Chapter 9

The SW_{HEL} System for High-Resolution Analysis of In Vivo Antigen-Specific T-Dependent B Cell Responses

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Abstract

T cell-dependent B cell responses generate optimal antibodies to combat foreign antigens. Naïve B cells responding to antigen undergo a complex series of differentiation events and cell fate decisions to provide long-lived memory B cells and plasma cells. Historically, B cell biologists have been challenged by the task of investigating rare antigen-specific B cells in an in vivo setting such that their interactions with antigen, regulation and migration may be accurately tracked. We have developed the SW_{HEL} experimental system capable of accurately monitoring B cells that interact with a protein antigen and then subsequently undergo isotype switching, somatic hypermutation, and affinity maturation within germinal centers (GC) to generate high-affinity antibodies. Here we provide a comprehensive description of the procedures involved in establishing and using the SW_{HEL} system to assess B cell responses to a foreign antigen. This system can provide a valuable measure of the functional capabilities of T follicular helper cells, whose role is ultimately to support and shape long-term humoral immunity.

Key words B cells, Antibodies, T-dependent, In vivo, Affinity maturation, Affinity-selection, Flow cytometry, Single cell sorting

1 Introduction

The ability to study antigen-specific B cell responses in vivo has been paramount in the quest to better understand humoral immunity. Many of the obstacles initially associated with investigating rare antigen-specific B cell clones in the primary repertoire have been overcome by the advent of transgenic and knock-in mice bearing high frequencies of lymphocytes with defined antigen specificities. We have developed the SW_{HEL} experimental system for high-resolution analysis of antigen-specific B cells that respond to hen egg lysozyme (HEL) within their normal cellular and molecular environments. The gene-targeting approach used to generate SW_{HEL} mice results in ~10–20 % of B cells carrying the anti-HEL specificity of the HyHEL10 monoclonal antibody.

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Importantly, SW_{HEL} B cells have the ability to undergo normal class switch recombination (CSR) and somatic hypermutation (SHM) of their immunoglobulin variable region genes.

The SW_{HEL} system is a means of examining numerous aspects of the B cell response following antigen encounter and activation: clonal expansion, cell fate decisions, cell migration and commitment into either extrafollicular antibody secreting cell (ASC) or early memory or GC B cell compartments. Since HEL-specific B cells in SW_{HEL} mice are capable of undergoing normal CSR and SHM, a major feature of the model is the ability to monitor affinity maturation and clonal selection as it occurs within a dynamic GC environment.

To mirror the low precursor cell frequencies typically expected in a primary response, small numbers of SW_{HEL} B cells are adoptively transferred, challenged with HEL, and identified in recipient mice using CD45 congenic approaches (Fig. 1). To assess B cell responses with physiologically relevant affinities for antigen, we have engineered recombinant HEL proteins carrying amino acid substitutions such that the B cell antigen receptor (BCR) interacts with antigen within a $K_a = 1.1 \times 10^7 - 2 \times 10^{10}$ (M^{-1}) affinity range. By challenging SW_{HEL} B cells with these lower-affinity HEL mutant proteins, the influence of initial BCR antigen affinity on key differentiation decisions may be examined. At various time points

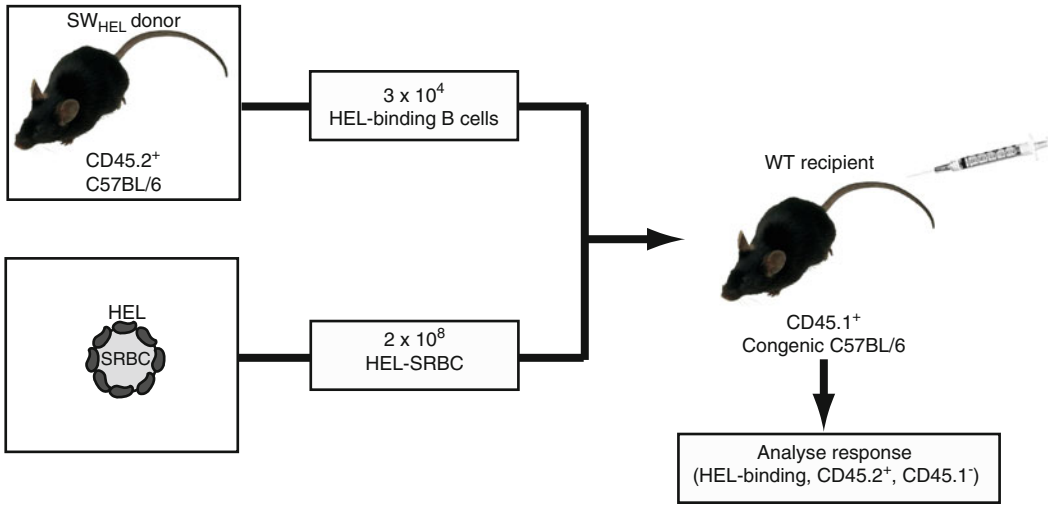


Fig. 1 Adoptive transfer strategy. SW_{HEL} donor ($CD45.2^+$) splenocytes were harvested and 3×10^4 HEL-binding B cells adoptively transferred into congenic SJL.*Ptprc*^{a} ($CD45.1^+$) recipients. These were simultaneously challenged with 2×10^8 HEL-conjugated SRBCs. Recipient spleens were harvested at various timepoints and analysed by flow-cytometry or immunofluorescent histology

post-immunization, spleens and serum from recipient mice may be assessed by (1) flow cytometry to quantitate and phenotype B cell subsets, (2) immunofluorescence histology to track their anatomical localization, and (3) ELISA to determine the circulating levels of antigen-specific antibody. Affinity maturation can be accurately monitored since the initial BCR specificity is known, and thus, any changes to the HyHEL10 variable region that occur via SHM are readily identified. Clonal selection can be tracked by flow cytometric sorting of single GC B cells to precisely identify BCR mutation patterns positively selected for survival within the GC. A powerful and versatile use of this system is that genetic deficiencies may be incorporated such that the role of virtually any gene of interest predicted to regulate the B cell response can be accurately investigated. In this chapter, we detail the reagents needed and procedures involved in genotyping SW_{HEL} mice, performing the adoptive transfer process and investigating the phenotype and affinity maturation of B cells.

