# Chapter 12

# A Systems Biology Approach of Quantifying Signal Transduction to B-Cell Proliferation and Differentiation

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## Abstract

Combining mathematical modeling with experiments enables quantitative understanding of cell signaling, transcriptional regulation, and cell fate decisions. Here, we provide a systems biology approach to link signal transduction with B cells fate decisions, to enable quantitative prediction of B-cell proliferation, and differentiation. We describe methodology to run simulations that reveal how signal transduction regulates gene expression and predicts cell fate decision. We describe how to quantitively validate modeling predictions with wet-lab experiments.

**Key words** B cell, Cell Signaling, Proliferation, Plasma Cell, Mathematical Modeling

## 1 Introduction

The activation and consequent processes of proliferation and differentiation of naïve mature B cells are initiated by the binding of an antigen or ligand to cell surface receptors. Upon receptor engagement, signaling transduction cascades enable the activation of transcription factors, which control the expression of target genes to govern the processes of cell survival, proliferation, and differentiation of naïve mature B cells into antibody-secreting plasma cells (PCs) [1, 2]. The processes of B-cell activation, division, death, and differentiation are determined by the signaling and regulatory networks within individual B cells [3–5]. The differentiation of naïve mature B cells to PCs is a cell division-dependent process, as highly proliferating cells have a higher propensity to differentiate into PCs. In each cell division cycle, proliferating cells experience progressive changes in their gene regulatory networks, which potentiate their differentiation into PCs [6, 7].

These processes can be modeled ex vivo through stimulation of mature B cells with lipopolysaccharide (LPS). Challenging mature B cells with LPS allows the activation of the transcription factor

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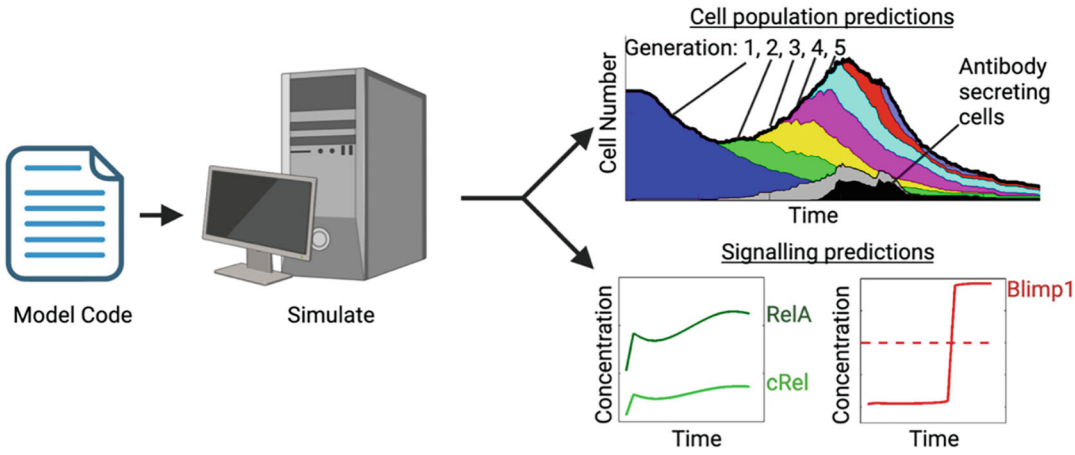


**Fig. 1** Schematic of computational methods**.** Computational models are used to generate predictions that can be tested with experimental methods. **Upper Panel**: Computational predictions of cell numbers, cell phenotypes, and cellular differentiation states can be generated and tested using flow cytometry. **Lower Panel**: Concentrations/abundances of individual biomolecules can be generated with single-cell resolution or averaged to generate cell-population estimates. In response to stimuli upregulation of transcription factor NFkB cRel and RelA leads to cellular proliferation, and terminal differentiation in a portion of cells (through upregulation of Blimp1)

NFκB, which is a key regulator of B-cell proliferation and survival, and is often hyper-activated in hematological malignancies and other B-cell disorders [2, 4, 7, 8]. Within minutes of stimulation, NFκB proteins translocate to the nucleus and induce the transcription of NFκB-target genes responsible for regulating B-cell survival, proliferation, and differentiation [5, 9–11]. Considering the heterogeneous responses of individual B cells to stimulation, it is essential to understand the signaling and transcriptional networks within individual cells to enable predictions of cell fate decisions to death, proliferation, and differentiation to PCs.

