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Somatic hypermutation and affinity maturation analysis using the 4-hydroxy-3-nitrophenyl-acetyl (NP) system

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Running head: NP-system in analysis of affinity maturation

Abstract

Somatic hypermutation of immunoglobulin variable region (IgV) genes and affinity maturation of the antibody response are the hallmarks of the germinal center (GC) reaction in T cell-dependent immune responses. Determining the consequences of the experimental manipulation of the GC response on somatic hypermutation and affinity maturation requires the availability of a system that allows measuring these parameters. Immunization of mice of the C57/Bl6 genetic background with the hapten 4-hydroxy-3-nitrophenyl-acetyl (NP) coupled to a carrier protein leads to the predominant usage of one particular IgV heavy chain gene segment, V186.2, among the responding B cells. Moreover, a specific somatic mutation in codon 33 of V186.2 that leads to a tryptophan to leucine amino-acid exchange increases the affinity of the corresponding antibody by ~10-fold, thus representing a molecular marker for affinity maturation. In addition, due to the simplicity of the antigen and the virtual absence of NP-specific plasma cells prior to immunization, NP-based immunizations represent ideal tools to quantify the plasma cell response by measuring NP-specific antisera by ELISA and the generation of NP-specific plasma cells by ELISPOT analysis. We here describe approaches to i) measure the anti-NP plasma cell response by ELISA and ELISPOT analysis, and to ii) amplify and sequence V186.2 rearrangements from GC B cells and plasma cells to determine the level of somatic hypermutation and the extent of affinity maturation in the anti-NP response.

Key Words

B cell; plasma cell; Ig variable region gene; germinal center; somatic hypermutation; affinity maturation; T cell-dependent; T cell-independent

1. Introduction

Antigen-specific memory B cells and plasma cells are generated during the germinal center (GC) reaction of T cell-dependent immune responses in secondary lymphoid tissues (1, 2). Within the GC microenvironment, antigen-activated B cells undergo somatic hypermutation of the rearranged IgV genes with the aim to generate high-affinity antibodies that effectively bind to the invading pathogen, resulting in its elimination. In a defined area within the GC called dark zone, the rapidly proliferating 'dark zone' cells hypermutate their IgV genes and then differentiate into 'light zone' cells that are positively selected for improved antigen-binding in the GC light zone (2-5). Selected light zone B cells recirculate to the dark zone to undergo additional rounds of mutation and selection to further improve antigen-affinity before they eventually differentiate into memory B cells or plasma cells that exit the GC. The descendants of the GC reaction thus carry somatically mutated IgV genes and have often switched from IgM to other Ig classes which have different effector functions.

Understanding the molecular mechanism of the GC response is critical for the development of improved vaccines against microorganisms. Thus, for studies aimed at manipulating the GC response, which e.g. may include the deletion or overexpression of GC-associated genes or the use of particular adjuvants, it is imperative to have tools that allow the investigation of the processes that underlie the generation of antigen-specific memory B cells and plasma cells, i.e. somatic hypermutation and antibody affinity maturation. In C57/Bl6 mice, the immune response against the hapten 4-hydroxy-3-nitrophenyl-acetyl (NP) coupled to a carrier protein frequently results in a specific, affinity-enhancing hypermutation in codon 33 of the V186.2 gene segment that leads to an amino acid exchange resulting in a ~10-fold increase in affinity against NP (6-8), allowing for the generation of NP-specific GC B cells, memory B cells

and plasma cells to be tracked at the molecular level. The resulting antibodies are mostly of the IgG1 class (6-8). The characterization of the B-cell response against NP at the molecular level by several groups (9-11) provided the baseline for numerous studies on the extent of affinity maturation in situations where certain genes were either knocked out in the mouse germ-line or conditionally deleted in GC B cells (12-16).

By immunizing mice with NP coupled to a carrier protein such as chicken gammaglobulin (CGG) or keyhole limpet hemocyanin (KLH), the characteristics and dynamics of the immune response against the hapten in the presence or absence of a certain signaling pathway or transcription factor, or a specific treatment, can be investigated by i) determining the somatic hypermutation frequency in V186.2 gene rearrangements, ii) the fraction of B cells with the affinity-enhancing tryptophan to leucine amino-acid exchange, iii) the quantity of NP-specific serum IgG1 secreted by GC-derived plasma cells by ELISA, and iv) the frequency of plasma cells in lymphoid tissues by ELISPOT analysis. The use of the NP system is not confined to T cell-dependent antibody responses, as the coupling of NP to lipopolysaccharide (LPS) or the polysaccharide Ficoll allows the study of the plasma cell response in T cell-independent responses type I and II, respectively, by ELISA.

