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# Flow cytometric characterization of murine B cell development

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**TITLE:**

# Flow cytometric characterization of murine B cell development

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**KEYWORDS:**

B cells, lymphocytes, development, differentiation, peritoneum, bone marrow, spleen, flow cytometry, mice

**SUMMARY:**

We describe herein a simple analysis of the heterogeneity of the murine immune B cell compartment in the peritoneum, spleen, and bone marrow tissues by flow cytometry. The protocol can be adapted and extended to other mouse tissues.

**ABSTRACT:**

Extensive studies have characterized the development and differentiation of murine B cells in secondary lymphoid organs. Antibodies secreted by B cells have been isolated and developed into well-established therapeutics. Validation of murine B cell development, in the context of autoimmune prone mice, or in mice with modified immune systems, is a crucial component of developing or testing therapeutic agents in mice and is an appropriate use of flow cytometry. Well established B cell flow cytometric parameters can be used to evaluate B cell development in the murine peritoneum, bone marrow, and spleen, but a number of best practices must be

adhered to. In addition, flow cytometric analysis of B cell compartments should also complement additional readouts of B cell development. Data generated using this technique can further our understanding of wild type, autoimmune prone mouse models as well as humanized mice that can be used to generate antibody or antibody-like molecules as therapeutics.

**INTRODUCTION:**

Monoclonal antibodies have increasingly become the choice therapy for many human diseases as they become part of mainstream medicine[1,2]. We have previously described genetically engineered mice which efficiently produce antibodies harboring fully human variable regions with mouse IgH constants[3,4]. Most recently, we have described genetically engineered mice that produce antibody-like molecules that have distinct antigen-binding[5]. Antibodies are secreted by B cells and form the basis of adaptive humoral immunity. There are two distinct types of B cells, B-1 and B-2. In mammals, B-1 cells originate in the fetal liver and are enriched in mucosal tissues and the pleural and peritoneal cavities after birth, while B-2 cells originate in the fetal liver prior to birth and thereafter in the bone marrow (BM). B-2 cells are enriched in secondary lymphoid organs including the spleen and blood[6-8]. In the BM, B-2 hematopoietic progenitors start to differentiate to pro-B cells upon the initiation of Ig mu heavy chain rearrangement[9,10]. Successful rearrangement of Ig heavy chain and its assembly into the pre-B cell receptor (pre-BCR), along with signaling and proliferative expansion, leads to differentiation to pre-B cells. After pre-B cells rearrange their Ig kappa (Igκ), or if unproductive, Ig lambda (Igλ) light chains, they pair with μ heavy chain, resulting in surface IgM BCR expression. It is important to point out that IgM surface expression is known to be reduced under conditions of autoreactivity, thus contributing to self tolerance in functionally unresponsive or anergic B cells[11,12]. Immature B cells then enter a transitional stage, where they begin to co-express IgD and migrate from the BM to the spleen. In the spleen, IgD expression increases further and the cells mature into a second stage of transitional B cells, followed by completion of their maturation status and development into either marginal zone (MZ) or follicular (Fol) cells[13,14,15]. In adult mice, in a non-diseased setting, the number of mature B cells remains constant despite 10-20 million immature B cells being generated daily in the BM. Of these, only three percent enter the pool of mature B cells. The size of the peripheral B cell compartment is constrained by cell death, due in part to several factors including self-reactivity and incomplete maturation[16-18]. Flow cytometric analysis has been extensively used to characterize and enumerate many immune cell sub-compartments in humans and mice. While there are some similarities between human and murine B cell compartments, this protocol applies only to the analysis of murine B cells. This protocol was developed with the purpose of phenotyping genetically engineered mice, to determine whether genetic manipulation would alter B cell development. Flow cytometry has also been hugely popular in many additional applications, including in measuring cell activation, function, proliferation, cycle analysis, DNA content analysis, apoptosis and cell sorting [19,20].

Flow cytometry is the tool of choice to characterize various lymphocyte compartments in mice and humans, including in complex organs such as the spleen, BM and blood. Due to widely available mouse-specific antibody reagents for flow cytometry, this technique can be used to investigate not only cell surface proteins but also intracellular phosphoproteins and cytokines, as well as functional readouts[21]. Herein we demonstrate how flow cytometry reagents can be used

to identify B cells subsets as they mature and differentiate in secondary lymphoid organs. After optimization of staining conditions, sample handling, correct instrument set up and data acquisition, and finally data analysis, a protocol for comprehensive flow cytometric analysis of the B cell compartment in mice can be utilized. Such comprehensive analysis is based on a decades old nomenclature devised by Hardy and colleagues, where developing BM B-2 cells can be divided into different fractions (Fraction) depending on their expression of B220, CD43, BIP-1, CD24, IgM and IgD[22]. Hardy et al., showed that $B220^+$ CD43 BM B cells can be subdivided into four subsets (Fraction A-C') on the basis of BP-1 and CD24 (30F1) expression, while $B220^+$ $CD43^{-(dim\ to\ neg)}$ BM B cells can be resolved into three subsets (Fraction D-F) based on differential expression of IgD and surface IgM[23]. Fraction A (pre-pro-B cells) are defined as $BP\text{-}1^-$ $CD24\ (30F1)^-$, Fraction B (early pro-B cells) are defined as $BP\text{-}1^-$ $CD24\ (30F1)^+$, Fraction C (late pro-B cells) are defined as $BP\text{-}1^+$ $CD24\ (30F1)^+$, and Fraction C' (early pre-B cells) are defined as $BP\text{-}1^+$ and $CD24^{high}$. Furthermore, Fraction D (pre-B cells) are defined as $B220^+$ $CD43^-$ $IgM^-$ B cells, and Fraction E (newly generated B cells, combination of immature and transitional) are defined as $B220^+$ $CD43^-$ $IgM^+$ B cells and Fraction F (mature, recirculting B cells) are defined as $B220^{high}$ $CD43^-$ $IgM^+$ B cells. In contrast, the majority of naïve B cells found in the spleen can be divided into mature ($B220^+$ $CD93^-$) B cells and transitional (T1, T2, T3) cells depending on expression of CD93, CD23 and IgM. Mature B cells can be resolved into marginal zone and follicular subsets based on expression of IgM and CD21/CD35, and follicular subsets can be further divided into mature follicular type I and follicular type II B cell subsets depending on the level of their IgM and IgD surface expression[24]. These splenic B cell populations express predominantly Igκ light chain. Finally, B-1 B cell populations, which originate in the fetal liver and are mainly found in the peritoneal and pleural cavities of adult mice, have been described in the literature. These peritoneal B cells can be distinguished from the previously described B-2 B cells by their lack of CD23 expression. They are then further subdivided into B-1a or B-1b populations, with the former defined by the presence of CD5 and the latter by its absence[25]. B-1 cell progenitors are abundant in the fetal liver, but are not found in adult BM. While B-1a and B-1b cells originate from different progenitors, they both seed the peritoneal and pleural cavities[24]. In contrast to B-2 cells, B-1 cells are uniquely capable of self-renewal and are responsible for production of natural IgM antibodies.

