

1. Biosafety hood.

2. Centrifuge with swinging bucket rotor.

3. Safety lids for centrifuge buckets.

4. 37 °C incubator.

5. 2–8 °C refrigerator or cold room

6. Vortex.

7. ELISA reader.

3   Methods

3.1   Processing Human Blood Samples

1. Extract 3 mL of blood from healthy individuals into 15 mL conical tubes.

2. Keep the blood for 30 min at 25 °C and then for 1 h at 4 °C.

3. Centrifuge the tubes at $1600 \times g$ for 10 min.

4. Separate the serum from the clot under the hood and aliquot with a micropipette. Prepare small enough aliquots to avoid freeze-thaw cycles of samples (*see* **Note 1**).

5. Store serum samples in 1.5 mL vials at −80 °C for later use.

3.2   ELISA to Detect Recognition of Gangliosides by Human Sera

1. Dissolve lyophilized NeuGcGM3 and NeuAcGM3 gangliosides in chloroform: methanol 2:1 (v:v) at 1 mg/mL to prepare the stock solutions in glass containers (*see* **Note 2**).

2. Sonicate the stock solutions during 5 min to guarantee the complete dissolution of the ganglioside.

3. Prepare gangliosides' working solutions, at 4 μg/mL, by diluting the stock solutions with methanol.

4. Coat PolySorp microtiter plates with 50 μL per well of 4 μg/mL NeuAcGM3 and NeuGcGM3 working solutions (200 ng) or just 50 μL of methanol (background detection) and allow drying at 37 °C (approximately 2 h) (*see* **Note 3**).

5. When plates are completely dry, add 200 μL per well of blocking buffer and block the plates during 2 h at 4 °C.

6. Meanwhile, thaw a human serum aliquot. Once thawed, vortex the serum thoroughly and dilute it 1:50 in sample buffer to a final volume of 500 μL (10 μL of serum for 490 μL of PBS-HSA 4%).

7. After the 2 h of blocking, remove the blocking buffer manually (inverting the plate over the sink) or using a multichannel pipet and drain the plates carefully over a filter paper to remove remaining buffer. No washing is needed after this step. Do not let the plate dry and immediately start the next step. If there are several plates, remove the blocking solution just before adding the samples to each one.

8. Vortex the diluted sera and add 50 μL per well in triplicate into wells coated with NeuGcGM3, NeuAcGM3, or methanol. Add sample buffer to three wells coated with each ganglioside or methanol. These last will be blank wells. Incubate the plates overnight at 4 °C. Incubating overnight at 4 °C instead of 2 h at 37 °C decreases significantly the background.

9. Wash the plates six times manually with a multichannel pipet (200 μL per well) or using an automated washer, with wash buffer. After the last wash, drain the plates carefully over a filter paper to remove remaining buffer. Do not let the plate dry and

immediately start the next step. If there are several plates, remove the washing solution just before the next step. This applies for all washing steps.

10. Add 50 μL per well of the secondary antibody, biotin-conjugated goat anti-human IgG or IgM diluted 1:10,000 in sample buffer and incubate the plates during 1.5 h at room temperature.

11. Wash as described in **step 9**.

12. Add 50 μL per well of alkaline phosphatase conjugated streptavidin diluted 1:20,000 in sample buffer and incubate the plates for additional 1.5 h at room temperature.

13. Wash as described in **step 9**.

14. Finally, add 100 μL of the substrate solution to the plates. The reaction develops optimally at 45 min.

15. Measure the absorbance at 405 nm in an ELISA reader.

16. To consider that a serum sample has a positive reaction to a particular ganglioside, values of absorbance have to be ≥ 0.25 and at least three times the absorbance value obtained by incubating the serum in wells containing no ganglioside (only methanol dried on the wells). The optical densities (ODs) of the blanks should be lower than 0.1.

17. When the assay involves working with more than one plate, an internal control antibody (in our case an anti-NeuGcGM3 antibody) should be used to normalize the results obtained in all the plates and to avoid differences due to the delay between plates. In this case, the results will be reported as ELISA Units, which will be calculated as:

ELISA Units = Absorbance of Human sample/Absorbance of Control Ab.