2. Materials

2.1 Immunization

- Sterile phosphate-buffered saline (PBS).
- NP-KLH (e.g. NP_{28}-KLH, Biosearch Technologies; of note, the number of NP molecules conjugated to KLH varies among batches).

- For T-independent immunizations (Notes 1), NP-LPS and NP-AECM-FICOLL (Biosearch Technologies).
- Freud's adjuvant complete.
- Freud's adjuvant incomplete.
- 1 ml syringes.
- Needles (18G1½ and 25G$^{5}/_{8}$).
- Vortex.
- Sonicator.

2.2 Acquisition of blood samples and serum preparation

- Razor blade or scalpel.
- 1 ml or 2 ml syringes.
- 21G1 needles.
- 1.5 ml collection tubes.
- Centrifuge.

2.3 ELISA

- 96-well immune plates (Thermo Fisher Scientific).
- 96-well culture plates.
- Parafilm.
- PBS.
- 2% fetal bovine serum (FBS) in PBS.
- Wash buffer: PBS containing 0.05% Tween-20.
- Capture antibody: anti-mouse Ig(H+L) (Southern Biotec).

- NP-bovine serum albumin (BSA) with low and high hapten coating (e.g. NP_9-BSA and NP_{25}-BSA).
- Mouse Ig (e.g. IgM, IgG1).
- Detection antibody: alkaline phosphatase (AP)-conjugated anti-mouse Ig (e.g. IgM, IgG1).
- PNPP (p-nitrophenylphosphate; Southern Biotec).
- Substrate buffer (500 ml): ddH_2O + 24.5 mg $MgCl_2x6H_2O$ + 48 ml diethanolamine; adjust pH to 9.8 with 5N HCl.
- Microplate reader.

2.4 Sample generation from mouse tissues following NP immunization

- PBS/0.5% BSA
- Glass slides; alternatively 40 µm cell strainer, 50 ml falcon tube and plunger of a 5 ml syringe.
- 10 ml syringes.
- 22G1 needles.
- RBC lysis buffer (e.g. Red Blood Cell Lysis HybriMax; Sigma Aldrich); alternatively, prepare lysis buffer: ammonium chloride (NH_4Cl) 0.15 M, 8.29 g per l; potassium bicarbonate ($KHCO_3$) 10 mM, 1 g per l; EDTA 0.1 mM, 0.037 g per l; H_2O, 1 l; filter solution with 0.45 µm filter, store at 4ºC.
- 15 ml Falcon tubes.
- Refrigerated centrifuge.
- Optional: cell counter (e.g. Countess, Invitrogen).

2.5 ELISPOT

- 96-well filtration plates (Millipore, Cat# MSIPS4510).
- 96-well culture plates.
- Plastic wrap.
- 0.2 μm filter.
- 35% ethanol (diluted in molecular grade H_2O).
- Sterile PBS.
- RPMI culture medium supplemented with 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin and 0.1% β-mercaptoethanol.
- Wash buffer: PBS containing 0.05% Tween-20.
- PBS/2% BSA.
- NP-BSA with high hapten coating (e.g. NP_{25}-BSA).
- Detection antibody: AP-conjugated anti-mouse IgG1.
- Nitro blue tetrazolium chloride-5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Roche).
- NBT/BCIP substrate buffer: 0.1 M Tris + 0.1 M NaCl + 0.05 M $MgCl_2$; pH 9.5.
- Optional: cell counter.
- Microplate reader (alternatively, colonies can be counted using a magnifying glass and pictures of wells can be taken with a camera that has a macro function).

2.6 Isolation of B-cell subsets from immunized mice by FACS

- RBC lysis buffer (see above).
- PBS/0.5% BSA.

- Glass slides; alternatively 40 μm cell strainer, 50 ml falcon tube and plunger of a 5 ml syringe.
- 15 ml Falcon tubes.
- MidiMACS Separator (Miltenyi Biotech).
- MidiMACS Separation Units (columns) (Miltenyi Biotech).
- 'Untouched' B-cell isolation kit (Miltenyi Biotech).
- Antibodies: B220-PercP, CD95-PE, CD138-PE (conjugates available from various companies), PNA-biotin (Vector Laboratories), streptavidin-FITC (or streptavidin-APC if mouse cells are GFP^+ or YFP^+).
- Fluorescent-activated cell sorter (FACS; e.g. provided by the institution's staff-operated Flow Cytometry Core Facility).
- 15 ml, 5 ml or 1.5 ml collection tubes.
- Collection buffer: PBS/BSA with 20% fetal bovine serum (FBS).
- Refrigerated centrifuge.