Mathematical modeling is a crucial tool in predicting interactions between key regulatory molecules that control cellular outputs in any given condition [12]. The use of in-silico experiments, alongside wet-lab experiments is key to comprehensively understand how signaling regulates cell fate decisions [3–5, 13]. Here, we provide an approach that integrates mathematical modeling with experimental data, to connect signal transduction, gene regulation, and cell fates to predict decisions of cell death, proliferation, and differentiation (Figs. 1 and 2). Briefly, we explain how to run computational simulations that allow predictions of B-cell fate decisions, and how to experimentally test modelling predictions of both signaling with immunoblotting, and cellular phenotypes with flow cytometry.

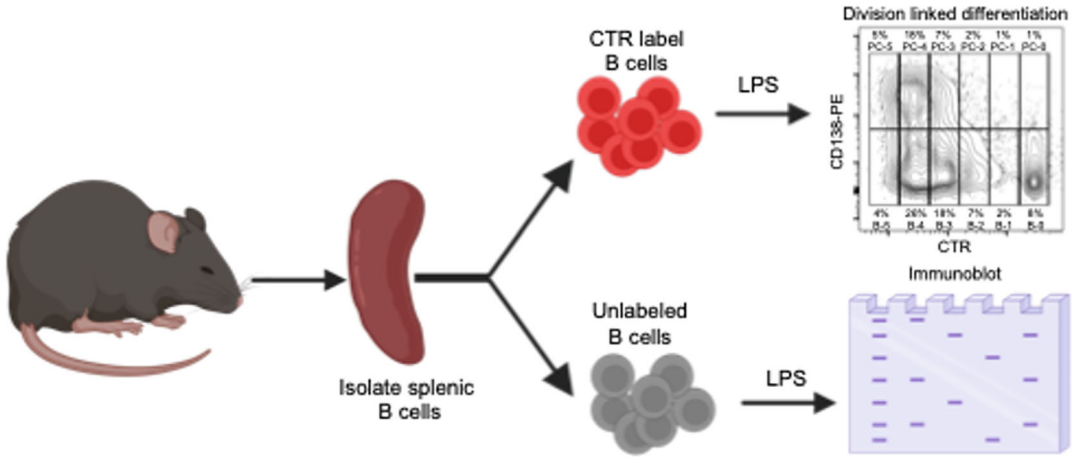


**Fig. 2** Schematic of experimental design. Splenic mature B cells were isolated from mice using magnetic sorting. **Upper Panel**: Mature B cells were labeled with CTR and stimulated with LPS (10 μg/mL) for 24 h, 48 h, 72 h, and 96 h. Cells were labeled with CD138-PE to quantify cell division-dependent PC generation (division-linked differentiation). PCs are defined as CD138 positive cells (PCs are low in B220 expression. Confirm B220 expression in CD138 positive cells) and B cells (B) are defined as CD138 negative cells. B-0, B-1, B-2, B-3, B-4, and B-5 define B cells in generations 0, 1, 2, 3, 4, and 5, respectively. Similarly, PC-0, PC-1, PC-2, PC-3, PC-4, and PC-5 define PCs in generation 0, 1, 2, 3, 4, and 5, respectively. The percentage above each population is the proportion of cells in each population within the total population, e.g., "8% B-0" defines 8% generation "0" B cells and "1% PC-0" defines 1% generation "0" PCs. **Lower Panel**: Mature B cells were stimulated with LPS (10 μg/mL) for 24 h, 48 h, 72 h and 96 h. Total cellular proteins were isolated to measure expression key regulators by immunoblot. A schematic describing immunoblotting with multiple antibodies for different protein size

## 2 Materials

### *2.1 Computational Models*

1. MATLAB, MathWorks, Inc. (*see* **Note 1**).
2. MATLAB Parallel Computing Toolbox.
3. A computational server with ≥16 cores of speed ≥2GHz, and 64GB of random-access memory.
4. ≥10GB of hard disk/solid-state storage, to simulate 100 cells in 2 conditions, scale up as appropriate for size of simulation.
5. Computational modeling code for multiscale model of B cells (*see* **Note 2**).