4 Notes

1. According to unpublished data by Ravindranath et al. [31], most IgM antibodies against major gangliosides (GM2, GD3, GD1a, and GD1b) are not affected by five freeze-thaw cycles.

2. Both solvents, especially methanol, should not get moisture, since this strongly increases the background of the test. We usually decant both solvents in smaller glass containers in order to avoid introducing pipets into the original organic solvent bottles and the continue opening. For the same reason the humidity of the room should be low and the temperature kept at or below 25 °C. Prepare the chloroform: methanol 2:1 stock solution and later add this solution to dry powdered ganglioside using glass pipets instead of plastic tips. The same applies when preparing the working solutions from the stock

solutions, whenever possible. When the volumes are small we recommend using glass Microliter Syringes, for example from Hamilton, Switzerland. Since the working solution is based fundamentally in methanol, which is less corrosive, we use pipets and plastic tips to coat the plates.

3. We usually used a volume of 50 µL of ganglioside working solutions to coat the plates. Lately, we have tried 25 µL at 20 µg/mL (200 ng), which dries much faster. The results are the same at least with NeuAcGM3 and NeuGcGM3.

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Chapter 15

Evaluating the Impact of Natural IgM on Adenovirus Type 5 Gene Therapy Vectors

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Abstract

Natural IgM antibodies have an innate ability to recognize many viruses and viral-based gene therapy vectors. Naive mice have natural IgM antibodies that bind to adenoviruses, and these antibodies can profoundly affect the biodistribution and efficiency of gene delivery by adenovirus type 5 vectors. Here, we present protocols for isolating IgM from mouse serum, for assaying the concentration and adenoviral reactivity of mouse IgM, and for evaluating how natural antibodies and complement can synergize to neutralize adenovirus vectors.

Key words Natural IgM antibodies, Adenovirus type 5 vectors, Mouse, Complement, Gene delivery, Virus

1 Introduction

Adenoviral vectors are the most widely used class of gene therapy vectors in clinical trials [1]. Adenoviral infections are common during childhood, which leads to a high seroprevalence of IgG antibodies against common serotypes such as adenovirus type 5 (Ad5). These acquired antibodies can neutralize vectors and greatly impair gene therapy [2–5]. However, it is less well appreciated that natural antibodies can also inhibit viral vectors even in the absence of prior exposure to the virus [6].

When non-replicating Ad5 vectors are injected intravenously, they are rapidly cleared from the circulation by macrophages of the reticuloendothelial system, especially Kupffer cells (KCs) in the liver [7, 8]. This clearance of vectors by KCs and other macrophages reduces gene delivery and causes pathology, including rapid necrosis of KCs [9–11]. The mechanism of vector clearance by KCs does not involve typical virus-receptor interactions, since KCs can efficiently accumulate mutant Ad5 vectors that have lost the ability to bind to their normal cellular receptors (integrins and the coxsackie and adenovirus receptor) [12]. Instead, KCs use broadly

reactive mechanisms to remove Ad5 from the circulation, including scavenger receptors, natural antibodies, and complement [13]. Natural antibodies have a major inhibitory effect on gene delivery by Ad5 vectors—when mice are injected intravenously with Ad5 vectors, KCs in wild-type mice accumulate much higher amounts of vector than KCs in antibody-deficient mice [13]. The inhibitory effects of natural antibodies are driven by IgM rather than IgG; natural IgM antibodies bind directly to Ad5 in ELISAs [13–15], and injection of natural IgM into antibody-deficient mice is sufficient to inhibit Ad5 liver transduction [14, 16].