Defects in B cell development can arise in many instances, including deficiencies in the components of the BCR[26,27], perturbations of signaling molecules that impact BCR signaling strength[14,28,29], or disruption of cytokines that modulate B cell survival[30,31]. Flow cytometry analysis of the lymphoid compartments has contributed to the characterization of the B cell development blocks in these mice and many others. One advantage of flow cytometric analysis of lymphoid compartments is that it offers the ability to make measurements on individual cells obtained from live dissociated tissue. The availability of reagents in an ever-expanding range of fluorophores allows for the simultaneous analysis of multiple parameters and enables the assessment of B cell heterogeneity. Furthermore, enumeration of B cells by flow cytometric analysis complements other immunological assays such as immunohistochemistry methods that visualize cell localization within lymphoid organs, detection of circulating antibody levels as a measure of humoral immunity, as well as two photon microscopy to measure B cell responses in real space and time[32].

**PROTOCOL:**

All mouse studies were overseen and approved by Regeneron's Institutional Animal Care and Use Committee (IACUC). The experiment was conducted on tissues from three C57BL/6J female mice (17 weeks of age) from Jackson Laboratories. Titrate all antibodies prior to starting the experiment to determine ideal concentration. When using compensation beads for single-color compensation, ensure they stain as bright or brighter than your samples. Keep all buffers, antibodies, and cells on ice or at 4 °C. After the addition of viability dye, perform all steps and incubations at 4°C in either low light or in the dark.

**1. Peritoneal cell harvest and single cell isolation**

1.1. Euthanize the mouse using $CO_2$ or according to approved protocol.

1.2. Lay the mouse on its back, spray with 70% ethanol, and cut outer abdominal skin with scissors, being careful not to cut the peritoneum.

1.3. Inject 3 mL of ice-cold wash buffer (0.5% bovine serum albumin (BSA) in DPBS [vol/vol]) into the peritoneal cavity with a 3 mL syringe fitted with a 25 gauge needle.

1.4. Gently massage the peritoneum with fingertips.

1.5. Repeat steps 1.3 and 1.4.

1.6. Insert a 3 mL syringe fitted with an 18 G needle through the peritoneum, being careful to avoid organs and fat.

1.7. Extract the wash buffer, now containing peritoneal cells, and transfer to 15 mL conical tube on ice.

1.8. Repeat steps 1.3 and 1.4.

1.9. Cut a small hole in the peritoneum while holding up with tweezers.

1.10. Insert a disposable transfer pipette into the hole and collect the remaining wash buffer, once again avoiding fat and organs.

1.11. Transfer the collected remaining peritoneal cells to the 15 mL conical tube on ice.

NOTE: Discard sample if blood contamination is evident.

1.12. Incubate the cells on ice until spleen and bone extraction are complete.

1.13. Centrifuge the cells at 300 x *g* for 8 min at 4 ˚C. Aspirate the supernatant.

1.14. Resuspend the cell pellet in 1 mL of wash buffer.

1.15. Filter the cells through a 70 µM cell strainer into a clean 15 mL conical tube on ice.

1.16. Determine the cell concentration using a cell counter instrument or hemocytometer.

**2. Spleen harvest and single cell isolation**

2.1. Lay the mouse on its belly and cut through the peritoneum on the left backside using clean scissors. Cut out the spleen, removing fat and connective tissue.

2.2. Transfer the spleen to a 1.5 mL microcentrifuge tube containing 1 mL of wash buffer on ice.

2.3. Incubate the spleen on ice until bone extraction is complete.

2.4. Move the spleen to automated dissociation tube with 5 mL of red blood cell lysis buffer. Place the tube on the tissue dissociator instrument and dissociate for 60 s to create a single cell suspension.

NOTE: It is also permissible to use other routine methods of obtaining single-cell spleen suspensions such as smashing between frosted glass slides in wash buffer. If another method of dissociation is used, follow the dissociation with centrifugation, aspiration, and then resuspension in 5 mL of red blood cell lysis buffer before continuing to step 2.5.

2.5. Incubate the cells at room temperature for 3 min.

2.6. Add 10 mL of 4 ˚C wash buffer containing 2mM EDTA.

2.7. Transfer to a clean 15 mL conical tube.

2.8. Centrifuge the cells at 300 x *g* for 8 min at 4 ˚C. Aspirate the supernatant.

2.9. Resuspend the cell pellet in 5 mL of 4 ˚C wash buffer.

2.10. Filter the cells through a 70 µM cell strainer into a clean 15 mL conical tube on ice.

2.11. Determine the cell concentration using a cell counter instrument or hemocytometer.

**3. BM harvest and single cell isolation**

3.1. Remove the skin from the lower half of the mouse body. Trim the excess muscle from leg. Remove the entire leg with scissors, being careful not to cut the femur. Clean the femur and tibia by removing remaining muscle, fat, and feet.

3.2. Transfer the bones to a 1.5 mL microcentrifuge tube containing 1 mL of wash buffer on ice.

3.3. Perforate the bottom of a 0.5 mL microcentrifuge tube, leaving a hole small enough for leg bones not to protrude. Insert the 0.5 mL tube into a clean 1.5 mL microcentrifuge tube. Snip off the end of the femur and tibia proximal to the knee and place the cut ends facing down into the 0.5 mL tube.

3.4. Centrifuge the cells at 6,780 x *g* for 2 min at 4 ˚C.

3.5. Resuspend the cell pellet in 1 mL of red blood cell lysis buffer and transfer to a 15 mL conical tube containing an additional 3 mL of red blood cell lysis buffer.

3.6. Incubate at room temperature for 3 min.

3.7. Add 10 mL of 4 ˚C wash buffer containing 2mM EDTA.

3.8. Centrifuge the cells at 300 x *g* for 8 min at 4 ˚C. Aspirate the supernatant.

3.9. Resuspend the cell pellet in 3 mL of 4 ˚C wash buffer.

3.10. Filter cells through a 70 µM cell strainer into a clean 15 mL conical tube on ice.

3.11. Determine the cell concentration using a cell counter instrument or hemocytometer.

**4. Stain cells and prepare compensation**

4.1. Aliquot $10^6$ cells of each cell type from each animal to a 96 well U bottom plate.

4.1.1. Make sure to include enough wells for all samples and controls, including full stain, fluorescence-minus-one (FMO), unstained, and finally the optional single-color compensation for each fluorophore used.