### *2.2 Isolation of B Cells*

1. Phosphate buffer saline (PBS, pH 7.4): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4.
2. B-cell Media: 1640 RPMI, 10% FBS, 1X pen-strep, 5 mM glutamine, 1 mM sodium pyruvate, 1 mM MEM nonessential amino acid, 20 mM HEPES and 55 μM 2-mercaptoethanol (*see* **Note 3**).
3. Room temperature (RT) PBS (*see* **Note 4**).

4. RT media (*see* **Note 5**).
5. 1.5 mL polypropylene centrifuge tube.
6. Tabletop centrifuge.
7. Cold FBS.
8. Cell Counter.
9. CD43 (Ly-48) Microbeads (Miltenyi Biotec GmbH, 130-049-801) mouse.
10. Red blood cell (RBC) lysis buffer (eBioscience, 00-4333-57).
11. Magnetic-assisted cell sorting (MACS) buffer: PBS containing 0.5% BSA with 2 mM EDTA.

### 2.3 Labeling of Lymphocytes

1. 37 °C PBS.
2. RT media.
3. 1.5 mL polypropylene centrifuge tube.
4. Rotator for mixing.
5. 37 °C incubator.
6. Tabletop centrifuge.
7. Cold FBS.
8. CellTrace™ Far Red (CTR) Cell Proliferation Kit (Thermo Fisher Scientific, C34572). Add 20 μL of DMSO to one vial of CTR. Mix it by either mild vortexing or pipetting up-and--down, followed by a short spin (*see* **Note 6**).
9. Cell counter.

### 2.4 Division Linked Differentiation

1. Prepare $2.5 \times 10^5$ CTR label cells/ mL in media.
2. Lipopolysaccharide (LPS).
3. 48-well tissue culture plate.
4. Flowcytometry buffer (1X PBS, 2.5% FBS, 2 mM EDTA, and 0.02% Sodium Azide).
5. Purified anti-mouse CD16/32 Antibody.
6. Dead cell marker (7AAD, 7-Aminoacetinomycin D).
7. Plasma cell markers (B220-FITC, CD138-PE).
8. Flow cytometry (CytoFlex, Beckman Coulter).

### 2.5 Immunoblot (Western Blot)

1. Phosphate buffer saline (PBS, pH 7.4): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4.
2. 1X RIPA cell lysis buffer (25 mM Tris-HCl, pH 7.6; 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS).
3. 1.5 mL polypropylene tube.
4. 15 mL polypropylene tube.

5. Protease inhibitor cocktail (Millipore Sigma, 11873580001).
6. 4 °C tabletop centrifuge.
7. RC DC™ Protein Assay (Biorad, 5000122).
8. 4x Laemmli buffer (Biorad, 1610747).
9. Plate reader.
10. Vortex mixer.
11. Mini-PROTEAN® TGX™ Precast Gels (Biorad, 4561081).
12. Transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol).
13. Methanol.
14. PVDF membrane.
15. Protein gel running apparatus.
16. Western blot transfer apparatus.
17. Whatman paper (VWR, 28137-858).
18. Mesh Filter.
19. TBS-T (20 mM Tris, 150 mM Sodium Chloride, 0.1% Tween 20).
20. 1% BSA TBS-T.
21. Antibody.
22. Western ECL substrate (Biorad, 170-5060).
23. Restore™ Western Blot Stripping Buffer (Cat# 21059).

## 3 Methods

### *3.1 Simulate Terminal B-Cell Differentiation Driven by NFκB*

1. Unzip all downloaded modeling files to an empty folder on your computational server.
2. Open "AllAnalysis.m" in the MATLAB editor window.
3. Check conditions and adjust based on computational power/accuracy required. Defaults are as used in [4] and may be left unchanged.
4. To change number of cells (default 100) edit this parameter: conditions.numSamples = 100.
5. To simulate cells for a longer time (default 96 h), edit this parameter: conditions.maxTime = 96.
6. To repeat the entire simulation multiple times (default triplicate), edit this parameter: conditions.replicates =3.
7. To run a simulation of NFκB-control over of B-cell terminal differentiation select all commands in allAnalysis.m from the beginning of the file up to and including this line: [x2,cumulativeSumNFkBv1,allTimeCoursesNFkBv1,startingStatesNFkBv1,v2,rememberActivationStrengths] = runNFkBv1(conditions);

Use MATLAB's evaluate selection to run the highlighted code only.