Mouse strains differ genetically in their levels of natural IgM—for example, BALB/c mice have approximately twofold higher circulating IgM concentrations than C57BL/6 mice [17]. We have found a strong negative correlation between IgM concentration and the efficiency of gene delivery to the liver with Ad5 vectors [15]. When natural antibody concentrations in mice are lowered either genetically (using knockout mouse strains) or experimentally (by splenectomy), the efficiency of Ad5 liver transduction increases markedly [14, 15, 18]. Interestingly, some vectors derived from non-Ad5 serotypes are less susceptible to natural IgM, and it has been shown that Ad6 vectors are less sensitive to IgM because of differences in the major capsid protein hexon [16].

IgM activates the classical complement pathway very efficiently [19], and binding of natural IgM to Ad5 can activate complement [20, 21]. Interestingly, the Ad5 hexon protein specifically binds to another plasma protein, coagulation factor X (FX), resulting in a layer of FX that covers most of the capsid surface [22–24]. We have found that FX protects Ad5 both in vitro and in vivo from the effects of natural IgM and complement. In vitro, Ad5 vectors are stable in serum as long as the vector can bind FX. When Ad5 vectors are unable to bind FX, however, IgM induces strong activation of the classical complement pathway, eventually leading to vector neutralization [20]. IgM by itself has little or no ability to neutralize Ad5; rather, neutralization depends on both IgM and complement. The ability of FX to "shield" Ad5 vectors has consequences in vivo as well. FX protects Ad5 vectors from complement in wild-type mice, but FX is not essential for protecting Ad5 vectors in knockout mice that lack natural antibodies or that lack the classical complement proteins C1q or C4 [20].

Because we have found commercially available mouse IgM to be expensive and insufficiently pure, the first protocol describes how to isolate highly pure IgM from mouse serum. The next two protocols describe ELISAs that measure the total concentration of IgM and the concentration of Ad5-binding IgM. The Ad5-binding ELISA can be easily adapted to measure binding of IgM to any other antigen simply by changing the protein that is attached to the plate. Finally, the last protocol explains how to evaluate the stability of Ad5 vectors in mouse serum. Mouse complement is highly labile

and challenging to work with, but we have optimized this protocol to produce reliable results. This protocol can easily be adapted to use serum from other species, including guinea pig, rat, and human [20, 25, 26], as long as the serum is collected and stored in a manner that preserves complement activity.

2 Materials

2.1 IgM Purification from Mouse Serum

1. CaptureSelect™ IgM Affinity Matrix (ThermoFisher Scientific) (*see* **Note 1**).
2. Phosphate buffered saline (PBS).
3. Elution buffer: 0.1 M glycine–HCl, pH 3.0, 150 mM NaCl (*see* **Note 2**).
4. Neutralization buffer: 1 M Tris–HCl, pH 9.0.
5. 20% Ethanol.
6. Large syringe with luer lock.
7. Syringe pump, for example Harvard Apparatus PHD 2000 infusion pump (*see* **Note 3**).
8. Empty 5 mL chromatography column with luer-lock connections at both ends (MoBiTec) (*see* **Note 4**).
9. Pressure tubing with male/female luer lock connections (MedOne).
10. Three-way large bore stopcock with rotating male luer lock adapter (Baxter).
11. Centrifugal filter unit with 100 kDa molecular weight cutoff (Millipore Amicon Ultra-15).
12. BALB/c mice.
13. Ketamine and xylazine. For the anesthetization of mice.
14. Pyrogen-free 1.5 mL polyethylene tubes.

2.2 IgM ELISAs

1. Clear flat-bottom 96-well high binding microplate (Thermo Scientific).
2. Mylar plate sealer (Thermo Scientific).
3. Coating buffer (CB): 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6. To enable the long-term storage of this and other buffers, the preservative Proclin-200 may be added at a final concentration of 0.1% if desired.
4. Blocking buffer: PBS containing 2% globulin-free bovine serum albumin (BSA).
5. Wash buffer (WB): PBS containing 0.05% Tween-20.
6. Dilution buffer: PBS containing 2% globulin-free BSA and 0.05% Tween-20.

7. IgM Standard: Mouse IgM (Rockland).

8. Purified Ad5 (e.g., American Type Culture Collection) or Ad5 vector (*see* **Note 5**).

9. Capture antibody: Goat anti-mouse IgM antibody, affinity purified, cross-absorbed against mouse IgG and IgA (Southern Biotech).