2 Materials

2.1 Genotyping Materials

1. 10× Isolation Buffer: 670 mM Tris pH 8.8, 166 mM ammonium sulfate, 65 mM magnesium chloride, 10 % β-mercaptoethanol, 5 % Triton X-100. Combine 134 ml of 1 M Tris pH 8.8, 4.39 g ammonium sulfate, 13 ml of 1 M magnesium chloride, 20 ml β-mercaptoethanol, 10 ml Triton X-100, and dissolve in 23 ml Baxter Irrigation H_2O.
2. Proteinase K: Make up at 50 mg/ml in Proteinase K diluent.
3. Proteinase K diluent: 50 mM Tris–HCl (pH 8.0) and 10 mM calcium chloride in water.
4. GoTaq™ Green (or other equivalent Taq polymerase) and corresponding buffer.
5. dNTPs.
6. Genotyping Primers: Resuspend oligonucleotides at 200 μM. SW_{HEL} Heavy Chain (SHH) primer sequences are as follows: Upper primer (SHH-U) = gtctctgcaggtgagtcctaacttct, Lower primer 1 (SHH-L1) = caactatccctccagccataggat and Lower primer 2 (SHH-L2) = gttgattcttgtgtgacaccag. SW_{HEL} Light Chain (SHL) primer sequences are as follows: Upper primer 1 (SHL-U) = cagggccagccaaagtattg, Lower primer 1 (SHL-L) = tccaacctcttgtgggacagtt, Upper primer 2 (Sonic-U) = ctggctgtggaagcaggttt, and Lower primer 2 (Sonic-L) = cctgccagggactttctgaat.
7. DNA molecular weight marker.
8. Thermocycler/PCR machine.

2.2 Preparing Recombinant HEL

1. PD-10 columns.
2. Conjugation Buffer: 0.35 M D-mannitol, 0.01 M sodium chloride.
3. Retort stand.
4. Cuvette 50–2,000 μl.
5. Spectrophotometer.

2.3 HEL-SRBC Conjugation Materials

1. Hen Egg Lysozyme (HEL): Lysozyme from chicken egg white or recombinant HEL^{2X} and HEL^{3X} mutant proteins produced by the Brink laboratory.
2. Sheep red blood cells (in Alsever's Solution).
3. Dulbecco's PBS (DPBS).
4. Conjugation Buffer: 0.35 M D-Mannitol, 0.01 M sodium chloride.
5. EDCI: *N*-(3-Dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride.
6. HyHEL9 monoclonal antibody: HyHEL9 antibody was derived from a hybridoma and purification performed in the Brink laboratory.
7. Alexa Fluor® 647 Monoclonal Antibody Labeling Kit (Invitrogen Molecular Probes) or equivalent.
8. Hemocytometer.
9. Flow cytometer.

2.4 Reagents/ Buffers for Preparing SW_{HEL} Splenocytes and Adoptive Transfer

1. 2FR: 2 % (v/v) fetal bovine serum, 55 μM β-mercaptoethanol, 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, made up in RPMI.
2. RBC Lysis Buffer: 0.15 M ammonium chloride (NH_4Cl), 10 mM potassium hydrogen carbonate (KHCO3), 0.1 mM sodium EDTA (Na_2EDTA), pH 7.2–7.4. Weigh 8.29 g NH_4Cl, 1.0 g KHCO3, 37.2 mg Na_2EDTA and dissolve in 800 ml water. Check pH and then top up with water to 1 l.
3. PBA (FACS) Buffer: 0.1 % (w/v) bovine serum albumin, 0.1 % (w/v) sodium azide, made up in PBS.
4. Fluorochrome-conjugated anti-B220 (RA3-6B2) antibody.
5. $OVA_{323–339}$ peptide (CGGISQAVHAAHAEINEAGR). Required only if using HEL-OVA to investigate T cell-B cell collaboration.
6. Succinimidyl-6-([ß-maleimidopropionamido] hexanoate) (SMPH). Required only to conjugate HEL-OVA.
7. Alum. Required only if investigating HEL-OVA responses.

2.5 Antibodies and Reagents for Flow Cytometry

1. Anti-mouse monoclonal antibodies: PE-conjugated anti-CD45R/B220 (RA3-6B2), PerCP-Cy5.5-conjugated anti-CD45.1 (A20), PE-Cy7-conjugated anti-CD45.2 (104), FITC-conjugated anti-CD38 (90). Use additional antibody clones and conjugates according to project aims.
2. Anti-CD16/32 (2.4G2) for Fc-blocking.
3. Fetal calf serum/fetal bovine serum (FCS/FBS).
4. 50 ml tubes.
5. 96-well round bottom plates.
6. 35 μm filter round-bottom FACS tubes.
7. FlowJo or equivalent software to analyze flow cytometry data.

2.6 Additional Reagents for Affinity Maturation Analysis

1. Antibodies as in Subheading 2.5 to detect B cell subsets of interest.
2. Recombinant HEL^{2X} and HEL^{3X} protein obtained from the Brink laboratory.
3. Biotinylated anti-mouse IgG1 (A85-1) monoclonal antibody or other isotype according to project aims.
4. Streptavidin Pacific Blue™ (SA-PB).
5. C57BL/6 mouse serum: Obtained either by cardiac puncture or purchased.

2.7 Single Cell FACS Sorting Materials

1. Digest Buffer (suitable for one 96-well plate): 100 μl 10× Taq PCR reaction buffer (no $MgCl_2$), 50 μl Proteinase K (10 mg/ml in water), 10 μl 10 mM EDTA, 10 μl Tween 20 (10 % solution), make up with 830 μl water to give a total of 1 ml (Table 2a). Allow an additional 10 % when calculating volumes for multiple plates.
2. 96-well Skirted PCR plates.
3. 96-well non-skirted PCR plates.

2.8 SHM Analysis Materials

1. Thermocycler/PCR Machine.
2. Adhesive plate seals (either foiled or non-foiled).
3. SHM Primary PCR Primers: Upper primer (SSC-U1) = gtt gta gcc taa aag atg atg gtg and Lower primer (SSC-L1) = gat aat ctg tcc taa agg ctc tga g (lower).
4. SHM Secondary PCR Primers: Upper primer (SSC-U2) = tct tct gta cct gtt gac agc cc. Upper primer (SSC-U2) = ttg tag ccta aaa gat gat ggt gtt aag tc. Lower primer (SSC-L2) = caa ctt ctc tca gcc ggc tc.
5. Taq polymerase or equivalent.
6. dNTPs.

7. illustra™ ExoStar™ or equivalent PCR Clean-up Reaction Kit.
8. Gel-doc/equivalent to visualize DNA under UV light.
9. Sequencing Primers: Same as SHM Secondary PCR Primers.
10. DNA Strider™ and Sequencher™ or equivalent sequence alignment software.