8. Calculate the number of cells that complete differentiation using: cells ThatSwitchNFkBv1 = find(squeeze(allTimeCoursesNFkBv1(2,end,:)) > 5).
9. Observe how the model is unable to capture substantial B-cell differentiation by comparing cellsThatSwitchNFkBv1 (the number of cells that complete differentiation and downregulate AID) with conditions.numSamples (the number of cells in total).

### 3.2 Predict the Impact of cRel Downregulation on Terminal B-Cell Differentiation

1. To predict the impact of adding cRel downregulation during B-cell differentiation, and run the model in which Blimp1 upregulation leads to downregulation of cRel run: [x4,cumulativeSumNFkBv2,allTCReplicatesWT] = runNFkBv2 (conditions);
   allTimeCoursesNFkBv2 = allTCReplicatesWT{1}.
2. Visualize the predicted number of B cells that complete differentiation with and without cRel downregulation and observe the prediction that cRel downregulation is required for terminal B-cell differentiation. plotV1VsV2shadedErrorBars2 (cumulativeSumNFkBv1,cumulativeSumNFkBv2);

### 3.3 Generate Quantitative Testable Predictions of B-Cell Numbers Through Multiscale Modeling (Fig. 1)

1. Open the "Multiscale" folder.
2. Open "SolveBigAllSpecies.m" in the MATLAB editor.
3. At the top of the file adjust the number of cells, and parallel computing options based on number of computing cores available.
4. Default parameters will simulations as published (250 cells, on 32 cores), [4].
5. Suggested parameters for larger simulation are 500 cells ($N = 500$), and 64 cores (parpool('local',32)).
6. Suggested parameters for smaller simulation are 125 cells ($N = 125$), and 12 cores (parpool('local',12)).
7. Run the multiscale simulation, with heterogeneity as published [3]: SolveBigAllSpecies(0.072,0.112) (*see* **Note 7**).
8. Upon completion of simulation results will be saved in the folder "Metabolite TimeCourses."
9. Identify the appropriate CSV file for the protein abundance/activity prediction required (e.g., IRF4.csv contains the concentration of IRF4 in individual cells) (*see* **Note 8**).
10. These concentrations can be validated using western blot (below).

11. Calculate cell numbers and differentiation state of cells non-zero concentrations of Blimp1 (Blimp1 > 4 = Plasma Cells).
12. These cell number predictions can be validated using flow cytometry.

### 3.4 Isolation of B Cells

1. Isolate spleens from 10- to 12-week-old C57BL/6 mice (*see* **Note 9**).
2. Immediately keep the spleen in cold B-cell media on ice.
3. Isolate splenocytes by maceration, using strainer and plunger.
4. Centrifuge at 450 rcf, 4 °C for 5 min and discard supernatant.
5. Resuspend the pellet in 5 mL RBC lysis buffer (*see* **Notes 10** and **11**) and keep in RT for 5 min.
6. Add 5 mL of RT PBS and centrifuge at 450 rcf, 25 °C for 5 min (*see* **Note 12**).
7. Resuspend the cell pellet in 10 mL MACS buffer and count the splenocytes.
8. Centrifuge at 450 rcf, 4 °C for 5 min and discard the media.
9. Resuspend $10^7$ splenocytes in 90 μL cold MACS buffer and add 10 μL CD43 (Ly-48) MicroBeads mouse (*see* **Note 13**).
10. Incubate for 15 min on ice, or in a cold chamber (4 °C), with continuous shaking.
11. Adjust the volume to 10 mL by adding MACS buffer and centrifuge at 450 rcf, 4 °C for 5 min.
12. Equilibrate the LS column 3 times with 1 mL of cold MACS buffer, as the cells are centrifugating.
13. Discard the MACS buffer after centrifugation and resuspend pellet in 500 μL MACS buffer per $10^7$ splenocytes (*see* **Note 14**).
14. Pass sample through the LS column and collect it in 15 mL polypropylene centrifuge tube. Wash the column 3 times with 1 mL cold MACS buffer.
15. Centrifuge cells at 450 rcf, 4 °C for 5 min.
16. Resuspend the pellet in 1 mL of B-cell media and count cells.
17. Verify the purity and viability of B cells with flow cytometry, by staining with B220-FITC and 7AAD.

