# 2

# Characterization of B-Cell Maturation in the Peripheral Immune System

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**Summary**

In the periphery different populations of B cells can be identified, corresponding to subsequent stages of B-cell development. Transitional 1 B cells are recent bone marrow emigrants traveling with the blood to the spleen. Here they further develop to transitional 2 and mature B cells. Marginal zone B cells are a sessile population only found in the spleen. The distinction of these cell types is only possible by three- and four-color flow cytometry, analyzing the relative expression of several developmentally regulated markers. We describe the method for the staining of the cells and the analysis of the collected data and show examples of the results obtained in normal and mutant mice.

**Key Words**

B cells; development; T1; T2; spleen.

## 1. Introduction

B cells are generated from hematopoietic stem cells (HSC) in the bone marrow during adult life. Their development can be divided into several stages, identified on the basis of their distinct phenotype and characterized by the progressive acquisition of the B-cell antigen receptor (BCR) components. The fitness of developing B cells is continuously verified. Only B cells with a signaling BCR and able to appropriately respond to growth factors derived from microenvironment of the bone marrow progress along the differentiation pathway ADDIN ENRfu ***(1–6)***

B cells leave the bone marrow at the transitional 1 (T1) stage when they are still functionally immature. With the blood they reach the spleen, where they further develop to transitional 2 (T2), mature (M), and marginal zone (MZ) B cells. As in the bone marrow, this phase of development in the spleen is

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also dependent on the signaling function of the BCR, on the availability of microenvironment-derived factors, and on the adequate response of B cells to these factors ADDIN ENRfu *(7–11)*.

The accurate discrimination and quantification by flow cytometry of the B-cell populations in the periphery allows (a) the identification of alterations and blocks of B-cell development; (b) the separation of the different B-cell developmental stages, an indispensable tool for their molecular and genetic comparison both in normal and mutant mice.

## 2. Materials

1. Medium for single-cell suspensions: Iscove's Dulbecco's modified Eagle's medium (DMEM) (GIBCO-BRL) supplemented with 2% heat-inactivated fetal calf serum (FCS; GIBCO-BRL), 2% L-glutamine (GIBCO-BRL), $5 \times 10^{-5}$ *M* 2-β-mercaptoethanol (Sigma, St. Louis) and 20 mg/mL gentamycin (GIBCO-BRL).
2. Gey's solution: 14 mL sterile $H_2O$, 4 mL solution A, 1 mL solution B, 1 mL solution C. Solution A (for 1 L $H_2O$): 35 g $NH_4Cl$, 1.85 g KCl, 1.5 g $Na_2HPO_4 \cdot 12H_2O$, 0.119 g $KHI_2PO_4$, 5.0 g glucose, 25 g gelatin, 0.05 g phenol red. Autoclave at 120°C. Store at 4°C. Solution B (for 100 mL $H_2O$): 0.14 g $MgSO_4$ 7 $H_2O$, 0.42 g $MgCl_2$. 6 $H_2O$, 0.34 g $CaCl_2$. 2 $H_2O$. Autoclave at 120°C. Store in dark at 4°C. Solution C (for 100 mL $H_2O$): 2.25 g $NaHCO_3$. Autoclave at 120°C. Store in dark at 4°C.
3. FACS buffer: 1X phosphate-buffered saline (PBS) without $Ca^{2+}$ and $Mg^{2+}$, supplemented with either 1% bovine serum albumin (BSA) or 3% dialyzed FCS and containing 0.1% $NaN_3$ (*see* **Notes 1** and **4**).
4. Antibodies: anti-IgM (clone 2911), anti-IgD (clone 11.26c), anti-CD21 (clones 7G6 and 7E9), anti-CD23 (clone B3B4), anti-B220 (clone RA3-6B2), and anti-heat-stable antigen (HSA; clone M1/69) were obtained from Pharmingen (San Diego, CA). Dead cells were excluded from analysis by side/forward scatter gating and/or propidium iodide (PI) staining.