4.1.2. For the BM maturation panel and the spleen maturation panel, aliquot cells into 2 wells, $10^6$ cells per well, for each full stain sample. For the single-color compensation viability controls, add 2 x $10^6$ cells of each cell type to individual wells.

4.2. Centrifuge the plate at 845 x *g* for 2 min at 4 ˚C. Decant the supernatant by quickly inverting and flicking the plate over a sink, being careful not to cross-contaminate wells.

4.3. Resuspend the cells in 200 µL of DPBS (without BSA or FBS). This step is important to remove protein before staining with amine-reactive viability dye.

4.4. Repeat steps 4.2 and 4.3.

4.5. Repeat step 4.2.

4.6. Resuspend the cells in 100 µL viability dye diluted 1:1,000 in DPBS.

NOTE: If using cells for single-color compensation, do not add viability dye to those wells.

4.6.1. For each stain set, leave several unstained wells for a completely unstained sample and any other controls you might need.

4.6.2. For each stain set, leave an additional unstained well for the viability FMO control.

4.6.3. For the single-color viability compensation controls: Resuspend the 2 x $10^6$ cells, aliquoted in step 4.1, in 200 µL of diluted viability dye. Transfer 100 µL of cells to a 1.5 mL microcentrifuge tube, heat cells for 5 min at 65 ˚C, and transfer the 100 µL of heat-killed cells back to the original well with the 100 µL remaining live cells.

4.7. Incubate cells at 4 ˚C, protected from light, for 30 min.

4.8. Centrifuge the plate at 845 x *g* for 2 min at 4 ˚C. Decant the supernatant by quickly inverting and flicking the plate over a sink, being careful not to cross-contaminate wells.

4.9. Resuspend the cells in 200 µL of DPBS (without BSA or FBS).

4.10. Repeat steps 4.8 and 4.9.

4.11. Repeat step 4.8.

4.12. Resuspend the cells in 50 µL of Fc block diluted 1:50 (final concentration=10 µg/mL) in stain buffer (0.5% BSA in DPBS [vol/vol]).

4.12.1. For peritoneal cells – also add 5 µL of monocyte blocker to reduce non-specific staining.

4.13. Incubate the cells at 4 ˚C, protected from light, for 15 min.

4.14. Prepare full stain master mixes and FMOs in stain buffer for a final volume of 100 µl per $10^6$ cells. Refer to **Table 1-Table 4** for the antibody lists.

NOTE: FMOs are made by including all antibodies in a stain set except one. Prepare an FMO for each antibody in a stain set. When a stain set contains multiple brilliant dyes, substitute 50 µL of brilliant stain buffer for stain buffer per sample

4.15. Without removing Fc block, add 100 µL of full stain mixes and FMOs to selected wells.

4.16. Prepare single-color compensation controls for each antibody in a stain set.

4.16.1. If using compensation beads follow the manufacture's directions for use.

4.16.2. If using cells, add titrated antibody to $10^6$ cells, reserved previously in step 4.6.1 without viability dye, in 100 µL stain buffer. If all cells in the sample are positive for a particular marker, set aside unstained cells to be used when acquiring compensation data on the flow cytometer.

4.17. Incubate the cells and beads at 4 ˚C, protected from light, for 30 min.

4.18. Centrifuge the plate at 845 x *g* for 2 min at 4 ˚C. Decant the supernatant by quickly inverting and flicking the plate over a sink, being careful not to cross-contaminate wells.

4.19. Resuspend the cells and beads in 200 µL of stain buffer.

4.20. Repeat steps 4.18 and 4.19 two times.

4.21. Repeat step 4.18.

4.22. To fix the samples for analysis within 48 h, resuspend cells and beads in 200 µL of 2% paraformaldehyde in DPBS.

CAUTION: Parafomaldehyde is a serious health hazard and flammable. Refer to the Safty Data Sheet before use.

4.23. Incubate the cells and beads at 4 ˚C, protected from light, for 30 min.

4.24. Repeat steps 4.18 and 4.19 two times.

4.25. Place a filter plate over a clean 96 well U-bottom plate. Using a multi-pipette, transfer each sample to a well of the filter plate.

4.26. Centrifuge the filter plate–96 well U-bottom plate setup at 845 x *g* for 2 min at 4 ˚C. Remove the filter plate and decant the supernatant by quickly inverting and flicking the plate over a sink, being careful not to cross-contaminate wells.

4.27. For the BM and spleen maturation panels resuspend the fully stained cells in 100 µL of stain buffer. Combine the 2 wells for each animal into 1 well. Resuspend the remaining panels, FMOs, and controls in 200 µL of stain buffer.

4.28. Incubate fixed cells and beads at 4 ˚C, protected from light, overnight.

**5. Flow cytometric data acquisition**

5.1. Initialize and QC the flow cytometer as per manufacturer instructions.

5.2. Load the template specific for each panel.

5.3. Prior to recording data, ensure all events for each sample are on scale and visible on the dot plots.

5.4. Record compensation controls for each stain panel using single stain compensations prepared in step 4.16. Set positive and negative gates for each sample. Have the software calculate the compensation matrix.

5.5. Start acquiring the first sample, and ensure gates are set appropriately.

5.6. Set the machine to record at least 50,000 B cell events for the peritoneal B cell panel and spleen Igκ and Igλ panel; 150,000 B cell events for the BM maturation panel; and 300,000 B cell events for the spleen maturation panel.

5.7. For each stain panel, run and record the fully stained samples for each animal, an unstained sample, and the FMOs.

**6. Analyze data**

6.1. Proceed with data analysis using flow cytometry analysis software. Follow gating strategies outlined in **Figure 1-Figure 4**.

**REPRESENTATIVE RESULTS:**

Here we present the gating strategy for characterizing B cell development in mouse peritoneum, BM and spleen. The basis of the analysis is formed around the concept of staining with viability dye, then gating out doublets based on the Forward-Scatter-Area (FSC-A) and Forward-Scatter-Height (FSC-H), and finally gating out debris by selecting cells according to their FSC-A and Side-Scatter-Area (SSC-A) characteristics, referred to here as the size gate, which are reflective of relative cell size and cell granularity, before gating on population of interest.

Flow cytometric analysis of peritoneal B cells shows the frequencies of viable peritoneal cells, total B cells, B-1 and B-2 subsets, as well as B-1a and B-1b cells in C57BL/6J mice (**Figure 1**), using a staining panel outlined in **Table 1**. Average absolute cell number of these frequencies are shown in **Table 5**. Perturbations in B-1 cells could be delineated by distribution of cell subsets, either by cell frequency or absolute cells number per mouse.