2.7 Sequencing of V186.2 gene rearrangements amplified from RNA

- TRIzol Reagent (Invitrogen).
- 26G½ needles and 1 ml syringes.
- Chloroform.
- Isopropanol.
- Glycogen.
- DEPC-treated water.

- UV spectrometer (e.g., NanoDrop ND-8000 Spectrophotometer; NanoDrop Technologies).
- cDNA synthesis kit (e.g. from Invitrogen or an equivalent kit).
- PCR thermocycler.
- PCR tubes.
- dNTPs.
- Gene-specific primers (Table 1).
- Taq polymerase and reagents for PCR amplification.
- Gel electrophoresis unit with appropriate buffer.
- Agarose and agarose plates.
- Ethidium bromide.
- Gel imaging system.
- TA-cloning vector; pGEM-TEasy (Promega).
- Competent bacteria (DH5α).
- SOC medium.
- Ampicillin.

3. Methods

3.1 Primary and secondary immunization with NP

The general approach is summarized in Fig. 1.

3.1.1 Preparation of a NP-KLH emulsion

1. Dissolve NP-KLH in sterile PBS at a concentration of 1 mg/ml.

2. For primary immunizations, NP-KLH is mixed with complete Freud's adjuvant at a 1:1 ratio (for the use of an alternative adjuvant, alum, see Notes 2). Prepare 2.5–3x of the volume needed to ensure sufficient immunization emulsion for all experimental mice.
3. Vortex the mixture for 10 min and subsequently sonicate 3x for 5 min at 30 sec intervals.
4. For secondary challenges, mix NP-KLH at a 1:5 ratio with sterile PBS. The diluted NP-KLH solution is then mixed with incomplete Freud's adjuvant at a 1:1 ratio and processed as described.
5. For T-independent immunizations, see Notes 1.

3.1.2 Immunization

1. Fill a 1 ml syringe with the NP-KLH emulsion by using a 18G1½ needle. Use the same syringe for all mice. If immunizing many mice, fill as few syringes as possible.
2. Remove the needle and compress the mixture by drawing the punch up and down several times to remove the air.
3. For injection, attach a 25G$^{5}/_{8}$ needle and intraperitoneally inject 200 μl per mouse.

3.2 Detection of NP-specific Ig by ELISA

3.2.1 Collection and preparation of serum from immunized mice

1. Prior to and at different time-points following NP immunization (7 days before immunization, day 14 and day 28 after immunization), take a few drops of blood from the tail vein of the mice using a razor blade or scalpel or by an alternative

IACUC-approved method from live mice and collect into 1.5 ml collection tubes (important: no addition of heparin). At the final time point, withdraw peripheral blood (any volume from a drop to several hundred µl is fine) with a 21G1½ needle and a 1 or 2 ml syringe from the heart of sacrificed mice and transfer to a 1.5 ml reaction tube.

2. Keep reaction tube at RT for the remainder of the day and at 4ºC o/n to allow clotting.
3. Centrifuge at 12,000xg for 5 min and carefully transfer the clear supernatant (serum) to a new 1.5 ml reaction tube.
4. Repeat the process to remove any clotted material and store serum at –20 ºC until use.

3.2.2 Detection of NP-specific Ig

1. Coat one or more 96-well immune plates with 50 µl per well of 2.5 µl/ml NP_9-BSA, and the same amount of plates with NP_{25}-BSA diluted in PBS (the latter is necessary only if one plans to determine also the specificity of the secreted antibodies for an affinity maturation analysis; see 3.2.2.12 below). In doing so, skip the first row of each plate and coat instead with 2.5 µl/ml capture antibody diluted in PBS to later generate a standard curve. Seal the plates with parafilm and incubate over night at 4°C. Discard the solution and wash the wells 3x with 200µl wash buffer.
2. Apply 200µl of PBS/2% FBS per well, seal the plates with parafilm and incubate at room temperature for at least 1 hour.
3. Discard the solution and wash the wells 3x with 200 µl wash buffer.