## 3. Methods

### 3.1. Cell Suspensions and Basic Staining Procedure

1. Remove the organs surgically and keep them in a 15-mL plastic Petri dish containing 5 mL of cold medium for single-cell suspensions on ice. Make a single-cell suspension by pushing the organs with the piston of a syringe through a metal sift. Keep tubes on ice.
2. Remove red blood cells from samples using Gey's solution. Centrifuge single-cell suspension for 5 min at 290*g* in a refrigerated centrifuge (4°C), remove supernatant, and add 5 mL ice-cold Gey's solution. Underlay with 1 mL FCS (**Caution:** Use a 1-mL pipet or a Pasteur pipet and keep on ice for 5 min. A longer incubation time may result in reduced lymphocyte vitality.
3. Centrifuge as in **step 2** and remove supernatant. Wash with 10 mL FACS buffer and resuspend in 5 mL FACS buffer.

4. Count cells.
5. Use for each staining $5 \times 10^5$ to $3 \times 10^6$ cells per sample. The stainings can be done either in round-bottom 96-microwell plates or in FACS tubes. Washing volume is 250 µL (three times) or 2.5 mL (once), respectively.
6. After the last washing resuspend the pellet in 10 µL of antibody solution and mix gently (*see* **Notes 2** and **3**).
7. Incubate for 15 min on ice.
8. Wash (as indicated in **step 5**) and resuspend in 200–300 µL FACS buffer for acquisition.
9. For live/dead discrimination, add 10 µL PI solution (stock at 10 µg/mL). If fixing cells before analysis, do not add PI (*see* **Note 5**).

### 3.2. Four-Color Fluorescence Staining

Three very important things have to be kept in mind when making multiple color stainings: (a) The biotin-labeled antibody has to be added one step before the streptavidin-bound fluorochrome. If both reagents are added together, complexes are immediately formed between excess antibody and fluorochrome, and the cells will remain unstained. (b) Always use the smallest fluorochromes first to avoid steric hindrance. Fluorescein isothiocyanate (FITC), biotin, peridinin chlorophyll protein (PerCP), and Cy5 are small. PE, allophycocyanin (APC), and all tandem conjugates are big molecules. (c) Use the brightest fluorochromes for the less well-expressed markers. PE, Cy5, and most tandem conjugates are bright; PercP is very weak. Fluorochrome choice can really make a difference. For example, CD25 on pre-B cells and on regulatory T-cells can be detected with PE- or biotin-labeled antibodies, but it is almost undetectable with FITC.

1. Start from the pellet obtained in **step 5** of **Subheading 3.1.** Resuspend the cells in the antibody mix (10 µL) containing FITC and biotin-labeled antibodies.
2. Follow **steps 7** and **8**, but after centrifugation add the second-step antibodies to the pellet—for example, those labeled with Cy5 and PE.
3. Repeat **steps 7** and **8**, and after centrifugation resuspend the cells in the solution containing the streptavidin-bound fluorochrome (normally a tandem conjugate).
4. Proceed as in **step 8**.
5. To identify T1, T2, MZ, and M B cells, we normally use CD21-FITC, CD23-biotin, IgD-PE, and IgM-Cy5 plus streptavidin-cychrome. Keep in mind that CD23 is the weakest marker.

### 3.3. Flow-Cytometric Analysis

The identification of T1, T2, MZ, and M B cells in the spleen is only possible through the combination of several markers, which are differentially expressed during development (*see* **Table 1**). The best combination includes surface immunoglobulin IgM and IgD, the complement receptor type 2, CD21, and the low-affinity receptor for IgE, CD23 ***(10).***

**Table 1**
**Phenotype and Location of Different B-Cell Populations in the Spleen**

| | T1 | T2 | M | MZ |
|---|---|---|---|---|
| sIgM | +++ | +++ | + | +++ |
| sIgD | – | +++ | +++ | – |
| CD23 | – | ++ | ++ | – |
| CD21 | – | +++ | ++ | +++ |
| HSA | +++ | +++ | + | + |
| CD62L | – | + | ++ | – |
| 493 | + | + | – | – |
| Location | red pulp, outer PALS | follicle | follicle | marginal zone |