Flow cytometric analysis of BM B cells shows the frequencies of viable BM cells, total B cells, Fraction A (pre-pro-B cells and contaminating lymphocytes), pre-pro-B cells, Fraction B, Fraction C, Fraction C', Fraction D, immature (subset in Fraction E), transitional (subset in Fraction E), and Fraction F B cells in C57BL/6J mice (**Figure 2**), using a staining panel outlined in **Table 2**. Average absolute cell number of these frequencies are shown in **Table 6**. Perturbations in BM B cells could be delineated by distribution of cell subsets, either by cell frequency or absolute cells number

per leg(s).

Flow cytometric analysis of splenic B cells shows the frequencies of viable spleen cells, total B cells, transitional B cells, T1, T2, T3 cells, mature B cells, follicular I cells (Fol I), follicular II (Fol II) cells, marginal zone (MZ) precursor cells, mature MZ cells, and B-1 cells in C57BL/6J mice (**Figure 3**), using a staining panel outlined in **Table 3**. Average absolute cell number of these frequencies are shown in **Table 7**. Perturbations in splenic B cells could be delineated by distribution of cell subsets, either by cell frequency or absolute cells number per spleen.

Similarly, flow cytometric analysis of the spleen shows the frequencies of $Ig\kappa^+$ and $Ig\lambda^+$ B cells in C57BL/6J mice (**Figure 4**), using a staining panel outlined in **Table 4**. Average absolute cell number of these frequencies are shown in **Table 8**. Perturbations in $Ig\kappa^+$ and $Ig\lambda^+$ B cells could be delineated by distribution of cell subsets, either by cell frequency or absolute cells number per spleen.

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**FIGURE AND TABLE LEGENDS:**

**Table 1: Peritoneal B Cell Panel**

**Table 2: Bone Marrow Maturation Panel**

**Table 3: Spleen Maturation Panel**

**Table 4: Spleen Igκ and Igλ Panel**

**Table 5: Absolute Cell Numbers of Peritoneal B Cell Subsets**

**Table 6: Absolute Cell Numbers of Bone Marrow B Cell Subsets**

**Table 7: Absolute Cell Numbers of Splenic B Cell Subsets**

**Table 8: Absolute Cell Numbers of Igκ and Igλ B Cell Subsets**

**Figure 1: Characterization of B cell populations in the peritoneum.** Viable, single cell, size gated peritoneal B cells are first separated from contaminating cells by gating on $IgM^+$ cells. B-1 and B-2 cells are then distinguished from each other by absence (B-1) or presence of CD23 (B-2). Next CD5 expression is used to delineated B-1a cells ($CD5^+$) from B-1b cells ($CD5^-$). FMOs were used to empirically determine where to draw gates. Numbers are percentages of each population within the same density plot.

**Figure 2: Characterization of B cell subsets in the BM.** Viable, single cell, size gated BM B cells are separated from non-B cells by gating on $B220^+$ $dump^-$ (where dump refers to CD3/GR-1/CD11b/TER119) cells. CD43 and B220 expression further defines Hardy Fraction A-C' ($CD43^+$ $B220^+$) and Hardy Fraction D-F ($CD43^{low/neg}$ $B220^{+/++}$). Fraction A-C' is further separated by expression of BIP-1 and CD24. Fraction A ($BIP\text{-}1^-$ $CD24^-$) corresponds to pre-pro-B cells along with contaminating cells. To separate pre-pro-B cells from contaminating cells in Fraction A, the expression of CD93 and the absence of CD19 are utilized. Fraction B ($BIP\text{-}1^-$ $CD24^{int}$) and Fraction C ($BIP\text{-}1^+$ $CD24^{int}$) correspond to early and late pro-B cells, respectively, and Fraction C' ($BIP\text{-}1^{+/-}$ $CD24^+$) corresponds to early pre-B cells. To separate Fraction D-F, expression of IgM and IgD are utilized. Fraction D corresponds to late pre-B cells ($IgM^{-/low}$ $IgD^-$); Fraction E (blue gate, $IgM^{int/high}$ $IgD^-$) to both immature (Imm, $IgM^{int}$ $IgD^-$) and transitional (Tran, $IgM^{high}$ $IgD^-$) B cells; and Fraction F ($IgM^{int/high}$ $IgD^+$) to recirculating mature B cells. FMOs were used to empirically determine where to draw gates. Numbers are percentages of each population within the same density plot

**Figure 3: Characterization of splenic B cell maturation.** Viable, single cell, size gated splenic B cells are separated from non-B cells by gating on $B220^+$ cells. In order to identify the B-1 subset, $CD23^-$ $CD19^+$ cells are identified and defined by expression of CD43. To classify B-2 populations, $CD19^+$ cells are separated into transitional ($CD93^+$ $B220^+$) and mature ($CD93^-$ $B220^+$) B cells. Transitional ($CD93^+$ $B220^+$) cells are further divided into T1 ($IgM^+$ $CD23^-$), T2 ($IgM^+$ $CD23^+$), and T3 ($IgM^{int}$ $CD23^+$) populations. Mature ($CD93^-$ $B220^+$) cells are separated into marginal zone ($CD21/35^+$ $IgM^+$) and follicular ($CD21/35^{int}$ $IgM^{int/+}$) B cells. The expression of CD23 is further used to separate MZ precursor ($CD23^+$ $B220^+$) cells from more mature MZ ($CD23^-$ $B220^+$) cells. Follicular populations are then delineated into Fol I ($IgD^+$ $IgM^{int}$) and Fol II ($IgD^+$ $IgM^+$) cells. FMOs were used to empirically determine where to draw gates. Numbers are percentages of each population within the same density plot

**Figure 4: Igκ and Igλ expression of splenic B cells.** Viable, single cell, size gated splenic B-cells are separated from non-B-cells by gating on $B220^+$ $CD3^-$ cells. B cells are then distinguished by the expression of Igλ and Igκ. Numbers are percentages of each population within the same density plot.

**DISCUSSION:**

Flow cytometric analysis of lymphoid and non-lymphoid tissues has enabled simultaneous identification and enumeration of B cell sub-populations in mice and humans since the 1980's. It has been used as a measure of humoral immunity and can be applied further to evaluate B cell

functionality. This method takes advantage of reagent availability to assess different stages of B cell maturation in mice and humans, by way of simultaneous analysis of multiple parameters enabling the assessment of B cell heterogeneity, even in rare populations. If used to measure complex heterogenous samples, it can detect sub-populations within minutes, on individual cells[33]. Sequential gating analysis strategy, most often applied to flow cytometric analysis, can be simple and intuitive when a specific population has to be identified[34]. Finally, another advantage of flow cytometry is that it is easily adaptable in most academic labs, while under guidance of experienced users. Our protocol successfully describes assessment of B cell populations in the peritoneum, BM, and spleens of mice, by describing and enumerating B-1 populations and delving into the development of B-2 pro-B cells, pre-B cells, immature, transitional, and mature B cells, as well as their surface expression of Igκ or Igλ light chains. Flow cytometry is the most widely used, and easiest method to apply, when investigating B cell development in mice.