4. In the first row of each plate, generate a standard curve (of e.g. IgM, IgG1) with each 50 µl of 400 - 200 - 100 - 50 - 25 - 12.5 - 6.25 - 0 ng/ml.
5. In a separate 96-well plate, generate 1:2 serial dilutions in PBS/2% FBS of each serum sample, starting with a 1:8,000 dilution and ending with a 1:64,000 dilution. Do every condition in duplicate. Add 50 µl of each sample dilution to one test plate coated with NP_9-BSA, and one test plate coated with NP_{25}-BSA (use the same order of samples and dilutions on both plates!). Seal the plates with parafilm and incubate for 2 hours at room temperature.
6. Discard the samples and wash the wells 3x with 200 µl wash buffer.
7. Add 50 µl of detection antibody diluted 1:2,000 in PBS/2% FBS to each well. Seal the plates and incubate at room temperature for 1 hour.
8. Discard the solution and wash the wells 5x with 200 µl wash buffer.
9. Dissolve one PNPP 5 mg tablet in 5 ml substrate buffer and apply 50 µl to each well. Incubate at RT for 10-30 min, depending on the intensity of color development.
10. Read the plates at 405 nm OD. Save the 'raw data' as well as the 'blanked data' and 'calculated concentrations' in an Excel data sheet.
11. For analysis of the amount of secreted NP-specific antibodies, plot the standard curve and include all sample values of the NP_9-BSA-coated plate that lie within the linear range of the standard curve, indicated by similar Ig concentrations at different sample dilutions. An average of at least two different dilutions in duplicate is recommended for analysis.

12. To estimate the specificity of the secreted antibodies, analyze the NP_{25}-BSA-coated plate in the same way as described above, and calculate the ratio of high-affinity (NP_9-BSA):total NP-specific antibodies (NP_{25}-BSA) for each sample and dilution in the linear range of the standard curve. If for example in a hypothetical knockout genotype the NP_9/NP_{25} ratio was lower compared to the proficient controls, this would indicate that affinity maturation in this genotype is impaired.
13. For T-independent immunization, see Notes 1.

3.3 Detection of antibody secretion by ELISPOT

All steps on day 1 and day 2 have to be performed under the hood to keep the plates sterile. Do every condition in duplicate. The general approach is summarized in Fig. 1.

3.3.1 Preparation of the plates (day 1)

1. Pre-wet each well of a filtration plate with 15 µl of 35% ethanol for a maximum of 1 min.
2. Wash each well of the plate 3x with 200 µl sterile PBS.
3. Coat each well with 100 µl of 5 mg/ml NP-BSA (e.g. NP_{25}-BSA) diluted in PBS. Wrap the plate tightly in plastic wrap and incubate at 4°C o/n.

3.3.2 Sample preparation (day 2)

4. At least 2 hours before addition of the samples, discard the coating solution and wash the plate 3x with sterile PBS.
5. Block the plate with 100 µl per well with culture medium (RPMI) for at least 2 hours at 37°C.

6. In the meantime, collect the spleen and/or bone marrow samples from NP-immunized mice at day 14 or day 28, respectively. Isolate splenic mononuclear cells (SMC) in PBS/0.5% BSA by mincing the spleen between glass slides (rough surfaces) or using a 40 μm Falcon cell strainer placed on top of a 50 ml Falcon tube and a plunger from a 5 ml syringe to mince the spleen with occasionally flushing with ice-cold PBS/0.5% BSA. Bone marrow cells are isolated by flushing the femurs with PBS/0.5% BSA. Spin down at 4°C in a 15 ml Falcon and resuspend in RBC lysis buffer for 5 min, then add 10 ml PBS/BSA and spin; after resuspending in 10 ml, let the "ghosts" settle and transfer supernatant into a new 15 ml tube.
7. Count the cells (e.g. using a cell counter) and resuspend at the desired cell density (e.g. $1x10^6$ cells/ml for SMCs, $8x10^6$ cells/ml for bone marrow) in pre-warmed culture medium (RPMI).
8. Generate 1:2 dilution series for each sample in a separate 96-well culture plate, starting with 250,000 cells for SMCs, and $2x10^6$ cells for bone marrow cells.
9. Decant the blocking medium of the filtration plate and add 100 μl of each sample dilution, resulting in a starting cell number of 100,000 for SMCs and 800,000 cells for bone marrow samples.
10. Incubate the samples over night at 37°C and 5% CO_2. During this time, do not open the incubator door, as it may result in smeared spots difficult to read.

3.3.3 Spot development (Day 3)

The following steps can now be performed on the benchtop.

11. Discard the cell solution and wash the plate 6x with wash buffer.

12. To each well, add 100 µl of AP-conjugated anti-mouse IgG1 diluted 1:2000 in PBS/2% BSA. Filter the detection antibody solution prior to use (0.2 µm filter). Incubate for 1 hour at RT.
13. Discard the detection antibody and wash the plate 3x with wash buffer, followed by 3 washes with PBS-only to avoid interference of Tween with spot development.
14. Take 200 µl NBT/BCIP stock solution per 10 ml NBT/BCIP substrate buffer, pass the solution through a 0.2 µm filter and apply 100 µl per well. Incubate for 5-10 min.
15. Stop spot development under running water and wash the plate extensively. While washing, remove the underdrain and continue rinsing.
16. Blot the plate on paper towels and leave it to dry under the hood in the dark.
17. Read the plate in a microplate reader or determine number of spots by alternative methods.