A plot of forward scatter (FSC) vs CD23 can be used to separate $CD23^{pos}$ and $CD23^{neg}$ cells of lymphocyte size (small cells, low FSC) (**Fig. 1A**). IgM and IgD expression in $CD23^{neg}$ cells discriminates non-B cells, negative for both markers, from $IgM^{pos}$ and $IgD^{neg}$ B cells, including both T1 and MZ B cells (**Fig. 1B**, $CD23^{neg}$). The relative size of these two compartments can be determined by combining IgM with CD21. T1 B cells will appear in the $CD21^{neg}$ region, whereas MZ B cells are bright for CD21 (**Fig. 1C**, $CD23^{neg}$). In the spleen of adult C57BL/6 mice T1 B cells are 5–10% and MZ 3–5% of all B cells. $CD23^{pos}$ B cells analyzed for the expression of IgD and IgM appear positive for both markers. T2 B cells, however, are bright for both IgM and IgD ($IgM^{bright}$ $IgD^{bright}$), whereas M B cells express high amounts of IgD and low amounts of IgM ($IgD^{bright}$ $IgM^{pos}$) (**Fig. 1B**, *right panel*). T2 can also be discriminated from M B cells based on the different levels of IgM and CD21: M B cells express moderate amounts of both markers ($IgM^{pos}$ $CD21^{pos}$), whereas T2 B cells are $IgM^{bright}$ $CD21^{pos\ bright}$ (**Fig. 1C**). T2 B cells are 15–20% of all B cells in the spleen.

The detection of IgM, IgD, and CD21 is sufficient to compare the different distribution of B cells in the spleen, blood, and lymph nodes. M B cells ($IgM^{pos}$, $IgD^{pos}$, $CD21^{pos}$) are present in all tissues. T2 ($IgM^{bright}$, $IgD^{bright}$, $CD21^{bright}$) and MZ B cells ($IgM^{bright}$, $IgD^{neg}$, $CD21^{bright}$) are present only in the spleen and T1 in spleen and blood (**Fig. 2A**). Because MZ B cells can only be found in the spleen, it is not indispensable to use CD23 in this type of analysis.

The frequency of T1 B cells in the blood can be, however, precisely calculated only by taking into consideration that IgM $^{bright}$, $IgD^{neg}$, and $CD21^{neg}$ pop-