While flow cytometry generates invaluable data, there are some limits to this technology when used to investigate the heterogeneity of the immune B cell compartment. Huge data sets can be overwhelming because 10 color staining allows the recognition of more than 1,024 different cell populations[34]. One must take into consideration that some commonly used lymphoid cell markers have proven to be less specific than originally thought. This can be resolved by employing a multitude of cell surface markers to ascertain gating on desired populations. While flow cytometric analysis can be simple and intuitive, another constraint to flow cytometric analysis is that it typically allows the visualization of only two parameters at a time, though data visualization tools such as t-SNE can be used to cluster cell populations more efficiently when using high parameter flow cytometry. Another important limitation is that the gates used during both the acquisition and analysis are sometimes dependent on the subjectivity of the operator.

For successful adaptation or replication of this protocol, there are several critical parameters that have to be taken into consideration[35]. Careful consideration must be taken into panel design and fluorochrome selection. It is imperative to pair dim or important antigens with bright fluorochromes. Antibody titration must be carried out to avoid excess antibody binding to cells non-specifically, potentially increasing background staining and decreasing resolution. Antibody titration is carried out by staining a known number of cells with decreasing concentrations of antibodies, to determine the best separation index[36]. This should be repeated for every lot of antibody. During sample preparation and staining, it is important to assure a single cell suspension by avoiding $Ca^{++}$ and $Mg^{++}$. Additionally, addition of EDTA can help prevent cell aggregation and enzymatic activity which can lead to antibody-mediated stimilulation and internalization of labeled markers. Prior to data acquisition, samples must be properly suspended, filtered and free of aggregates. Spillover of signal from one parameter to another is resolved by using compensation controls, in the form of single stained cells or commercially available compensation beads[35]. Another important consideration is to have proper controls in each experiment. Unstained cells establish the baseline of autofluorescence. Isotype controls are no longer considered appropriate controls for gating due to non-specific binding. The most important step in helping to make accurate gates is the use of FMO controls. In an FMO control, all conjugated antibodies are present in the stain except the one which is being controlled for. FMO controls enable the measurement of the spread of all the

fluorophores into the missing channel and hence allow for setting up gates accordingly. It is critical that enough cells are acquired for added accuracy. As a rule of thumb, at least 2,000 events of the population of interest should be collected. Lastly, compensation controls, whether beads or cells, should be exactly matched to the fluorochromes being utilized and controls must be at least as bright as the experimental samples[37].

Overall, using flow cytometric analysis of B cell compartments is widely used in the immunology field. This technique can be used to investigate perturbations in humoral immunity in both wild type and genetically modified mice, under non-disease states and upon immunological challenge.

**ACKNOWLEDGMENTS:**

We thank Matthew Sleeman for critical reading of the manuscript. We also thank the Vivarium Operations and Flow Cytometry Core departments at Regeneron for supporting this research.

**DISCLOSURES:**

All authors are employees and shareholders of Regeneron Pharmaceuticals, Inc.

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Figure 1: Characterization of B cell populations in the peritoneum.

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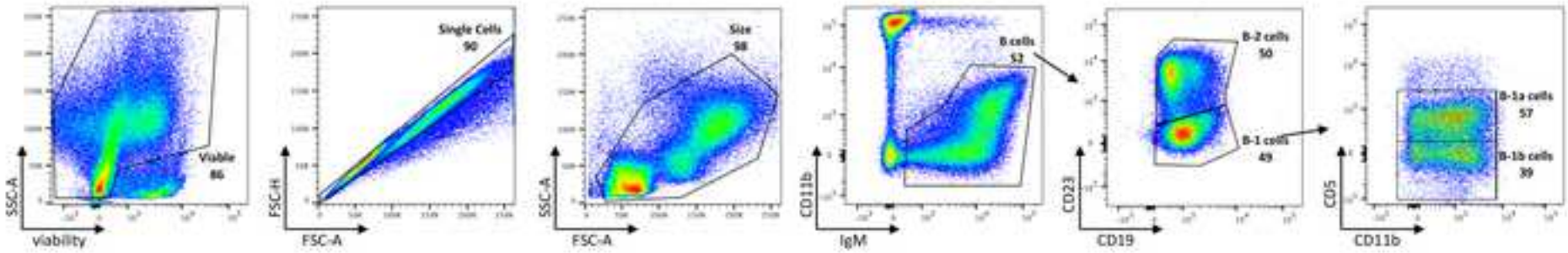

Figure 2: Characterization of B cell subsets in the BM.