3.4 Isolation of B-cell subsets from immunized mice by FACS

3.4.1 Isolation of mononuclear cells and B cells

1. Isolate splenic mononuclear cells in PBS/0.5% BSA at day 14 after immunization (Notes 3) by mincing the spleen between glass slides (rough surfaces) or using a 40 µm Falcon cell strainer placed on top of a 50 ml Falcon tube and a plunger from a 5 ml syringe to mince the spleen with occasional flushing with PBS/0.5% BSA.
2. Spin down in a 15 ml Falcon and resuspend in RBC lysis buffer for 5 min, then add 10 ml PBS/0.5% BSA and spin; after resuspending in 10 ml, let the “ghosts” settle

and transfer supernatant into a new 15 ml tube, count cells. This fraction should be used when flow-cytometrically isolating plasma cells ($CD138^{hi}B220^{low}$).

3. Isolate mouse B-cells by magnetic cell separation using the MidiMACS system with the "untouched B-cell isolation kit" (Miltenyi Biotech) according to the manufacturer's instructions or an equivalent system from another manufacturer; count cells.

3.4.2 Isolation of B-cell subsets by FACS

4. The cell count of B cells after magnetic cell separation is appr. 10^7 cells, although it can be highly variable. Staining for flow cytometric isolation should be performed in a 15 ml Falcon tube. Antibody concentrations need to be titrated which can be done on an analyzer (e.g. FACSCalibur or equivalent instrument) using less cells and antibody, which is then scaled up for sorting. As a rule of thumb, staining of 10^7 B cells can be performed in 100 μl total staining volume with each 5 μl of antibodies purchased from Pharmingen; antibodies from other companies including Biolegend or Bioscience usually require titration. For the isolation of splenic GC B cells, stain B cells isolated by magnetic cell separation with antibodies recognizing B220 and CD95 and PNA (Fig. 2; for alternative strategies, see Notes 4&5). For the isolation of splenic plasma cells, stain mononuclear cells with antibodies recognizing B220 and CD138 (Fig. 2). Staining is for 10 min on ice, followed by washing with PBS/0.5% BSA and resuspending the cells in appr. 2 ml PBS/0.5% BSA (inquire the appropriate cell density of the fraction to be sorted from the cell sorter operator).

5. Prepare collection tubes according to the following criteria: Depending on the number of cells that are expected to be recovered, either 15 ml Falcon tubes, 5 ml tubes or 1.5 ml reaction tubes should be used (inquire with the cell sorter operator about the best strategy). As a rule of thumb, for the isolation of GC B cells from immunized normal mice, 15 ml Falcon tubes are recommended; if the genotype of the mice is associated with the occurrence of reduced numbers of GC B cells, or if a subset of GC B cells (e.g. $eGFP^+$ GC B cells) are sorted, 5 ml or 1.5 ml tubes represent the best choices. Generally, plasma cells represent a minute population and should be sorted into 1.5 ml tubes. Regardless of the size of the tubes, fill the tubes completely with PBS/0.5% BSA and incubate for 10 minutes to coat the surface; this ensures that the sorted cells slide to the bottom early into the sort. After discarding the PBS/0.5% BSA, add 20% FBS in PBS/0.5% BSA (and up to 50% FBS if the expected cell number is very low) at appr. $1/5^{th}$ of collection tube's volume (e.g. 3 ml for a 15 ml tube).
6. For the isolation of GC B cells, set a lymphocyte scatter gate to exclude dead cells and small debris, a second gate to identify $B220^+$ cells, and a third gate to identify $CD95^{hi}PNA^{hi}$ GC B cells (Fig. 2). For the isolation of plasma cells, set a lymphocyte scatter gate (make certain to include large cells, as plasma cells are larger than B cells), and a second gate to identify $CD138^{hi}B220^{low}$ cells (Fig. 2). Sort cells into collection tubes. Optional: As control for unmutated V186.2 rearrangements, $CD95^{-}PNA^{lo}$ B cells can be isolated in parallel, although for this purpose we suggest the use of an alternative cell population as control (see Notes 6).

7. Spin down sorted cells, carefully remove the supernatant and lyse the cell pellet in 800 µl TRIzol, incubate for 10 min at RT and continue with step 3.5.1, or freeze at –20ºC if not continuing with the RNA isolation.
8. For an alternative approach that does not involve the isolation of B-cell subsets by FACS, see Notes 7. For studies where the specific analysis of peripheral blood memory B cells is of interest, please refer to ref. (12).