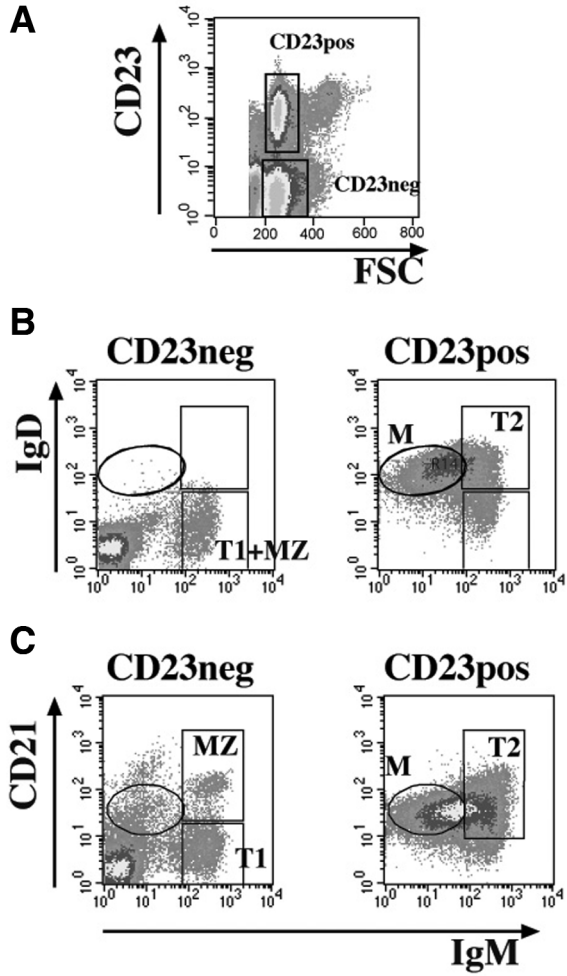


Fig. 1. Discrimination of T1, T2, M, and MZ B cells in the spleen by four-color fluorescence flow cytometry. (**A**) Cells stained for CD23, IgM, IgD, and CD21were first gated, based on size (FSC) and CD23 expression. The $CD23^{neg}$ population is shown in the *left panels* and the $CD23^{pos}$ in the *right panels* in (**B**) and (**C**). (**B**) The staining for IgM and IgD does not allow the discrimination of T1 and MZ B cells, which are both $IgM^{bright}$ and $IgD^{neg}$. M and T2 B cells can be recognized based on the relative amount of IgM and IgD: T2 B cells are bright for both IgM and IgD ($IgM^{bright}$ and $IgD^{bright}$), whereas M B cells express high amounts of IgD and low amounts of IgM ($IgD^{bright}$ and $IgM^{pos}$). (**C**) The high level of CD21 distinguishes MZ ($IgM^{bright}$ and $CD21^{bright}$) from T1 B cells ($IgM^{bright}$ and $CD21^{neg}$). Different levels of both IgM and CD21 are present in T2 ($IgM^{bright}$ and $CD21^{pos\ bright}$) and M B cells ($IgM^{pos}$ and $CD21^{pos}$).

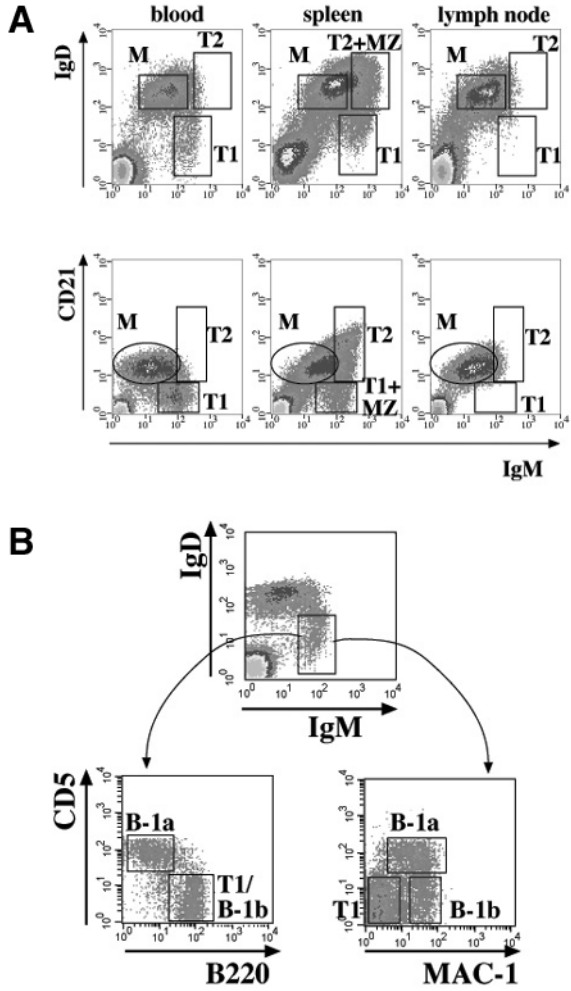


Fig. 2. Discrimination of T1, T2, M, and MZ B cells in the periphery. (**A**) Three-color fluorescence analysis of mononuclear cells isolated from blood, spleen, and lymph nodes. IgM and IgD staining is shown in the *top panels* and CD21 and IgM in the *lower panels*. (**B**) Four-color fluorescence flow cytometry of peripheral blood mononuclear cells IgD and IgM (shown in the *top panel*) in combination with either CD5 and B220 (*left panel*) or CD5 and MAC-1 (*right panel*).