2.9 ELISA Reagents

1. NPP Buffer: 34.9 mM sodium bicarbonate ($NaHCO_3$), 15 mM sodium carbonate (Na_2CO_3), 3.1 mM sodium azide (NaN_3), 1 mM magnesium chloride ($MgCl_2$). Weigh 2.93 g $NaHCO_3$, 1.59 g $NaCO_3$, 0.2 g NaN_3, 0.095 g $MgCl_2$. Make up to 1 l with water.
2. 96-well plates for ELISA.
3. Tween 20: 0.05 % (v/v) solution in PBS.
4. Humidity chamber/plastic container lined with wet paper towel on the bottom.
5. 1 % BSA buffer: 1 % (w/v) in PBS.
6. 0.1 % BSA buffer: 0.1 % (w/v) in PBS.
7. Skim milk powder: 1 % (w/v) in 0.1 % BSA buffer.
8. Recombinant HyHEL10 isotype-specific standards obtained from the Brink laboratory.
9. Biotinylated anti-mouse IgG1 (A85-1) monoclonal antibody (or other isotype of interest).
10. Streptavidin-Alkaline Phosphatase.
11. p-nitrophenyl phosphate.
12. ELISA microplate reader.
13. 37 °C dry air incubator.

2.10 Immunofluorescence Histology Materials

1. Cryostat.
2. Microscope slides.
3. Acetone: 100 % solution, stored at –20 °C until required.
4. Normal horse serum (NHS): 30 % solution made up in PBS.
5. HEL.
6. Polyclonal rabbit anti-HEL sera or other anti-HEL antibody.
7. Cy5-labelled anti-rabbit IgG antibody.
8. Conjugated monoclonal antibodies: FITC-labelled anti-IgD (11-26c.2a) antibody and biotinylated anti-CD3 antibody.
9. Streptavidin-Alexa Fluor® 350 antibody.
10. Additional reagents if investigating HEL-OVA responses: Biotinylated anti-Thy1.1 (HIS51) antibody, Cy3-tyramide staining kit, FITC-labelled anti-rabbit IgG antibody, Alexa Fluor® 647-labelled anti IgD antibody.

11. Anti-Fade Solution: Weigh 2.66 g of 1,4-diazabicyclo-[2.2.2] octane (DABCO), add 900 ml Glycerol and 100 ml PBS (or equivalent anti-fade product). Store at room temperature, protected from light.
12. Coverslips.
13. Fluorescence microscope and relevant software.

3 Methods

3.1 SW_{HEL} Mice and Genotyping

3.1.1 SW_{HEL} Ig Transgenic Mice

Gene-targeted V_H10 mice were produced by Prof. Robert Brink and have been previously described (1). These mice can be maintained either on a C57BL/6 or C57BL/6-SJL.*Ptprc*^{a} congenic background and are to be used as donor mice in the adoptive transfer. Congenic mice (either C57BL/6-SJL.*Ptprc*^{a} or C57BL/6 respectively) are used as recipient mice.

3.1.2 Genotyping of SW_{HEL} Mice by PCR Amplification

1. Extract genomic DNA by purifying from a 1–2 mm length of tail tip. Resuspend tail tips in 2 μl of 1× Isolation Buffer (diluted from 10× stock in water) containing 2 μl of Proteinase K (50 mg/ml in appropriate diluent) and incubate at 65 °C for 4–16 h. Quick spin and cool to room temperature. Use 1 μl of the resulting solution to determine the genotype by PCR.
2. PCR amplify the genomic DNA, extracted as described in Subheading 2.1. Set up PCR reactions using Go Taq, GoTaq™ buffer, dNTPS at 0.2 mM, and primers at 0.4 μM (Fig. 2a).
3. Run SW_{HEL} Heavy Chain and SW_{HEL} Light Chain PCRs on a thermocycler according to the following PCR conditions: 94 °C for 4 min, (94 °C for 30 s, 63 °C for 30 s, 72 °C for 1 min) × 35 cycles, followed by 72 °C for 10 min.
4. Separate the resulting PCR products on a 1–2 % agarose gel containing 1 % ethidium bromide (or equivalent) and visualize under UV light. Determine which mice carry the correct genotype (Fig. 2b, **Notes 1–2**).

3.2 Preparation and Desalting of Recombinant HEL Proteins

A comprehensive protocol for the transformation of plasmid DNA into *Pichia pastoris*, protein expression and purification has been excluded due to space limitations. Purified recombinant HEL^{WT}, HEL^{1X}, HEL^{2X}, HEL^{3X}, and HEL^{4X} proteins (2, 3) are stored in PBS aliquots at −80 °C for long-term storage, and once thawed, stored at 4 °C for a maximum of 8 months (*see* **Notes 3–5**). HEL mutant proteins are desalted into Conjugation Buffer prior to SRBC conjugation.

1. Equilibrate a PD-10 column with ~30 ml Conjugation Buffer.
2. Load 100 μg of protein onto the center of the column and allow to settle in prior to adding the remaining volume up to 2.5 ml with Conjugation Buffer. This flow-through can be discarded.

a SW_{HEL} Heavy Chain Genotyping PCR reaction

Reagent	Initial Conc.	Volume (µl)
DNA prep	Undiluted	1.00
tdH_2O	-	17.37
GoTaq Buffer	5X	5.00
dNTPs	10mM	0.50
Primer mix*	10µM	1.00
GoTaq	5U/µl	0.13
		25.00

*Primer mix is comprised of SHH-U, SHH-L1, and SHH-L2 primers diluted 1/20 in tdH_2O

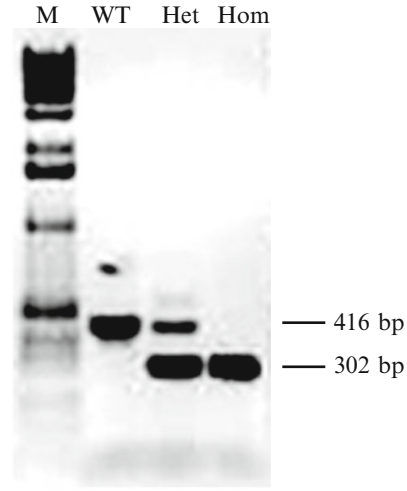
b SW_{HEL} Light Chain Genotyping PCR reaction

Reagent	Initial Conc.	Volume (µl)
DNA prep	Undiluted	1.00
tdH_2O	-	16.37
GoTaq Buffer	5X	5.00
dNTPs	10mM	0.50
Primer mix 1**	10µM	1.00
Primer mix 2***	10µM	1.00
GoTaq	5U/µl	0.13
		25.00