Click here to access/download;Figure;Figure 2.psd

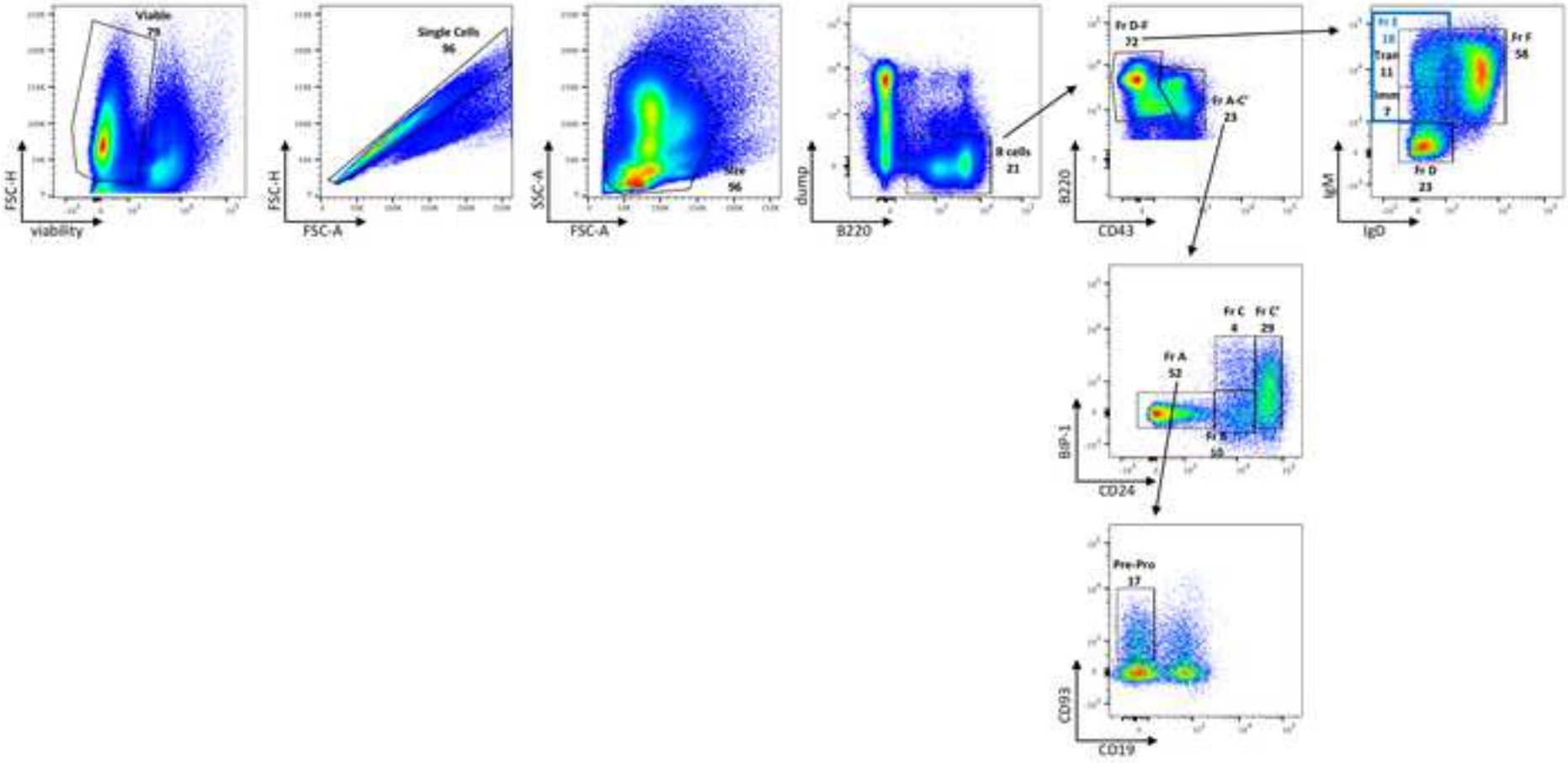

Figure 3: Characterization of splenic B cell maturation.

Click here to access/download;Figure;Figure 3.psd

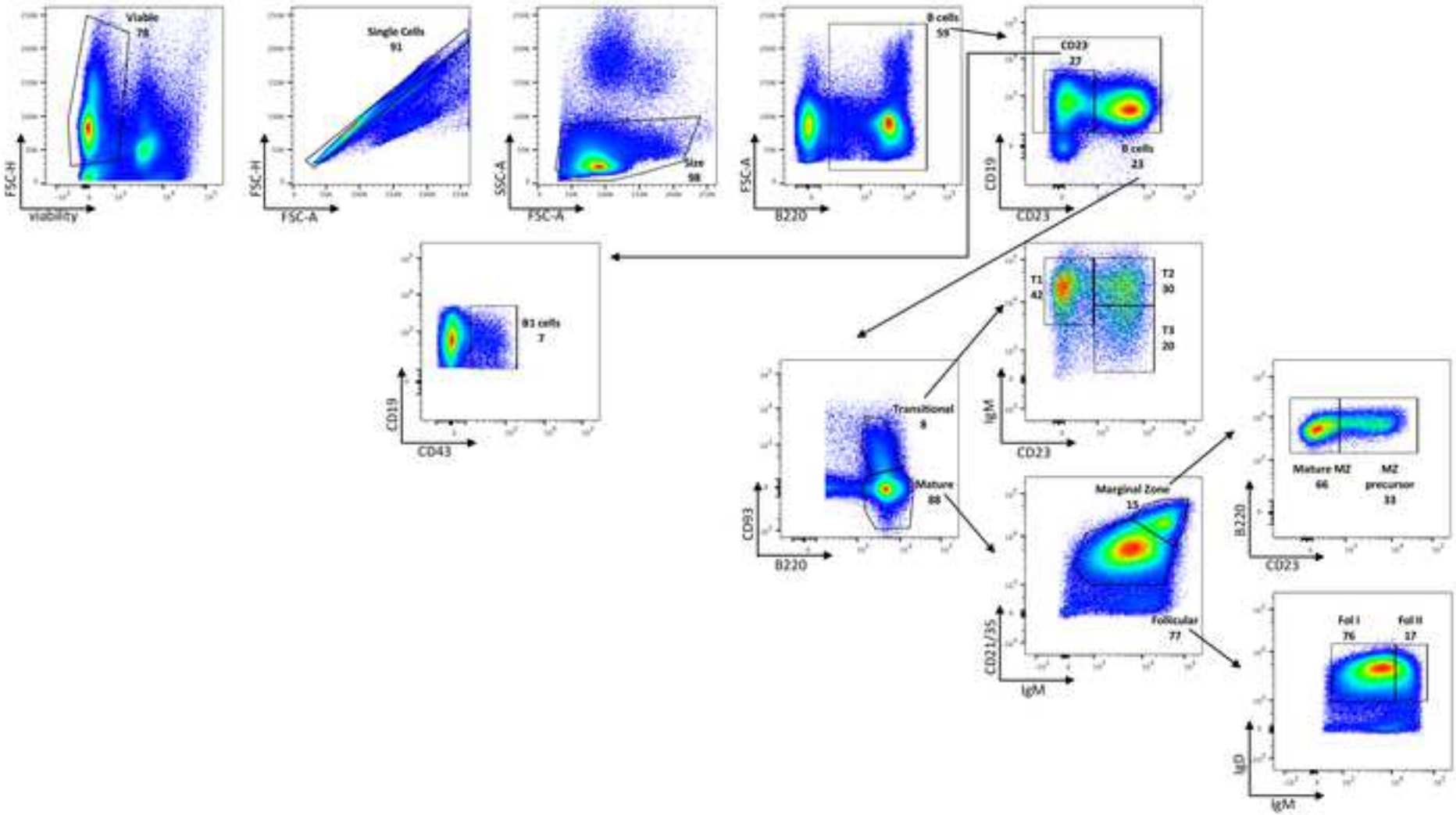

Figure 4: Igk and Igl expression of splenic B cells.