ulations contain not only T1 B cells but also B-1a and B-1b B cells ***(11).*** T1, B-1a, and B-1b B cells are identified by four-color fluorescence analysis using a combination of either IgD, IgM, CD5, and B220, or IgD, IgM, CD5, and Mac-1. A gate is set on $IgM^{bright}$ and $IgD^{neg}$ B cells ***(*****Fig. 2B**, top panel). By

plotting CD5 vs B220 only B-1a B cells appear as a defined population ($CD5^{pos}$, $B220^{dull}$, Fig. 2B, lower left panel), but T1 and B-1b B cells are both $CD5^{neg}$ and $B220^{bright}$. CD5 and MAC-1 instead separate B-1a ($CD5^{pos}$ and MAC-$1^{pos}$) from B-1b ($CD5^{neg}$ and MAC-$1^{pos}$) and T1 ($CD5^{neg}$ and MAC-$1^{neg}$) B cells (**Fig. 2B**, left panel).

An example of the analysis of mutant mice using this method is shown in **Fig. 3**. We studied the development of B cells in mice with genetic defects of molecules involved in the BCR signaling complex. In the spleen of mice lacking the phosphatase CD45 (CD45–/–) and of CBA/N mice lacking the natural mutant for the tyrosine kinase Btk, we observed an increase of the T2 population (**Fig. 3A** $CD23^{pos}$) and a reduction of the mature pool, with a normal frequency of both T1 and MZ B cells (**Fig. 3A** $CD23^{neg}$). We concluded that B-cell development is arrested at the T2 stage in both mouse strains and that a perfect signaling function of the BCR is important for the progression from T1 to T2 and M. This hypothesis was confirmed by the observation that B-cell development further than the T1 stage was completely abrogated in mice with a severely impaired BCR signaling function (mb1Δc/Δc–).

Several other mouse mutants have been analyzed using this method confirming the presence of important checkpoints at the T1 and T2 stage before the final differentiation of peripheral B cells. Based on the results of these studies we propose a model suggesting that the cooperation of signals from the BCR and from a growth factor produced and active in the spleen is indispensable in the late phases of B-cell development (**Fig. 4A**). Arrest of development at the T1 stage eliminates all subsequent B-cell types and can be caused either by a severe impairment of the BCR signaling function or by the lack of the B-cell activation factor (BAFF) or of its receptor (**Fig. 4B**) ADDIN ENRfu ***(10,12–14)***. The block at the T2 stage is observed in mice with qualitative defects of the BCR signaling function or overexpressing BAFF (**Fig. 4C**) ***(10,15–23)***. This model can be used as a framework to classify known and new mutations and will be continuously improved and modified by additional studies.

## 4. Notes

1. Dialysis of FCS helps to remove free biotin, which, if present in high amounts, binds streptavidin-coupled fluorochromes and impairs the detection through biotin-conjugated antibodies.
2. It is necessary to determine the proper concentration for each antibody used. For that, test antibody dilutions using the concentration of cells most often used—for example, $1 \times 10^6$. Choose the concentration that best separates positive from negative cells. For FITC-labeled antibodies, test dilutions from 1 : 10 to 1 : 80. It is possible to dilute all other antibodies much more; therefore, test dilutions up to 1 : 200, with the exception of PerCP-labeled antibodies, which may need to use undiluted.