10. Detection antibody: Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM, affinity purified, cross-absorbed against mouse IgG and IgA (Southern Biotech).

11. Tetramethylbenzidine (TMB) substrate reagent set (BD Biosciences).

12. Stopping solution: 1 M H_3PO_4.

2.3 Neutralization of Adenovirus by Mouse Serum

1. Clear Flat-Bottom 96-well cell culture plate (Corning).

2. Dulbecco's Modified Eagle Medium (DMEM).

3. Fetal bovine serum, heat inactivated at 56 °C for 30 min.

4. Gentamicin Sulfate (Cellgo).

5. 293 cells (American Type Culture Collection).

6. Ad5 vectors with luciferase expression cassette (*see* **Note 6**).

7. Serum separator Microtainer tube (BD Biosciences).

8. Globulin-free BSA.

9. Lysis buffer: 25 mM Tris phosphate-H_3PO_4, pH 7.6, 2 mM EDTA, 10% glycerol, 1% Triton X-100.

10. Luciferase Assay System (Promega).

11. Micro BCA protein assay kit (Thermo Scientific).

3   Methods

3.1 Purification of IgM from Mouse Serum

1. Anesthetize adult BALB/c mice (*see* **Note 7**) using an appropriate method, for example intraperitoneal injection of 150 mg/kg ketamine and 30 mg/kg xylazine.

2. Open the chest and collect blood from the heart using a needle and syringe, and immediately expel blood into pyrogen-free 1.5 mL polyethylene tubes.

3. Allow the blood to clot at room temperature for 30 min. Centrifuge the clotted blood at 3000 × *g* for 5 min at 4 °C.

4. Pool the serum and discard the clot. Serum can be frozen for later use, without any negative impact on IgM.

5. Pack an appropriate volume of IgM affinity matrix into an empty column (e.g., 4 mL of matrix to purify IgM from 10 mL of BALB/c serum).

6. Fill a syringe with PBS and connect it to the pressure tubing and three-way stopcock.

7. Prime the tubing and stopcock with PBS. Avoiding bubbles, connect the stopcock to the top of the column.

8. Place the syringe in the syringe pump and equilibrate the column with 5 column volumes of PBS at a rate of 0.5 mL/min.

9. Dilute the serum 1:1 with PBS. To evaluate the yield of the purification, a small sample may be retained for later measurement by IgM ELISA.

10. Load the diluted serum into a second syringe, connect to pressure tubing and the three-way stopcock, and load the diluted serum onto the column at a rate of 0.1 mL/min (*see* **Note 8**). If desired, the flow-through can be saved for later purification of natural IgG or other serum proteins, or to assess whether any IgM failed to bind to the matrix.

11. Wash the column using 10 column volumes of PBS, starting with a speed of 0.1 mL/min for the first volume and then 0.2 mL/min for the remaining 9 volumes.

12. Elute the IgM using 5 column volumes of elution buffer at a speed of 0.2 mL/min.

13. Collect the entire eluate, without fractionation. To store the column, wash with 5 volumes of 20% ethanol, cap the column, and store at 4 °C.

14. Neutralize the eluate using approximately 0.01 volumes of neutralization buffer. A pH indicator strip can be used to confirm that the eluate has been neutralized to a pH of between 7 and 8.

15. Transfer the neutralized eluate into the 100 kDa centrifugal filter (*see* **Note 9**). Centrifuge at $1500 \times g$ at 4 °C to reduce the volume to 50%, then add an equal volume of PBS.

16. Repeat adding PBS and centrifuging several more times to exchange the buffer, and then perform a final spin to achieve the final desired volume. If desired, the IgM can be sterilized by passing through a 0.45 μm filter.