3.5 Sequencing of V186.2 gene rearrangements amplified from RNA

3.5.1 RNA isolation and cDNA synthesis

1. Following incubation for 10 min at RT in 800 µl Trizol, pass 2x through a G26½ needle using a 1 ml syringe to shear the DNA.
2. Add 160 µl chloroform and shake vigorously with hand for 15 sec. Incubate for 3 min at RT. Centrifuge at 4ºC for 15 min at 12,000xg.
3. Carefully remove the upper layer of the interface and transfer to a new tube without touching the interphase. Add 2 µl glycogen to supernatant. Add 400 µl isopropanol, mix and incubate for 10 min at RT. Centrifuge at 4ºC for 15 min at 12,000xg.
4. Remove the isopropanol (leave ~100 µl); centrifuge again briefly and carefully remove remaining supernatant (the RNA pellet is transparent and thus difficult to see in isopropanol). Wash pellet with 1 ml 75% ethanol in DEPC-treated water (now pellet becomes visible).
5. Remove ethanol (the remaining drop can be removed using a G26½ needle placed on a pipette) and dry the pellet on the bench for 15 min, until ethanol has

evaporated. Dissolve RNA in 7 µl DEPC-treated water. RNA can be stored at –80ºC if not continuing with the cDNA synthesis.

6. Make 2 mM dilutions of the Cγ1-cDNA (and if applicable the Cµ-cDNA) primers (Table 1) using DEPC-treated water.
7. Follow the cDNA synthesis protocol according to the manufacturer's instructions using 7 µl RNA (in the case that the total reaction volume is 20 µl) or less depending on the number of sorted cells, and 1 µl of the Cγ1-cDNA primer and, if amplifying µ-transcripts in parallel (see Notes 8), 1 µl of the Cµ-cDNA primer. Perform the cDNA reaction as instructed up to the incubation with RNase H. cDNA can be stored at -20ºC if not continuing with the PCR reaction.

3.5.2 Semi-nested PCR amplification

1. For the first round of PCR, prepare a master mix consisting of 8 µl dNTP solution (1.25 mM), 5 µl 10x PCR reaction buffer, 5 µl $MgCl_2$ (25 mM), 1 µl V186.2-leader primer, 1 µl Cγ1-PCR primer (for PCR primers, see Table 1), and 27.5 µl H_2O. Add 2 µl of cDNA reaction mixture to master mix and then 0.5 µl of a mix of Taq polymerase with 1/60 Pfx polymerase (alternatively, a high fidelity polymerase that allows the amplification of low amounts of DNA can be used). The PCR program consists of 95ºC 2 min, 20x (95ºC 30 s, 70ºC 30 s, 72ºC 90 s), 72ºC 5 min, and 10ºC pause. There is no agarose gel analysis at this step.
2. For the second round of PCR, prepare a master mix consisting of 8 µl dNTP solution (1.25 mM), 5 µl 10x PCR reaction buffer, 3 µl $MgCl_2$ (25 mM), 1 µl V186.2-nested primer, 1 µl Cγ1-PCR primer (for PCR primers, see Table 1), and

28.5 µl H_2O. Add 3 µl of 1st round PCR product of each of the experimental samples and the water control (!) to master mix and then 0.5 µl of a mix of Taq polymerase with 1/60 Pfx polymerase (alternatively, a high fidelity polymerase that allows the amplification of low amounts of DNA can be used). The PCR program consists of 95ºC 2 min, 30x (95ºC 30 s, 70ºC 30 s, 72ºC 90 s), 72ºC 5 min, and 10ºC pause.

3. Run 5 µl of the PCR product on an agarose gel. Of note: there should be two water controls run along with the experimental samples, one which went through the nested PCR to control for potential contamination in the 1st round of amplification, and one that controls the 2nd round of amplification.
4. For a genomic DNA-based strategy to amplify V186.2 rearrangements, see Notes 9.

3.5.3 Cloning of V186.2 segments

1. Gel isolation: Prepare a 1.5% agarose gel with wells large enough for loading of 50 µl per well. Add 5 µl loading buffer to the remaining 45 µl PCR reaction and run on the agarose gel; load marker to identify correct length of PCR product. Cut out band with a scalpel using a UV lamp to visualize band and transfer into a 1.5 ml reaction tube. Isolated band can be stored at 4ºC if not continuing with the gel purification.
2. Isolate PCR product using a suitable gel extraction method such as the QIAquick Gel Extraction kit (Quiagen) or an equivalent kit using the manufacturer's instructions. Elute PCR product with 30 µl H_2O. Measure the DNA concentration

e.g. using Nanodrop methodology or agarose gel-based quantification methods. Optional: 20 ng of DNA can be loaded onto an agarose gel to verify that all bands appear at the same intensity.