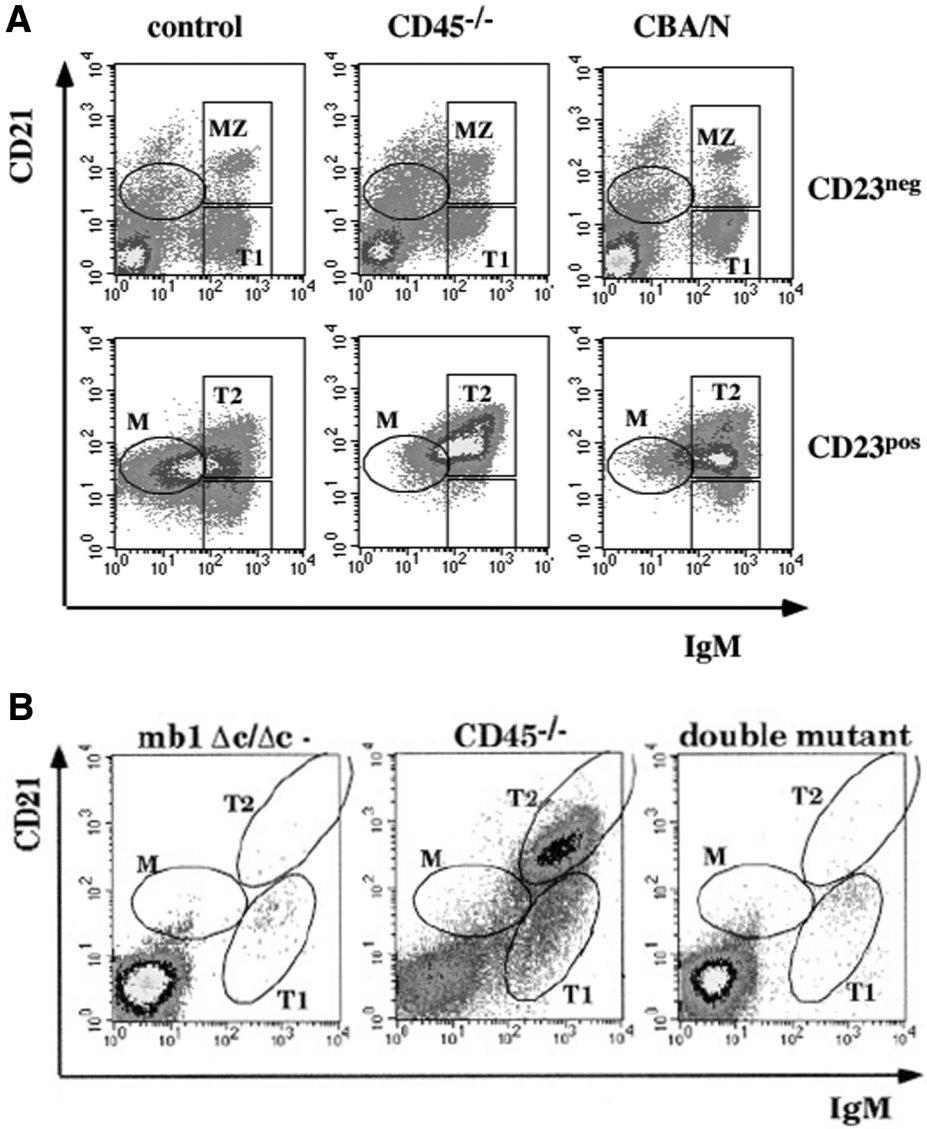


Fig. 3. Identification of blocks of B-cell development through the analysis of peripheral B cells. Four-color fluorescence flow cytometry using CD23, IgM, IgD, and CD21 in control and mutant mice. Block at the T2 stage. Block at the T1 stage.