Click here to access/download;Figure;Figure 4.psd

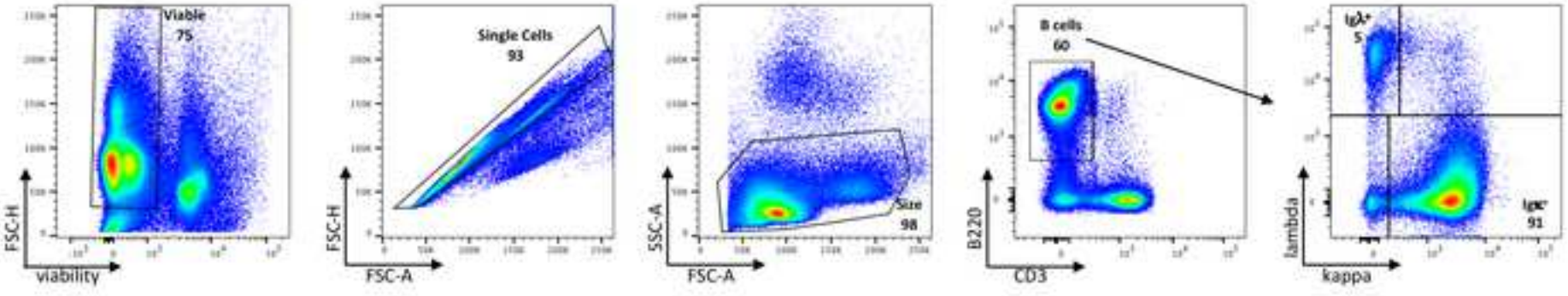

Table 1. Peritoneal B Cell Panel

| **Antibody** | **Fluorophore** | **clone** |
|---|---|---|
| CD19 | APC-H7 | 1D3 |
| B220 | APC | RA3-6B2 |
| IgM | PeCy7 | II/41 |
| IgD | PerCpCy5.5 | 11-26c.2a |
| CD43 | FITC | S7 |
| CD23 | BUV395 | B3B4 |
| CD11b | BV711 | M1/70 |
| CD5 | BV605 | 53-7.3 |

Table 2. Bone Marrow Maturation Panel

| **Antibody** | **Fluorophore** | **clone** |
|---|---|---|
| CD19 | APC-H7 | 1D3 |
| B220 | APC | RA3-6B2 |
| IgM | PeCy7 | II/41 |
| IgD | PerCpCy5.5 | 11-26c.2a |
| CD43 | FITC | 1B11 |
| CD24 (HSA) | PE | 30-F1 |
| C-Kit | BUV395 | 2B8 |
| BP-1 | BV786 | BP-1 |
| CD93 | BV711 | AA4.1 |
| **dump channel** | | |
| CD3 | AF700 | 17-A2 |
| CD11b | AF700 | M1/70 |
| GR1 (Ly6C/6G) | AF700 | RB6-8C5 |
| Ter119 | AF700 | TER-119 |

Table 3. Spleen Maturation Panel

| **Antibody** | **Fluorophore** | **clone** |
|---|---|---|
| CD19 | APC-H7 | 1D3 |
| B220 | APC | RA3-6B2 |
| IgM | PeCy7 | II/41 |
| IgD | PerCpCy5.5 | 11-26c.2a |
| CD43 | FITC | S7 |
| CD23 | BUV395 | B3B4 |
| CD21/35 | BV421 | 7G6 |
| CD11b | AF700 | M1/70 |
| CD5 | BV605 | 53-7.3 |
| CD93 | PE | AA4.1 |

Table 4. Spleen Igκ and Igλ Panel

| **Antibody** | **Fluorophore** | **clone** |
|---|---|---|
| CD19 | APC-H7 | 1D3 |
| B220 | APC | RA3-6B2 |
| IgM | PeCy7 | II/41 |
| IgD | PerCpCy5.5 | 11-26c.2a |
| CD3 | PB | 17-A2 |
| Kappa | FITC | 187.1 |
| Lambda | PE | RML-42 |

Table 5. Absolute Cell Numbers of Peritoneal B Cell Subsets

| | Absolute Cell Number | | | | |
|---|---|---|---|---|---|
| Animal Number | Viable peritoneal cells | B cells | B-1a cells | B-1b cells | B-2 cells |
| 1 | 1.02E+07 | 4.67E+06 | 1.28E+06 | 8.95E+05 | 2.35E+06 |
| 2 | 9.92E+06 | 4.52E+06 | 1.49E+06 | 9.60E+05 | 1.91E+06 |
| 3 | 1.15E+07 | 4.56E+06 | 1.71E+06 | 9.19E+05 | 1.78E+06 |
| Average | 1.05E+07 | 4.58E+06 | 1.49E+06 | 9.25E+05 | 2.01E+06 |

TABLE 6: Absolute Cell Numbers of Bone Marrow B Cell Subsets



Table 6. Absolute Cell Numbers of Bone Marrow B Cell Subsets

| Animal Number | Absolute Cell Number | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Viable bone marrow cells | B cells | Fraction A | Pre-pro | Fraction B | Fraction C | Fraction C' | Fraction D | Immature | Transitional | Fraction F |
| 1 | 5.05E+07 | 9.70E+06 | 1.13E+06 | 1.95E+05 | 2.22E+05 | 9.14E+04 | 6.31E+05 | 1.59E+06 | 4.56E+05 | 7.81E+05 | 4.03E+06 |
| 2 | 5.39E+07 | 1.03E+07 | 1.14E+06 | 2.29E+05 | 2.89E+05 | 1.22E+05 | 8.40E+05 | 2.11E+06 | 5.39E+05 | 8.07E+05 | 3.67E+06 |
| 3 | 5.93E+07 | 1.01E+07 | 1.10E+06 | 2.12E+05 | 2.84E+05 | 1.05E+05 | 9.02E+05 | 2.72E+06 | 5.94E+05 | 7.62E+05 | 2.59E+06 |
| Average | 5.46E+07 | 1.00E+07 | 1.12E+06 | 2.12E+05 | 2.65E+05 | 1.06E+05 | 7.91E+05 | 2.14E+06 | 5.29E+05 | 7.83E+05 | 3.43E+06 |

TABLE 7: Absolute Cell Numbers of Splenic B Cell Subsets



Table 7. Absolute Cell Numbers of Splenic B Cell Subsets

| Animal Number | Absolute Cell Number | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Viable Spleen Cells | B cells | Transitional B cells | T1 cells | T2 cells | T3 cells | Mature B cells | Follicular I cells | Follicular II cells | Precursor marginal zone cells | Mature marginal zone cells | B-1 cells |
| 1 | 9.16E+07 | 4.61E+07 | 3.66E+06 | 1.55E+06 | 1.10E+06 | 7.16E+05 | 4.06E+07 | 2.39E+07 | 5.27E+06 | 2.17E+06 | 3.98E+06 | 8.83E+05 |
| 2 | 9.97E+07 | 5.18E+07 | 4.88E+06 | 1.97E+06 | 1.57E+06 | 1.00E+06 | 4.49E+07 | 2.68E+07 | 7.33E+06 | 3.42E+06 | 3.84E+06 | 8.15E+05 |
| 3 | 1.02E+08 | 5.34E+07 | 4.64E+06 | 1.98E+06 | 1.41E+06 | 8.54E+05 | 4.62E+07 | 2.81E+07 | 5.84E+06 | 3.58E+06 | 4.02E+06 | 1.01E+06 |
| Average | 9.77E+07 | 5.04E+07 | 4.39E+06 | 1.83E+06 | 1.36E+06 | 8.58E+05 | 4.39E+07 | 2.63E+07 | 6.15E+06 | 3.06E+06 | 3.94E+06 | 9.02E+05 |