** Primer mix 1 is comprised of SHL-U, SHL-L diluted 1/20 in tdH_2O.

*** Primer mix 2 is comprised of Sonic-U, Sonic-L diluted 1/20 in tdH_2O.

c SW_{HEL} Heavy Chain Result



d SW_{HEL} Light Chain Result

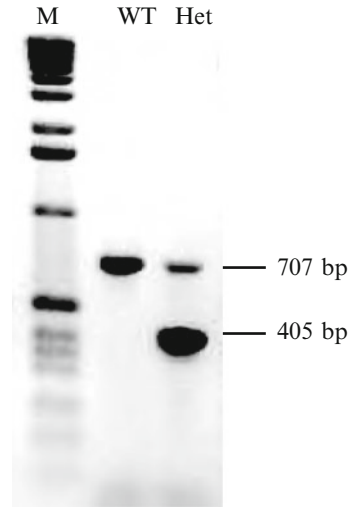


Fig. 2 Determination of SW_{HEL} mouse genotype. PCR reactions for determining the SW_{HEL} Heavy Chain (**a**) and Light Chain (**b**) genotype of SW_{HEL} mice, with expected PCR product sizes to determine zygosity for Heavy Chain (**c**) and Light Chain (**d**). Note that SW_{HEL} Light Chain is homozygous lethal and thus homozygous mice are not viable. *M* molecular weight marker X (Roche), *WT* wild-type, *Het* heterozygote, *Hom* homozygote

3. Elute the protein by adding 3.5 ml Conjugation buffer and keep the eluate as this will contain the HEL protein. Collect the eluate as fractions in the following volumes; Fraction 1 as 250 µl, Fraction 2 as 1,000 µl (this will contain the majority of protein), Fractions 3–7 as 250 µl.
4. Determine the absorbance/optical density of each fraction by spectrophotometry at 280 nm. Calculate the protein concentration.

3.3 Conjugation of HEL to Sheep Red Blood Cells

This protocol describes the conjugation of HEL to SRBC at 10 μg HEL/10×10^9 SRBC in a 1 ml reaction. All centrifugation steps for SRBC are performed at $1{,}111 \times g$ for 5 min at 4 °C.

1. Sterile aliquot approximately 8–10×10^9 SRBC into a 50 ml tube. Count SRBCs and adjust accordingly if SRBC number does not fall into this range (*see* **Notes 6** and **7**).
2. Wash SRBC in 30 ml DPBS and centrifuge. Repeat this twice. After the third wash, resuspend SRBC in 10 ml Conjugation Buffer and centrifuge.
3. During centrifugation, perform the following calculations:

   x = the volume representing 10 μg of mutant HEL protein (desalted)

   y = volume of Conjugation Buffer to resuspend in = 1,000 μl – 100 μl – x.
4. Resuspend SRBCs in volume y of Conjugation Buffer (*see* **Note 8**). This volume will ensure that a concentration of 10 μg HEL/ml is obtained in the reaction (allowing for volume of EDCI to be added and neglecting pellet volume).
5. Add 10 μg of HEL mutant protein (=x) and mix on ice on a platform rocker for 10 min.
6. Add 100 μl of 100 mg/ml EDCI (made up in Conjugation Buffer) and continue to mix on rocker for a further 30 min on ice.
7. Wash SRBCs in DPBS four times or until cell lysis has ceased (a colorless supernatant should be obtained).
8. Count SRBCs and resuspend accordingly such that a cell concentration of 1×10^9 cells/ml in DPBS is obtained. This allows 2×10^8 SRBC to be injected per mouse in a 200 μl volume.
9. Confirm that HEL-SRBC conjugation was successful by flow-cytometric analysis using Alexa Fluor® 647-conjugated HyHEL9 antibody (Fig. 3a).

3.4 Preparation of SW_{HEL} Splenocytes and Adoptive Transfer

All centrifugation steps for splenocytes in 50 ml tubes are performed at $440 \times g$ for 5 min at 4 °C.

3.4.1 Transfer of SW_{HEL} Splenocytes

1. Harvest the spleen from a SW_{HEL} donor mouse according to local ethical guidelines and collect in 2FR medium (*see* **Note 9**).
2. Prepare a cell suspension by using a 70 μm cell strainer compatible for a 50 ml tube.
3. Wash splenocytes in 8 ml of 2FR.
4. Lyse RBC by resuspending splenocyte pellet in 5 ml RBC Lysis Buffer and underlaying without delay with 1 ml FBS.
5. Centrifuge and resuspend SW_{HEL} cells in 5 ml of 2FR medium prior to counting.

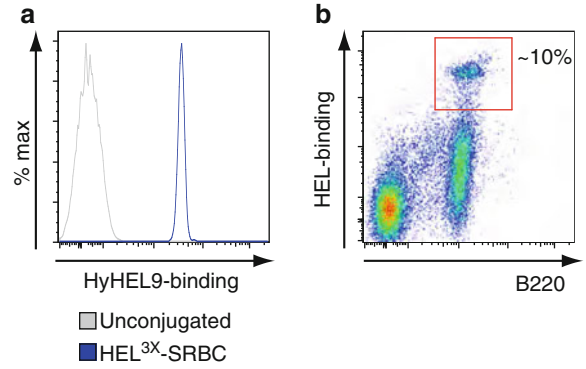


Fig. 3 Preparation of HEL^{3X}-SRBC and SW_{HEL} donor splenocytes. (**a**) Flow cytometry histogram showing HyHEL9-Alexa Fluor 647 staining of HEL^{3X}-conjugated Sheep Red Blood Cells. (**b**) Flow cytometry plot of splenocytes obtained from a SW_{HEL} mouse donor depicting ~10 % HEL-binding $B220^+$ cells

6. Quantitate HEL-binding B cells as follows: Stain 0.5×10^6 cells with 200 ng/ml HEL on ice for 20 min. Wash twice with PBA buffer followed by dual-staining with HyHEL9-Alexa Fluor® 647 and anti-B220-PE. Determine the percentage of HEL-binding B cells by flow cytometry (Fig. 3b, **Note 10**).
7. Adjust the concentration of splenocytes in suspension such that 3×10^4 HEL-binding B cells can be injected per recipient mouse in a 50 µl volume (6×10^5 HEL-binding B cells/ml).
8. Mix conjugated HEL-SRBC with SW_{HEL} cells in vitro immediately prior to tail vein injection. Each recipient mouse receives a total of 250 µl, which is comprised of 2×10^8 HEL-SRBCs in 200 µl and 3×10^4 HEL-binding B cells in 50 µl.
9. SW_{HEL} mice on the SJL/Ptprc^a ($CD45.1^+$) background are used as donors into 8-week-old C57BL/6 ($CD45.2^+$) recipient mice (or vice versa, *see* **Note 11**).