### 3.5 Labeling of Lymphocytes

1. Prepare 1 mM CellTrace™ Far Red (CTR) dye stock solution in DMSO. Aliquot 5 μL of stock solution in a 500 μL polypropylene centrifuge tube and store it in −80 °C (*see* **Note 15**).
2. A) Thaw 1 mM CTR stock at RT and prepare 2 μM working concentration by diluting in warm PBS (37 °C) (*see* **Note 16**). The volume of working concentration is 500 μL. B) Prepare

$10 \times 10^6$ cells/mL of working concentration in warm PBS (37 °C). Make single-cell suspension by pipetting up and down.

3. Add 500 μL of cells to 500 μL of CTR solution in a 1.5 mL polypropylene centrifuge tube. Mix immediately by inverting the tube 3–4 times. If necessary, mildly vortex the tube for 15–30 s. Final concentration of CTR and cells should be 1 μM and $5 \times 10^6$ cells/Ml, respectively (*see* **Note 17**).
4. Incubate the cells at 37 °C for 20 min with constant mixing. Alternatively, incubate the cells at RT for 25 min with constant mixing.
5. Quench the excess unreacted CTR by adding 500 μL (1 volume) cold FBS and mix well by inverting the tube.
6. Pellet the cells by centrifuge at 500 rcf, 4 °C for 3 min and rotate the tube upside down to centrifuge at 500 rcf, 4 °C for another 3 min.
7. Resuspend the cells pellet in 1 mL pre-warm (37 °C) media.
8. Keep the cells 10 min in pre-warm media. Pellet and resuspend the cells as described in step 3.5.6.
9. Check the efficiency and quality of CTR labeling by flow cytometry. If the histogram shows multiple peak or wide distribution discard the labeled cells and start to label new cells to achieve a log-fluorescence histogram with single peak and narrow distribution [14].
10. Count the cells.

### 3.6 Stimulation and Division Linked Differentiation

1. Add 1 mL media to $2 \times 10^5$ CTR label cells and prepare single-cell suspension.
2. Mix LPS, to a final concentration of 10 μg/mL, to the single cell suspension. Invert the tubes 3–4 times to thoroughly mix. Seed 250 μL of cells/ well in a 48-well plate and culture the cells in 37 °C with 5% $CO_2$ in humidified atmosphere for 24 h, 48 h, 72 h, and 96 h.
3. Gently pipette the cells in the respective well. Transfer the complete contents of each well to a new 1.5 mL polypropylene centrifuge tube.
4. Pellet the cells by centrifuge at 500 rcf, 4 °C for 3 min and rotate the tube upside down to centrifuge at 500 rcf, 4 °C for another 3 min.
5. Resuspend in 50 μL flowcytometry buffer.
6. Add purified antimouse CD16/32 antibody and incubate for 10 min in ice.

7. Add 50 μL flowcytometry buffer containing anti-mouse B220-FITC and CD138-PE antibody and incubate for 30 min in ice. Vortex each tube in every 10 min.
8. Add 900 μL cold flowcytometry buffer for washing.
9. Pellet the cells by centrifuge at 500 rcf, 4 °C for 3 min and rotate the tube upside down to centrifuge at 500 rcf, 4 °C for another 3 min.
10. Resuspend in 250 μL cold flowcytometry buffer and add 5 μL of 7AAD (*see* **Note 18**) to each tube and incubated for 5 min. Pass the cells through a 40 μm strainer. Acquire 100 μL (*see* **Note 19**) at each time point using the CytoFlex flow cytometer.
11. Division-linked differentiation analyzed in software FlowJo (Fig. 2).
12. CD138 positive cells are gated to define PCs and CD138 negative cells are gated to define B cells.
13. The highest CTR peak is defined as generation "0." CTR intensity halves during each division. The subsequent division is defined by halving of CTR intensity.
14. The generation-specific percentage of PCs was calculated using the equation:

$$\text{Proportion PC in generation (n)} = \frac{\text{Proportion of PCs in generation (n)}}{\text{Proportion of PCs in generation (n)} + \text{Proportion of B cells in generation (n)}}.$$