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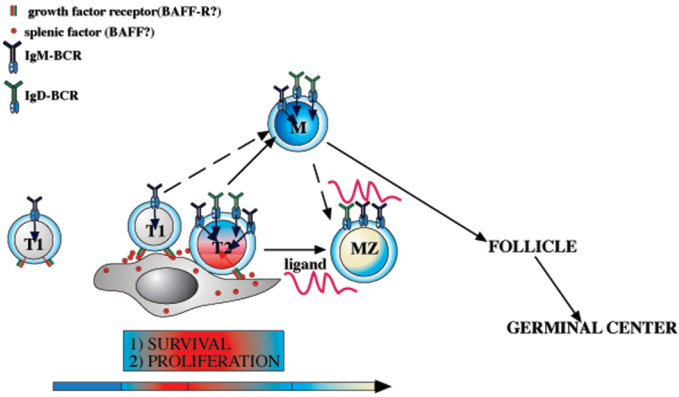
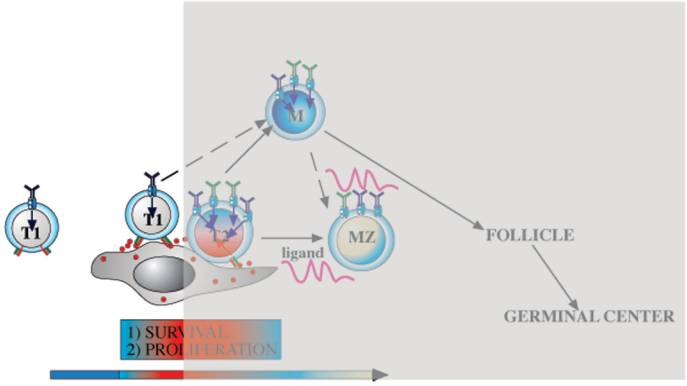
Fig. 4. *(see facing page)* Model of normal B-cell development in the spleen. The arrest of development at the T1 stage impairs the development of T2, M, and MZ B cells. The mutations causing this phenotype are indicated on the right. The block at the T2 stage is observed in the mutant mice indicated on the right.

## A NORMAL DEVELOPMENT



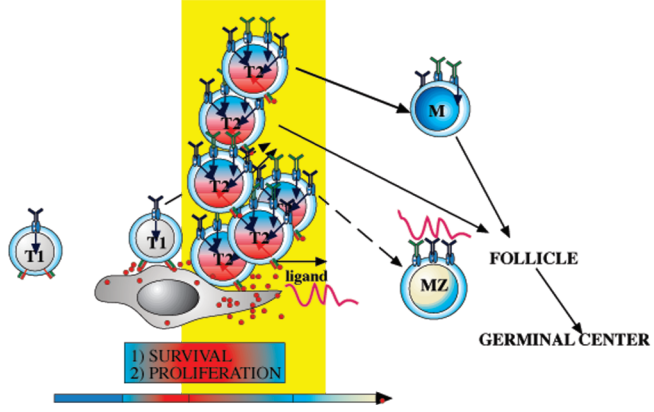

## B BLOCK AT THE T1 STAGE




**syk-/-**
**mb1 Δc/Δc-**
**Rel/RelA**

**BAFF-/-**
**A/WySnJ (BAFF-R)**

## C BLOCK AT THE T2 STAGE




**CD45 -/-**
**Btk-/-**
**Lyn-/-**
**Vav-/-**
**PI3K-/-**
**PLCγ-/-**
**BCAP-/-**
**BLNK-/-**

**BAFF transgenic**

3. Antibody concentration, not its quantity, is critical. When staining with 10 µL of antibody solution added to a dry pellet, spare a lot of antibody. If two antibodies are added together, each in 10 µL, consider that the final volume will be 20 µL, and the final antibody concentration will thus be half of what is really needed.
4. The concentration of $NaN_3$ in the FACS buffer may appear high, but it is necessary to block receptor modulation. If cells have to be functional after analysis or sorting, the medium should not contain $NaN_3$, and receptor modulation may only be controlled by keeping the cells cold during the whole procedure, including sorting (use a refrigerating chamber at 4°C).
5. In a two- or three-color fluorescence analysis, PI can be used for live/dead discrimination. Add 10 µL PI solution (stock at 10 µg/mL) just before acquisition. Remember that PI can be seen either in FL2 or FL3, and when fixing cells before analysis, you cannot add PI (all cells will be positive).

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