3. A-tailing to allow ligation of PCR product into pGEM-TEasy vector: Dilute sample to a concentration of 62.5 ng DNA in 6.4 µl. If the DNA concentration is too low, the volume can be reduced using a SpeedVac centrifuge. Add 1 µl 10x PCR reaction buffer, 0.8 µl $MgCl_2$ (25 mM), 1.6 µl dNTPs (1.25 mM), and 0.2 µl Taq polymerase. Mix and incubate for 20 min at 70ºC; place on ice.
4. Set up a ligation reaction using the pGEM-TEasy vector kit with 5 µl ligation buffer, 0.5 µl cloning vector, 2 µl PCR product, 1.5 µl H_2O and 1 µl T4 ligase, which should be added last. Mix and incubate for 1 hour at RT.
5. Using a standard transformation protocol with DH5α competent bacteria, add 2 µl of the ligation reaction to 50 µl DH5α. Following heat shock, add 500 µl SOC medium, of which after incubation for 1 hour at 37ºC, 100 µl are spread onto an agar plate that contains ampicillin. The remaining bacteria in SOC medium should be kept at 4ºC and can be spread onto agarose plates to obtain additional V186.2-containing clones.
6. Colony PCR: Carefully lift colony from plate and transfer into a 0.5 ml reaction tube containing 40 µl H_2O, vortex vigorously. Prepare a master mix consisting of 28.5 µl H_2O, 8 µl dNTP solution (1.25 mM), 5 µl 10x PCR reaction buffer, 3 µl $MgCl_2$ (25 mM), 1 µl V186.2-nested primer, 1 µl Cγ1-PCR primer (for PCR primers, see Table 1), and 0.4 µl Taq polymerase. Add 3 µl of the sample to the master mix. The PCR program consists of 95ºC 2 min, 32x (95ºC 30 s, 70ºC 30 s,

72ºC 90 s), 72ºC 5 min, and 10ºC pause. Determine DNA concentration in PCR product on agarose gel with suitable quantification marker. Send samples for sequencing together with an appropriate primer (e.g. T7 primer) using the sequencing company's guidelines; e.g. some companies require 4 ng/µl for direct sequencing of PCR products; usually, the company will purify the PCR product in house before sequencing, so that removal of excess PCR primers is not required.

3.5.4. Sequence analysis

1. The VBASE2 database (http://www.vbase2.org/) (17) is a convenient tool for the sequence analysis of V168.2 sequences. Following editing the sequence in the spherogram, which includes removing any primer sequences from the 5' and 3' ends and verifying the nucleotide sequence versus the A, G, T and C peaks, save the sequence in FASTA format and run it against the VBASE2 database. V186.2 corresponds to musIGHV057. If the sequence is more homologous to other V genes, it should be excluded from further analysis. The codon encoding the affinity enhancing tryptophan to leucine amino-acid exchange is at position 34 in the VBASE2 representation. Most somatic mutations lead to a G to T nucleotide exchange (TGG to TTG), but are not restricted to this particular exchange. To determine the number of somatic mutations in the V186.2 gene segment, all nucleotide exchanges relative to musIGHV057 should be counted with the exception to those that are directly at the junction to the diversity (D) gene segment, as those nucleotide differences could represent non-germline encoded (N) nucleotides added by terminal deoxynucleotidyl transferase (TdT). The V186.2 mutation frequency is determined by dividing the number of somatic

mutations through the number of V186.2 nucleotides sequenced, excluding N, D, and joining (J) sequences.

4. Notes

(1) For the study of T-independent type-I and type-II responses, mice are immunized i.p. with 20 µg NP-LPS or 30 µg of NP-AECM-FICOLL, respectively, dissolved in sterile PBS respectively. Serum is isolated from blood as described in section 3.2.1 7 days following immunization. ELISA is performed as described in section 3.2.2, except that the anti-NP serum response of IgM and IgG3 antibodies is measured.

(2) The adjuvant alum is an alternative for Freund's adjuvant and can be used interchangeably in the study of the T-dependent response to NP. Before deciding on an adjuvant, it is recommended to inquire with the institution's IACUC which adjuvant is acceptable.

(3) The analysis for somatic hypermutation in V186.2 rearrangements following NP-immunization is commonly performed around days 12 to 14 after immunization, which is considered to be at the height of the GC reaction.

(4) Different antibody combinations can be used to identify GC B cells, including the commonly used anti-CD95/anti-CD38 combination to identify $CD95^{hi}CD38^{lo}$ GC B cells (13), and anti-CD19 is a suitable alternative for anti-B220. GL7, which is highly expressed on GC B cells, can be used as an alternative for CD95, e.g. in the combination anti-GL7/anti-CD38.