### 3.7 Measurement of cRel and Other Key Regulators at the Protein Level

1. Add 10 mL B-cell media to $2 \times 10^6$ unlabeled (no CTR stained) B cells and stimulate with LPS (10 μg/mL) in 10 cm tissue culture grade nonadherent plate for 24 h, 48 h, 72 h, and 96 h.
2. Gently pipette the cells in the respective plate. Transfer the complete contents of plate to 15 mL polypropylene centrifuge tube.
3. Pellet the cells by centrifuge at 500 rcf, 4 °C for 5 min.
4. Resuspend in 1 mL cold PBS and transfer in a 1.5 mL polypropylene centrifuge tube.
5. Pellet the cells by centrifuge at 500 rcf, 4 °C for 3 min and rotate the tube upside down to centrifuge at 500 rcf, 4 °C for another 3 min.
6. Add 100 μL RIPA buffer containing cocktail of protease inhibitors and vortex for 15 s at maximum speed.
7. Incubate for 20 min at RT and vortex for 30 s at maximum speed.
8. Centrifuge at 21000 rcf, RT for 10 min and collect cell lysate in a 1.5 mL polypropylene tube.

9. Measure protein concentration by Biorad RC DC as described by manufacturer protocol.
10. Take 30 µL cell lysate in a 500 µL tube and add 10 µL of 4× Laemmli buffer containing 0.5 µL 2-mercapto ethanol. Mix well by pipetting.
11. Heat for 5 min in a 90 °C heating block.
12. Transfer to ice for 5 min and quick spin.
13. Load 2 µg protein in each well of a precast gel and add 5 µL of pre-stained protein marker into one well.
14. Run the gel for 10 min at 100 V and then run for 1 hr. at 150 V (*see* **Note 20**).
15. Rinse PVDF membrane in methanol for 15 s, then rinse with deionized water for 2 min. Add cold 1X Transfer buffer so the membrane does not dry up.
16. Wet two Whatman papers and two mesh filters in the cold 1× Transfer buffer.
17. Place the sandwich maker in tray and pour cold 1× Transfer Buffer.
18. Place first mesh filter and then Whatman paper on the on the black part of the sandwich maker.
19. Remove the gel from the plate with wet finger, and place on the Whatman paper carefully.
20. Place membrane on the gel carefully (*see* **Note 21**).
21. Place second wet Whatman paper on the membrane and second mesh filter on top of it.
22. Close the sandwich maker and place the sandwich into the transfer apparatus. The lock of the sandwich should be on the top. And the black part of the sandwich should be on the black side of the apparatus.
23. Place the apparatus into the chamber (Black part should be on the black side of the chamber).
24. Keep an ice pack into the chamber to cool down the buffer. Ice pack should be on the side of the sandwich.
25. Pour cold 1× Transfer Buffer.
26. Run for 1 h at 100 V.
27. Transfer the membrane in a closed box of appropriate size and cut one side at the top to mark which side is face up.
28. Wash with TBS-T three times for 5 min in a closed box.
29. Incubate 1% BSA TBS-T room temperature solution for 1 hr. on shaker to block the membrane (*see* **Note 22**).
30. Add primary antibody or antibodies diluted in 1% BSA TBS-T (*see* **Note 23**).