3.4.2 Co-transfer of SW_{HEL} Splenocytes and OT-II T Cells

1. To investigate collaborative responses between SW_{HEL} B cells and OT-II T cells, chemically conjugate HEL to $OVA_{323–339}$ peptide (CGGISQAVHAAHAEINEAGR) using the cross-linking agent SMPH (Fig. 4) (4).
2. Prepare a mixture of spleen cells from SW_{HEL} and OT-II mice containing 3×10^4 HEL-binding B cells and 3×10^4 $V\alpha2^+$ $CD4^+$ OT-II T cells.
3. Inject SW_{HEL} B cells and OT-II T cells intravenously into recipient mice, together with 30 µg HEL-$OVA_{323–339}$ conjugate.
4. Administer 100 µg OVA (Sigma) in Alum intraperitoneally on the same day of cell transfer.

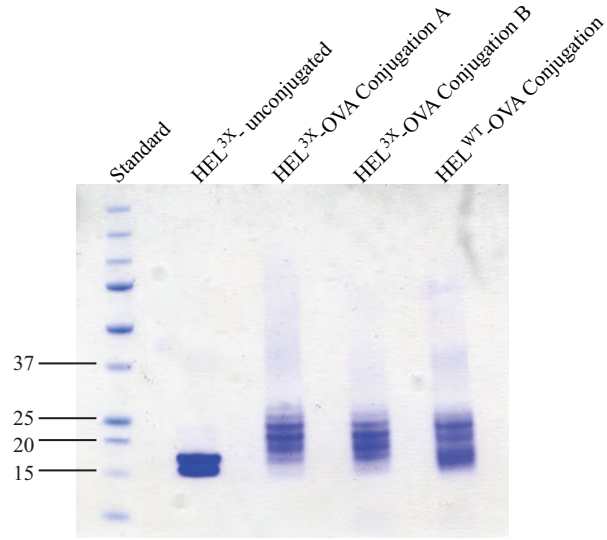


Fig. 4 Coomassie staining of HEL conjugated to OVA323–339 peptide, used as the immunizing antigen when studying HEL-specific B cell and Ovalbumin-specific T cell responses. Note the "ladder" of modified HEL proteins containing increasing numbers of conjugated peptides. Standard-BioRad Precision Plus™ Protein All Blue Standard

3.5 Flow-Cytometric Analysis of Antigen-Specific B Cell Subsets

This section describes the basic procedure involved to identify and quantitate B cell subsets by flow cytometry and is suitable for all time points post-immunization. For projects requiring affinity maturation analyses, please refer to Subheading 3.6. All centrifugation steps for tubes are performed at 440 × *g* for 5 min at 4 °C. All centrifugation steps for 96-well plates are performed at 863 × *g* for 10 s at 4 °C. All staining steps for flow cytometry are performed in 50 µl/well, in PBA buffer, on ice for 20 min. All wash steps for 96-well plates are performed by adding 200 µl PBA buffer to each well, centrifuging at 863 × *g* for 10 s at 4 °C to pellet the cells, then discarding the wash buffer by flicking and quickly inverting the plate on a paper towel.

3.5.1 Staining

1. Harvest recipient mouse spleens in 2FR at the desired time point according to project aims.
2. Prepare splenocyte suspensions using 70 µm cell strainers suitable for 50 ml tubes. Wash cell strainers with 2FR medium. Centrifuge tubes and remove supernatant.
3. RBC lyse by resuspending cell pellet in 5 ml RBC Lysis buffer and underlaying quickly with 1 ml FCS/FBS prior to centrifugation. Remove supernatant and resuspend in 5 ml of PBA buffer. Count cells.

Table 1
Staining protocol for flow cytometric analysis of responding SW_{HEL} B cells

Step	Antibody/reagent
(a)	
1	Fc-block (anti-CD16/32) HEL
2	HyHEL9-Alexa Fluor 647 Anti-B220 PE
3	Anti-CD45.1 PerCP/Cy5.5 Anti-CD45.2 PE/Cy7 Anti-CD38 FITC

Step	Antibody/reagent
(b)	
1	Fc-block (anti CD16/32) HEL^{3X}
2	Anti-IgG1 biotin
3	5 % mouse serum
4	HyHEL9-Alexa Fluor 647 Anti-B220 PE
5	Anti-CD45.1 PerCP/Cy5.5 Anti-CD45.2 PE/Cy7 Anti-CD38 FITC Streptavidin Pacific Blue

Dotted lines indicate wash steps are to be performed

4. Transfer 2.5×10^6 cells per well into 96-well round-bottom plates (*see* **Note 12**). Include the appropriate number of replicate wells by considering the desired number of events at acquisition on the cytometer and also the time point being analyzed. Centrifuge and wash twice in PBA.
5. Block Fc receptors with unlabelled anti-CD16/32. To detect antigen-specific cells, stain cells with saturating levels of HEL (200 ng/ml) (Table 1a). Wash twice.
6. Stain with HyHEL9-Alexa Fluor® 647 and PE-conjugated anti-B220 (*see* **Notes 13** and **14**). Wash twice.
7. Stain for congenic markers using PerCP Cy5.5-conjugated anti-CD45.1 and PE-Cy7-conjugated anti-CD45.2. Include FITC-conjugated anti-CD38 and any other stains according to project aims. Wash twice.

8. Following all staining and wash steps, cells are resuspended at a final concentration of approximately 10^7/ml prior to data acquisition on a flow cytometer.
9. Filter cells using a 35 μm filter round-bottom FACS tubes immediately prior to data acquisition on a flow cytometry analyzer. Collect ~2.5–4 × 10^6 events per sample.

3.5.2 Analysis

Analyze cytometer files with FlowJo software or equivalent.

1. Include light scatter gates on all samples to include lymphocytes but exclude dead cells and debris.
2. Apply a further doublet exclusion gate and exclude autofluorescent cells using a "dump" channel.
3. Separate donor-derived cells from endogenous recipient cells using CD45.1/ CD45.2 gates.
4. Apply subsequent HEL-binding gates prior to quantifying extrafollicular plasma cells, early memory B cells, Germinal center B cell, plasma cells, or memory B cells (Fig. 5a) (4).

3.6 Tracking Affinity Maturation Using the SW_{HEL} System

This section describes the procedures involved in tracking affinity maturation and is suitable for time points from day 7 onward, from which time point a mature GC has formed, and thus, affinity maturation may be readily identified.

1. Prepare splenocyte suspensions as in **steps 1–4** of Subheading 3.5.
2. For affinity maturation studies, stain cells with sub-saturating levels of HEL^{3X} (50 ng/ml) and unlabelled anti-CD16/32 (Table 1b) (*see* **Note 15**). Wash twice.