17. Determine the IgM concentration by ELISA (*see* Subheading 3.2) or by absorbance at 280 nm [14].

18. Determine the IgM purity by SDS-PAGE.

19. Aliquot the IgM and store at −80 °C. Freeze-thawing may cause aggregation and is not recommended.

3.2 ELISA Quantitation of Total IgM

1. Dilute the capture antibody to 0.5 μg/mL in CB, and add 100 μL per well. Seal the plate and incubate at 4 °C overnight.

2. Wash the plate with WB and add 200 μL of blocking buffer per well. Seal and incubate at room temperature for at least 1 h.

3. Prepare a twofold dilution series of IgM in dilution buffer, ranging from a concentration of 12.5–800 ng/mL. Dilute test samples to an appropriate concentration in dilution buffer.

4. Wash the plate three times with WB. Add 100 μL of each standard and test sample to duplicate wells. Seal the plate and incubate for 2 h at room temperature.

5. Wash the plate three times with WB. Dilute the detection antibody 1:16,000 with dilution buffer, and add 100 μL per well. Seal the plate and incubate for 1 h at room temperature.

6. Warm the TMB substrate reagent set to room temperature. Just before use, mix equal volumes of substrate solutions A and B. Wash the plate three times with WB. Add 100 μL of the TMB mixture per well. Incubate for 10 min at room temperature.

7. Add 50 μL of stop solution to each well. Within 30 min, read the absorbance using a 96-well spectrophotometer set at 450 nm.

8. Fit the standard curve using an appropriate function (such as four parameter logistic curve), and calculate the concentration of the test samples.

3.3 ELISA Quantitation of Mouse IgM Binding to Adenovirus

1. Dilute adenovirus to 3.0×10^{10} vector particles/mL in CB (*see* **Note 10**). Add 100 μL per well. Prepare an equal number of wells using 100 μL of CB, for later subtraction of background binding (*see* **Note 11**). Seal the plate and incubate at 4 °C overnight.

2. Wash the plate with WB and add 200 μL of blocking buffer. Seal the plate and incubate at room temperature for at least 1 h.

3. Wash the plate three times with WB. Dilute the test samples (mouse IgM or mouse serum) with dilution buffer to bring the IgM concentration into a range of 2–125 μg/mL (*see* **Note 12**). Add 100 μL to each well in duplicate. Seal the plate and incubate for 2 h at room temperature.

4. Wash the plate three times with WB. Dilute the detection antibody 1:16,000 with dilution buffer, and add 100 μL per well. Seal the plate and incubate for 1 h at room temperature.

5. Warm the TMB substrate reagent set to room temperature. Just before use, mix equal volumes of substrate solutions A and B. Wash the plate three times with WB. Add 100 μL of the TMB mixture per well. Incubate for 10 min at room temperature.

6. Add 50 μL of stop solution to each well. Within 30 min, read the absorbance using a microplate spectrophotometer set at 450 nm. Because background from IgM binding to uncoated wells may be substantial, the absorbance of corresponding control wells (CB-treated) should be subtracted from the absorbance of each adenovirus-coated well.

3.4 Adenovirus Neutralization by Mouse Serum

1. One day in advance, seed 293 cells in 96-well tissue culture plates at a density of 1.0×10^4 cells/well. For the culture medium, use DMEM with 10% FBS and 0.1% gentamicin sulfate, or other suitable medium.

2. Incubate the cells at 37 °C at a CO_2 concentration of 5%.

3. Anesthetize mice and collect blood from the heart using a needle and syringe, and immediately expel blood into serum separation Microtainer tubes.

4. Let the blood clot at room temperature for 30 min. Centrifuge the clotted blood at $12,000 \times g$ for 5 min at 4 °C. Separate and pool the serum, and hold on ice (*see* **Note 13**).

5. Prepare neutralization mixtures on ice by adding adenovirus vector to mouse serum, with a final reaction volume of 50 μL. The final concentration of vector in each mixture should be 2.0×10^{10} vp/mL, and we recommend that the concentration of serum should be at least 80% (the classical complement pathway is sensitive to dilution). Negative control samples consist of vector mixed with serum-free DMEM and 2% globulin-free BSA (*see* **Note 14**).