31. Keep the membrane on the shaker at 4 °C for overnight.
32. Wash with TBS-T three times for 5 min.
33. Add HRP conjugated secondary antibody diluted in 1% BSA TBS-T for 40 min on the RT shaker.
34. Wash with TBS-T three times for 5 min.
35. Prepare 1 mL ECL solution (Volume depends on the size of the membrane).
36. Cover the tube with aluminum foil.
37. Keep the membrane carefully on a Kimtech paper towel, clean the box with water, and wipe it.
38. Then place the membrane and add the substrate drop by drop with pipette.
39. Incubate the membrane in the substrate solution for 5 min.
40. Place membrane onto scanner and take the image.
41. Wash the membrane thrice for 5 min each with RT PBS.
42. Add 1:3 diluted (dilute in RT PBS) Restore™ Western Blot Stripping Buffer in the membrane for overnight on the RT shaker.
43. Wash twice in TBS-T for 10 min.
44. Block the membrane in blocking buffer before continuing with the next staining with antibodies.

## 4 Notes

1. Tested on MATLAB R2014.
2. Download all computational modeling code from published study ([4] https://www.signalingsystems.ucla.edu/models-and-code/Immunity2019/).
3. Prepare fresh B-cell media before (maximum 1 day earlier) isolation of B cells.
4. Keep the cold PBS at 37 °C water bath for 1 h to achieve (RT). Check the temperature of the media by touching the container using the palm of your hand. Only use it if the temperature is close to RT.
5. Keep the cold media for 2 h at RT. Check the temperature of the media by touching the container using the palm of your hand. Only use it if the temperature is close to RT.
6. Prepare freshly and store it at −80 °C in a 5 μL aliquot.
7. Simulation can take >5 h of wall-clock time depending on hardware. Monitor simulation progress to ensure multiple computational cores are being utilized. Monitor memory

usage and if memory becomes low repeat the simulation with fewer cells.

8. When cells undergo apoptosis the concentration of all molecular species is saved as zero. Therefore, calculating average concentrations should not consider zero values. This can be calculated using MATLAB's command: mean(nonzeros(x))
9. Usually, the color of the spleen is red-black. In some conditions, it is common to see that a portion of spleen is black in color. The black part of the spleen is primarily dead cells. Do not take the black part of the spleen for cell isolation to increase the proportion of viable B cells.
10. Use 5 mL/mice, if spleen size is normal, i.e., total splenocytes $<250 \times 106$.
11. Do not use cold RBC lysis buffer. Keep the RBC lysis buffer for a maximum of 30 min in a 37 °C water bath to reach RT. Check the temperature of the RBC lysis buffer by touching the container using the palm of your hand. Only use it if the temperature is close to RT.
12. If the pellet color is still red, then repeat steps 3.4.5 and 3.4.6.
13. Make a homogeneous suspension of the microbeads by vortexing.
14. Scale up the volume of the MACS buffer depending on the number of splenocytes.
15. CellTrace™ Violet/CellTrace™ Yellow/CFSE can be used as an alternate to CellTrace™ Far Red depending on the configuration of flow cytometry to be used. We observe that CellTrace™ conjugated dye shows more distinct peaks of different generations than CFSE. Also, the fluorescence decay rate of CFSE label is faster than CTR. CFSE-labeled B cells lose fluorescence intensity rapidly in the first 48 h and more slowly thereafter.
16. The freezing point of DMSO is 18.5 °C. If the RT is below 20 °C thaw it in >20 °C in a waterbath.
17. Adjust the number of cells and CTR solution depending on the experimental need; e.g., if the experiment needs $10 \times 10^6$ then add 1 mL of working concentration of cells to 1 mL CTR solution.
18. Propidium Iodide (PI) can also be used as a dead cell marker.
19. It is important to count cells in the same volume for each time point. If 100 μL contains too many or too few cells adjust the count volume to get a significant number of cells.
20. The gel running time might vary depending on the instrument. Stop the gel running when bromophenol blue just passes through the bottom of precast gel.

21. Make sure there is no bubble in between Whatman paper and gel and membrane. If you see bubble, use the roller and buffer to remove the bubbles.
22. If the blocking buffer is in 4 °C, keep the required volume of blocking buffer at room temperature when the transfer is running. Multiple primary antibodies targeting different protein can be used during staining step. The size of target protein must be different and monoclonal antibody is preferred when possible.
23. Multiple primary antibodies targeting different proteins can be used during the staining step. The size of target proteins must be different, and a monoclonal antibody is preferred when possible.

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