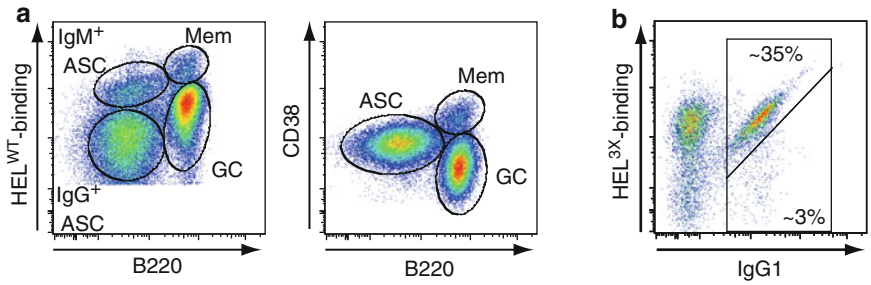


Fig. 5 Representative SW_{HEL} responses. (**a**) Day 5 response elicited by HEL^{2X}-SRBC. Forward and side light scatter gates were applied, doublets and autofluorescent cells excluded, and donor-derived cells revealed by gating on donor allotype-positive and recipient allotype-low cells. HEL-binding gates were applied prior to identifying SW_{HEL} responding B cell subsets at Day 5 as being Germinal Center B Cells (GC), Antibody-Secreting Cells (ASC), Early Memory B cells (Mem). (**b**) Day 14 response elicited by HEL^{3X}-SRBC. Light scatter gates, exclusion gates and donor-derived gates were applied as described for (**a**) prior to identifying SW_{HEL} responding B cells with high level HEL^{3X}-binding, indicative of an affinity-matured response

3. To identify isotype-switched cells, stain with biotinylated anti-mouse IgG1 or other desired antibody isotype (*see* **Note 16**). Wash twice.
4. Block with 5 % mouse serum (*see* **Note 17**). Wash twice.
5. Stain with HyHEL9-Alexa Fluor® 647 and PE-conjugated anti-B220. Wash twice.
6. Stain for congenic markers using PerCP Cy5.5-conjugated anti-CD45.1 and PE-Cy7-conjugated anti-CD45.2. Include FITC-conjugated anti-CD38 and Streptavidin Pacific Blue™. Wash twice.
7. Following all staining and wash steps, cells were resuspended at a final concentration of approximately 10^7/ml prior to data acquisition on a flow cytometer.
8. Filter cells and acquire data on a flow cytometry analyzer as described in Subheading 3.5. Collect ~3.5–7 × 10^6 events per sample.
9. Analyze cytometer files. For affinity maturation studies, apply GC B cell gates, and using the HEL^{3X}-binding stain counter-stained with $IgG1^+$, assess affinity-matured B cells according to their high level HEL^{3X}-binding (Fig. 5b) (*see* **Note 18**) (2, 5).

3.7 Single Cell FACS Sorting for SHM Analysis

This section describes the single cell sorting of donor-derived GC B Cells and should be modified to assess other B cell subsets according to project aims.

1. Prepare cell suspensions as previously described in Subheadings 3.5 and 3.6.
2. Block Fc receptors with unlabelled anti-CD16/32. Wash.
3. Stain cells with a FITC conjugated antibody directed against the relevant allotype marker, to detect donor-derived responders. Also stain with PE-conjugated anti-CD38 and APC-conjugated anti-B220 to identify B cell subsets.
4. Prepare 96-well skirted PCR plates for single cell sorting by adding 10 µl of Digest Buffer per well of a 96-well plate (*see* **Note 19**).
5. Single cell sort GC B cells (or other populations of interest) by applying appropriate sorting gates (Fig. 6).

3.8 SHM Analysis of HyHEL10 Heavy Chain Variable Region: PCR Amplification, DNA Visualization and Data Analysis

All centrifugation steps for single cells in 96-well plates are done at 1,500 rpm (440 × *g*) for 5 min.

1. After single cell sorting, cover cells with adhesive sealing foil and centrifuge without delay (*see* **Note 20**).
2. Heat plates to 56 °C for 40 min and 95 °C for 8 min, and recentrifuge prior to storage at –80 °C overnight (*see* **Note 21**).

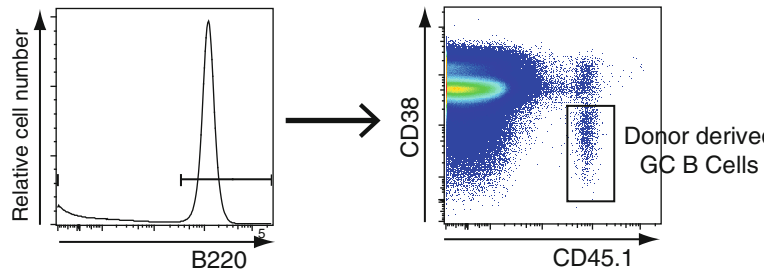


Fig. 6 Example Gating strategy employed for single-cell sorting and SHM analysis. Splenocytes from adoptive transfer recipients were stained as described in Subheading 3.7 and subjected to single-cell sorting into 96-well plates on a BD FACSAria. Forward and side light scatter gates were applied, doublets and autofluorescent cells excluded, prior to identifying B cells as $B220^{hi}$-expressing cells. Donor-derived Germinal Center B cells were then single cell sorted by applying gates on $CD45.1^{+}$ $CD38^{low}$ expressing cells

3. Thaw 96-well plates at room temperature for 5–10 min before subjecting to Primary Polymerase Chain Reaction (PCR) amplification of the V_H10 gene to analyze SHM.
4. Perform the Primary PCR reaction according to the volumes shown in Table 2b and the following PCR conditions: 94 °C for 3 min, (95 °C for 15 s, 55 °C for 1 min, 72 °C for 1 min) × 35 cycles, 72 °C for 10 min.
5. Perform the Secondary PCR reaction according to the volumes shown in Table 2c and PCR conditions: 94 °C for 3 min, (95 °C for 15 s, 62 °C for 40 s, 72 °C for 1 min) × 35 cycles, 72 °C for 10 min.
6. Determine which PCR wells contain the appropriately sized PCR product by subjecting to standard electrophoresis procedures, using 1–2 % agarose gels and detection via UV light (Fig. 7).
7. For wells containing amplified DNA, eliminate any unincorporated primers and inactivate nucleotides using illustra™ ExoStar™.
8. Dilute PCR products 1 in 10 prior to sequencing. Detect mutations by aligning sequence files with the HyHEL-10 sequence using DNA Strider™ or similar. Confirm sequence read by examination of the chromatogram trace files using Sequencher™ 3.11 or similar (*see* **Note 22**).

3.9 Determination of Antigen-Specific Serum Antibodies by ELISA

The serum levels of anti-HEL antibodies of the various Ig subclasses may be measured by ELISA. All wash steps are comprised of 3 × 300 µl 0.05 % Tween 20/PBS washes. Each step below is incubated in a humidity chamber for 1 h at 37 °C unless otherwise stated. This protocol is suitable for a 96-well plate format; however, volumes can be easily adapted to suit a 384-well plate format.