6. Incubate vector/serum mixtures and controls for 30 min at 37 °C and then place on ice.

7. Dilute vector/serum mixtures by 2000-fold in serum-free DMEM. Rinse the 293 cells with serum-free medium, and add 100 μL of diluted vector/serum mixtures to triplicate wells for 2 h at 37 °C. Replace the inoculum with DMEM medium containing 2% heat-inactivated fetal bovine serum.

8. After approximately 16 h, rinse the cells with 300 μL of PBS (*see* **Note 15**). Add ice-cold lysis buffer at 100 μL per well and shake the plate at 300 rpm for 10 min. Centrifuge the plate at $1000 \times g$ for 10 min at 4 °C. Harvest supernatant for determination of luciferase activity and protein concentration following the kit manufacturer's instructions. Normalize the luciferase activity by the protein concentration in each sample.

4   Notes

1. The CaptureSelect™ IgM affinity matrix is coupled with an antibody against a unique domain from the IgM Fc region (of human, mouse, and rat IgM). The manufacturer reports that this antibody has no cross-reaction to IgG or IgA. We have found that mouse IgM purified from this matrix contains no detectable mouse IgG. According to the manufacturer, the IgM binding capacity is greater than 2.5 mg of human IgM per mL of matrix, and we have found a similar binding capacity

for mouse IgM. We typically achieve an IgM yield of about 50%, relative to the initial amount of IgM in the serum.

2. The elution buffer is modified from the manufacturer's protocol (increased salt) to improve the yield of mouse IgM from serum.

3. Gravity flow is too fast and results in low yield. This protocol describes how to purify IgM using a syringe pump, but an FPLC system may be used instead.

4. If using an FPLC system, an appropriate column such as the GE Life Sciences Tricorn Empty High Performance column can be used to pack the matrix. Serum should be prefiltered and the flow speeds should be the same as indicated for the syringe pump protocol.

5. Adenoviruses and adenoviral vectors should be handled at an appropriate biosafety level.

6. Adenovirus vectors that express other marker proteins besides luciferase (such as β-galactosidase or fluorescent protein) may be used if the detection method is appropriately modified.

7. We have found that commercially collected mouse serum is sometimes contaminated with endotoxin, leading to endotoxin-contaminated IgM, so we obtain serum from mice in our lab. We prefer BALB/c mice because their serum has higher IgM concentrations than other mouse strains (about 1 mg/mL) [15, 17]. Approximately 0.8 mL of blood can be collected from the heart of each mouse.

8. A slow loading speed is critical for a good yield of IgM.

9. The 100 kDa filter not only allows buffer exchange, but will also remove any small contaminating serum proteins. To reduce the risk of IgM precipitation, always control the volume so that the IgM concentration does not exceed an estimated 3 mg/mL.

10. Adenovirus vector particles can be measured spectrophotometrically by the OD_{260}/SDS method [27].

11. IgM at high concentrations shows substantial binding even to BSA-coated wells, and it is essential that each adenovirus-coated well have a corresponding background control well.

12. To allow comparison among different experiments, it is advisable to establish a single lot of IgM to use as a reference material that can be run on every plate.

13. Mouse complement is very unstable [28]. Mouse serum must be freshly prepared on the day of each experiment and kept on ice until ready to use. Serum from other species such as humans is more stable and can be stored frozen for later use.

14. The inclusion of protein (2% BSA) in control DMEM samples helps to prevent nonspecific decrease in vector infectivity during the incubation of control samples. In serum samples, the impact of complement can be evaluated by inactivating the complement system by pretreating serum with heat (56 °C for 30 min), by adding EDTA (10 mM final concentration in the vector/serum mixture), or by pretreating serum with cobra venom factor (12 U/mL at 37 °C for 1 h).

15. After virus transduction, the cells will not be attached to the plate tightly. Rinse the cells gently. It is essential to rinse the cells with a PBS volume of 300 μL to remove all proteins present in the culture medium; otherwise, the protein concentration may be variable or inaccurate.

Acknowledgments

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INDEX