(5) When using an antibody-conjugate with PerCP, it need be ascertained that this fluorescence is compatible with the lasers and filters of the FACS sorter. As fluorescent colors, we suggest to

use FITC, PE and APC. If the cells are GFP-positive, consult with the operator of the flow cytometer which fluorescence can be used as an alternative to FITC.

(6) The most suitable control for unmutated V186.2 rearrangements from antigen-inexperienced naïve B cells are $B220^{+}IgM^{+}IgD^{+}$ splenic B cells (CD19 can be used as an alternative for B220). A suitable fluorochrome combination is B220-FITC, IgM-APC and IgD-PE. cDNA libraries generated with the Cμ cDNA primer as described in section 3.5.7 are amplified with the V186.2-leader and Cμ-PCR primer in the 1st round and the V186.2-nested and Cμ-PCR primer in the second round (for PCR primers, see Table 1).

(7) In principle, it is not necessary to flow-sort GC B cells or plasma cells for somatic hypermutation and affinity maturation analysis of rearranged V186.2 genes upon NP-immunization. cDNA generated from whole splenic mononuclear cells or B cells isolated by magnetic cell separation can be used for the amplification of IgG1-expressing V186.2 gene segments (18). There is one caveat, however. Since plasma cells have much larger amounts of antibody-encoding Ig transcripts than memory B cells or GC B cells, the cDNA-based V186.2 gene analysis is heavily skewed towards transcripts derived from plasma cells. To avoid this problem, V186.2 Cγ1-membrane transcripts can be selectively amplified using the following strategy (18): RNA is reverse-transcribed with an oligonucleotide hybridizing in the Cγ1-membrane-encoding exon (mCγ1-cDNA oligo: 5'-TGACAGCAGCGCTGTAGCAC-3') and amplified for 30 cycles with the amplification conditions described in section 3.5.2.1 using the V186.2-leader primer (Table 1) and a reverse primer hybridizing in the Cγ1-membrane-encoding exon (mCγ1-PCR oligo: 5'-CAGCACAGGTCTCGTCCAGTTG-3'). Generally, no nested PCR strategy is required, but can be implemented using the mCγ1-PCR and the V186.2-nested primer

described in section 3.5.2.2. All other cloning steps can be performed as described in sections 3.5.2-3.5.4.

(8) Somatic hypermutation analyses for V186.2 rearrangements are usually performed for IgG1 antibodies. Since somatic hypermutation occurs in both switched and non-switched GC B cells, V186.2 μ-transcripts may be analyzed as well. cDNA libraries generated with the Cμ-cDNA primer as described in section 3.5.7 are amplified with the V186.2-leader and Cμ-PCR primer in the 1st round and the V186.2-nested and Cμ-PCR primer in the second round (for PCR primers, see Table 1).

(9) A strategy has been published that allows the amplification of V186.2 rearrangements from genomic DNA isolated from B-cell subsets (14). In our own experience, this strategy leads to the amplification of a large percentage of non-V186.2 gene segments (~40%), and unless there is a particular requirement to amplify V186.2 rearrangements from genomic DNA, in our opinion the cDNA-based approach is more cost effective.

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Tables

Table 1: Primers for cDNA synthesis and PCR amplification.

Cγ1-cDNA	5'-CATGGAGTTAGTTTGGGCAG-3'
Cμ-cDNA	5'-CCACCAGATTCTTATCAGAC-3'
V186.2-leader	5'-AGCTGTATCATGCTCTTCTTGGCA-3'
V186.2-nested	5'-CATGCTCTTCTTGGCAGCAACAG-3'
Cγ1-PCR	5'-ATCCAGGGGCCAGTGGATAGAC-3'
Cμ-PCR	5'-CGCAGGAGACGAGGGGGAAGAC-3'

Figure legends

Fig. 1. Experimental flow: Analysis of NP-specific serum Ig by ELISA and ELISPOT for anti-NP-specific IgG1 plasma cells. The major experimental steps are indicated and representative examples for ELISA and ELISPOT results are shown. SMC, splenic mononuclear cells; BMC, bone marrow cells.

Fig. 2. Experimental flow: Analysis of V186.2-Cγ1 rearrangements for somatic hypermutation and affinity maturation. The major experimental steps are indicated. Representative examples for the isolation of $B220^{+}PNA^{hi}CD95^{hi}$ GC B cells and $B220^{lo}CD138^{hi}$ plasma cells are shown. A representative example for the analysis of somatic hypermutation and the analysis for codon 33 mutation using VBASE2 (17) are indicated; in VBASE2, codon 33 of V186.2 is found at position 34. FSC, forward scatter; SSC, side scatter.

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