Table 2
Volumes required for SHM analysis of HyHEL10 heavy chain variable regions

Reagent	Initial Conc.	Volume/plate (μl)	Volume/4.4 plates (μl)
(a)			
Taq buffer (no $MgCl_2$)	10×	100	440
Proteinase K	10 mg/ml	50	220
EDTA	10 mM	10	44
Tween 20	10 %	10	44
tdH_2O	–	830	3,652
		1,000	4,400

Reagent	Initial Conc.	Volume (μl)
(b)		
DNA in 1 × Taq buffer (no $MgCl_2$)	Undiluted	10.00
Taq buffer (no $MgCl_2$)	10×	1.50
$MgCl_2$	50 mM	1.00
dNTPs	10 mM	0.50
Upper primer	10 μM	0.10
Lower primer	10 μM	0.10
Taq DNA polymerase	5 U/μl	0.15
tdH_2O	–	11.65
		25.00

Reagent	Initial Conc.	Volume (μl)
(c)		
Primary PCR in 1 × Taq buffer	Undiluted	2.50
Taq buffer (no $MgCl_2$)	10×	2.25
$MgCl_2$	50 mM	1.00
dNTPs	10 mM	0.50
Upper primer	10 μM	1.00
Lower primer	10 μM	1.00
Taq DNA polymerase	5 U/μl	0.10
tdH_2O	–	16.65
		25.00

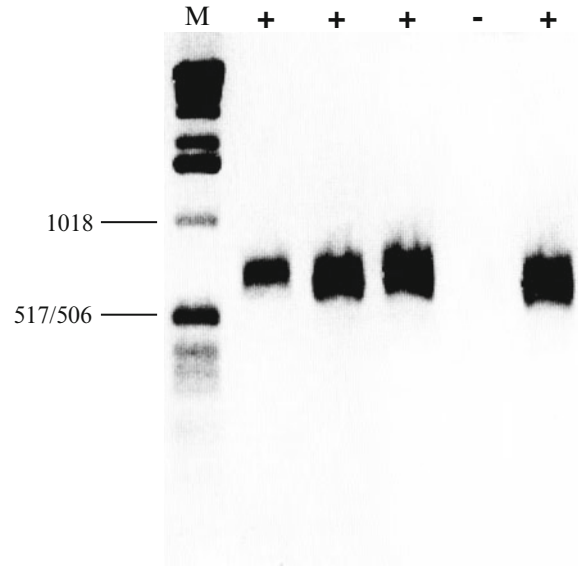


Fig. 7 Agarose gel bands showing wells containing (+) or not containing (–) secondary PCR product from single sorted SW_{HEL} B cells. *M* molecular weight marker X (Roche)

1. Coat 96-well ELISA plates with 10 μg/ml HEL in 60 μl NPP buffer and incubate overnight at 4 °C (*see* **Note 23**). Wash.
2. Block plate with 100 μl of 1 % BSA buffer. Incubate.
3. Perform serial twofold dilutions of sera in 50 μl 0.1 % BSA buffer in duplicate or triplicate. Use recombinant HyHEL10 standards (6) to quantitate antibody levels for each class of Immunoglobulin. Incubate.
4. Detect bound serum antibody using Ig heavy chain isotype-specific biotinylated antibody diluted in 50 μl 1 % skim milk powder diluted in 0.1 % BSA buffer. Incubate.
5. Add Streptavidin-Alkaline Phosphatase (SA-AP) diluted in 50 μl 0.1 % BSA buffer. Incubate.
6. Visualize with 1 mg/ml of substrate p-nitrophenyl phosphate in 100 μl NPP buffer by reading the absorbance at 405 nm on a microplate reader.
7. Construct a standard curve and determine the concentration of serum anti-HEL from the absorbance of dilutions that fall within the dynamic range of the curve (*see* **Note 24**).

3.10 Tracking Cell Localization by Immunofluorescence Histology

The following procedure is used to identify HEL-binding B cells within the context of surrounding B cell follicles and periarteriolar lymphoid sheath areas such that their migration can be accurately tracked (Fig. 8a). This procedure can be easily adapted to

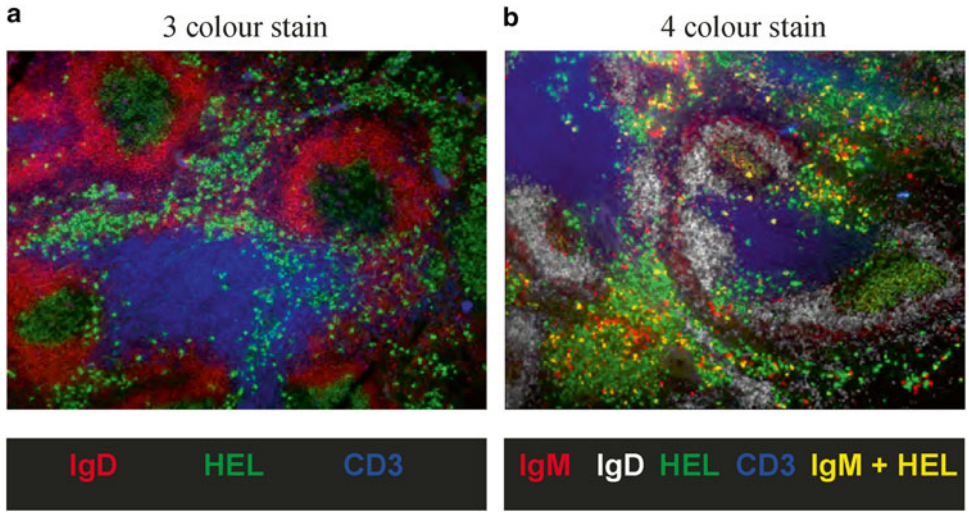


Fig. 8 Immunofluorescence Histology of SW_{HEL} responses. SW_{HEL} responses were elicited with HEL^{2X}-SRBC and recipients analyzed at Day 5 by spleen tissue sectioning and immunofluorescence histology. (**a**) Example of a 3-color stain. IgD^+ areas (*red*)—B cell follicles, IgD^- areas within B cell follicles—Germinal Centers, HEL-binding (*green*)—SW_{HEL} B cells, CD3 (*blue*)—T cells. (**b**) Example of a 4-color stain. IgM (*red*)—unswitched IgM+ cells, IgD (*white*) B cell follicles, HEL-binding (*green*)—SW_{HEL} B cells, CD3 (*blue*)—T cells, IgM + HEL (*yellow*)—colocalization of IgM and HEL-binding stains

accommodate different stains and fluorophores (Fig. 8b). Unless indicated otherwise, all stains are performed at room temperature for 20 min and all washes are of 3 × 5 min with PBS.

1. Cut mouse tissue into 6–7 μm sections using a cryostat.
2. Fix sections by submerging in chilled 100 % acetone for 20 min. At this point, store at −20 °C until the day of staining.
3. On the day of staining, thaw sections at room temperature for ~10 min (*see* **Note 25**). Block with 30 % NHS for 1 h at 37 °C. Wash.
4. To stain for HEL-binding cells, incubate sections firstly with 200 ng/ml HEL^{WT} (Sigma) (Table 3). Wash.
5. Stain sections with polyclonal rabbit anti-HEL sera. Wash.
6. Stain sections with Cy5-labelled anti-rabbit-IgG. Include a FITC-labelled anti-IgD (11-26c.2a) stain to identify follicular B cells and biotinylated anti-CD3-stain (eBiosciences; 500A2) to detect T cells in this step. Wash.
7. Stain with Streptavidin-Alexa Fluor® 350 (Molecular Probes) for 10 min.
8. Use an anti-fade solution to reduce photobleaching prior to mounting coverslips.
9. Visualize slides using a fluorescence microscope and appropriate software.

Table 3
Staining protocol for tracking antigen-specific responses by immunofluorescence histology

Step	Antibody/reagent	Concentration/dilution factor	Incubation duration (min)
1	PBS (rehydration)	Undiluted	20
2	Normal horse serum	30 %	30
3	HEL Anti-CD3 biotin	100 ng/ml 1:100	30
4	SA A350	1:1,000	20
5	Normal rat serum Rabbit anti-HEL	5 % 1:800	30
6	Anti-rabbit Cy5 Anti-IgD FITC	1:250 1:100	30

Dotted lines indicate wash steps are to be performed

For collaborative responses between SW_{HEL} B cells and OT-II T cells, detect OT-II transgenic T cells by staining with biotinylated anti-Thy1.1-biotin (HIS51) followed by a Cy3-tyramide staining kit. In this case, detect HEL-binding B cells using a FITC-labelled anti-rabbit-IgG and follicular B cells using an Alexa Fluor® 647-labelled anti-IgD-stain.

4 Notes

1. Expected PCR product sizes when genotyping are 302 bp for SW_{HEL} Heavy chain and 416 bp for WT Heavy Chain.
2. Expected PCR product sizes when genotyping are 405 bp for SW_{HEL} Light chain and 707 bp for WT Light Chain.
3. For long-term storage of mutant HEL proteins, keep at –80 °C and thaw when required. Aliquots in PBS may be stored for several months at 4 °C; however, HEL in Conjugation Buffer (mannitol/NaCl) should be stored for no greater than 2 months at 4 °C.
4. Confirm protein concentration of recombinant HEL by spectrophotometry at regular intervals, particularly post-thaw from –80 °C storage.
5. To calculate the concentrations of HEL^{1X}, HEL^{2X}, or HEL^{3X} use a molar absorption coefficient, Epsilon of 2.4.
6. As a rough guide, approximately 1.5–2 ml of SRBC (dependent on the supplier) will equate to $8–10 \times 10^9$ SRBC.

7. To count SRBC, perform several serial dilution steps to ensure greater accuracy of the count. Due to rapid settling of the SRBC, be sure to obtain an even suspension by inverting the tube several times before sampling.
8. Using this protocol, approximately 30 recipient mice can be injected after factoring in cell lysis and the loss associated with centrifugation steps. For recipient numbers >30 scale up the reaction as necessary but adhere to the same proportions of 10 μg HEL/10×10^9 SRBC per 1 ml conjugation reaction. By way of example if 60 recipients are to be injected, conjugate 20 μg of mutant HEL to 20×10^9 SRBC in a 2 ml reaction.
9. A spleen from a 6–10-week-old SW_{HEL} mouse will typically have ~10–20 % HEL-binding B cells. Younger mice will have a greater frequency of HEL-binding B cells.
10. When quantitating % HEL-binding B cells by flow cytometry, be sure to account for doublets and triplets by analyzing Forward Light Scatter.
11. Adoptive transfers can also be performed using SW_{HEL} mice on a C57BL/6 background and adoptively transferring these cells into C57BL/6.SJL.Ptprc^a recipients.
12. Round-bottom plates facilitate resuspension of cell pellets in buffer; however, V-bottom plates retain the original cell numbers with less associated cell loss. If cell numbers are limiting, V-bottom plates are recommended.
13. High-speed centrifugation of diluted antibody mixes can remove fluorophore aggregates, which when analyzed can appear as ultrabright false positives. Dilute antibodies at the required dilution factor as determined by antibody titration, then centrifuge in a benchtop microfuge at maximum speed (up to 25,000 × *g*) for 15 min at 4 °C.
14. Cells may be fixed and permeabilized as an alternative way of assessing plasma cells according to their high intracellular expression of immunoglobulins.
15. It is highly recommended to separate HEL^{WT} from lower affinity HEL mutant stains on a 96-well plate to avoid cross-well contamination during staining/centrifugation.
16. The HyHEL9 mAb is a mouse IgG1 antibody. Therefore, it is critical to block with 5 % normal mouse serum after staining with anti-mouse IgG1 to prevent false positive detection of high affinity IgG1 switched cells.
17. Mouse serum (used as 5 % v/v in PBA for blocking) can be purchased or obtained from the blood collection of a C57BL/6 mouse.
18. The canonical Y53D high affinity mutation can be read out by staining with 50 ng/ml of HEL^{3X}.

19. Prepare a master mix of Digest Buffer without Proteinase K, then add the Proteinase K just prior to single cell sorting if sorting large numbers of plates to ensure maximal enzyme digest efficiency.
20. Perform PCR Digest steps immediately following the sort.
21. Ideally perform the Primary PCR the day of or day following the sort. At this stage, the Primary PCR plates may be stored for several days at −80 °C.
22. For analyzing sequencing results, we recommend setting up an Excel spreadsheet to consolidate the results for easy visualization and graphing.
23. 384-well plates can be used instead of 96-well plates to accommodate a greater number of serum samples or when sample volume is limiting. Volumes for steps can be adjusted by using 30 µl instead of 60 µl, 40 µl instead of 100 µl, and 20 µl instead of 50 µl.
24. For affinity-matured antibody, serum samples should be titrated out to obtain a titration curve, with the relevant serum controls included such that endpoint titers may be calculated (7).
25. After thawing slides (if previously frozen), use a hydrophobic pen to outline the tissue section, such that smaller volumes of diluted antibody can be contained on top of the tissue section. As a rough guide, when making up diluted antibody mixes allow approximately 100 µl/tissue section.

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