Table 8. Absolute Cell Numbers of Igκ and Igλ B Cell Subsets

| | Absolute Cell Number | | | |
|---|---|---|---|---|
| Animal Number | Viable spleen cells | B cells | Igκ$^+$ B cells | Igλ$^+$ B cells |
| 1 | 9.16E+07 | 4.97E+07 | 4.51E+07 | 2.46E+06 |
| 2 | 9.97E+07 | 5.63E+07 | 5.08E+07 | 3.16E+06 |
| 3 | 1.02E+08 | 5.91E+07 | 5.33E+07 | 3.24E+06 |
| Average | 9.77E+07 | 5.50E+07 | 4.97E+07 | 2.95E+06 |

Table of Materials

Click here to access/download;Table of Materials;Materials_Table.xlsx

| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| 0.5 mL safe-lock Eppendorf tubes | Eppendorf | 22363611 | 0.5 mL microcentrifuge tube |
| 1.5mL Eppendorf tubes | Eppendorf | 22364111 | 1.5 mL microcentrifuge tube |
| 15 mL Falcon tubes | Corning | 352097 | 15 mL conical tube |
| 18 gauge needle | BD | 305196 | |
| 25 gauge needle | BD | 305124 | |
| 3 mL syringe | BD | 309657 | |
| 70 μM MACS SmartStrainer | Miltenyi Biotec | 130-110-916 | 70 μM cell strainer |
| 96 well U bottom plate | VWR | 10861-564 | |
| ACK lysis buffer | GIBCO | A1049201 | red blood cell lysis buffer |
| Acroprep Advance 96 Well Filter Plate | Pall Corporation | 8027 | filter plate |
| B220 | eBiosciences | 17-0452-82 | |
| BD CompBead Anti-Mouse Ig/κ | BD | 552843 | compensation beads |
| BD CompBead Anti-Rat Ig/κ | BD | 552844 | compensation beads |
| Bovine Serum Albumin | Sigma-Aldrich | A8577 | BSA |
| BP-1 | BD | 740882 | |
| Brilliant Stain Buffer | BD | 566349 | brilliant stain buffer |
| C-Kit | BD | 564011 | |
| CD11b | BD | 563168 | |
| CD11b | BioLegend | 101222 | |
| CD19 | BD | 560143 | |
| CD21/35 | BD | 562756 | |
| CD23 | BD | 740216 | |
| CD24 (HSA) | BioLegend | 138504 | |
| CD3 | BD | 561388 | |
| CD3 | BioLegend | 100214 | |
| CD43 | BD | 553270 | |
| CD43 | BioLegend | 121206 | |
| CD5 | BD | 563194 | |
| CD93 | BD | 740750 | |
| CD93 | BioLegend | 136504 | |
| DPBS (1x) | ThermoFisher | 14190-144 | DPBS |

| | | | |
|---|---|---|---|
| eBioscience Fixable Viability Dye eFluor 506 | ThermoFisher | 65-0866-14 | viability dye |
| Extended Fine Tip Transfer Pipette | Samco | 233 | disposable transfer pipette |
| FACSymphony A3 flow cytometer | BD | custom order | flow cytometer |
| Fc Block, CD16/CD32 (2.4G2) | BD | 553142 | Fc block |
| FlowJo | Flowjo | | flow cytometer analysis software |
| gentleMACS C Tubes | Miltenyi Biotec | 130-096-334 | automated dissociation tube |
| gentleMACS Octo Dissociator with Heaters | Miltenyi Biotec | 130-095-937 | tissue dissociator instrument |
| GR1 (Ly6C/6G) | BioLegend | 108422 | |
| IgD | BioLegend | 405710 | |
| IgM | eBiosciences | 25-5790-82 | |
| Kappa | BD | 550003 | |
| Lambda | BioLegend | 407308 | |
| paraformaldehyde, 32% Solution | Electron Microscopy Sciences | 15714 | |
| Ter119 | BioLegend | 116220 | |
| True-Stain Monocyte Blocker | BioLegend | 426103 | monocyte blocker |
| UltraPure EDTA, pH 8.0 | ThermoFisher | 15575038 | EDTA |
| Vi-CELL XR | Beckman Coulter | 731050 | cell counter instrument |

Oct 26, 2020

Dear Dr. Bajaj,

**Subject:** Re-submission of JoVE61565.

**Title:** Flow cytometric characterization of murine B cell development

**Authors:** Faith M. Harris, Karoline A. Meagher, Maggie Zhong, Benjamin J. Daniel, Mark Eckersdorff, Jesse A. Green , Vera Voronina, Chunguang Guo, Andre Limnander and Lynn E. Macdonald

Thank you for the invitation to submit the manuscript and for previously sending it out for editorial and peer review.

The authors would like to thank the reviewers for their time, comments and expertise. The reviewer's comments have helped us further strengthen our manuscript. The manuscript has been improved according to the suggestions of the Editor and reviewers. The specific responses to the reviewer's comments are below.

**Editorial comments:**
1. Manuscript was revised by proofreading.
2. Manuscript was re-formatted as per JoVE .
3. Manuscript was revised to include alternate methods in the protocol section.
4. Manuscript was revised by removing all commercial language, including trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. We have also removed VelocImmune® and Kappa-on-Heavy (KoH) terminology in the body.
5. Manuscript was revised to include a clear statement of the overall goal of this method, the rationale, advantages over alternative techniques, a description of the context of the technique in the wider body of literature, information to help readers to determine whether the method is appropriate for their application.
6. Manuscript was revised such that the protocol section is in the imperative tense.
7. Manuscript was revised such that the protocol only contains action items that direct the reader to do something.
8. Manuscript was revised by adding more details to the protocol steps.
9. Manuscript was revised to ensure that individual steps of the protocol only contain 2-3 actions sentences per step.
10. Manuscript was revised by highlighting the filmable content.
11. Manuscript was revised such that figure legends include a title and a short description of the data presented.
12. Manuscript was revised such that the results show a summary of the experiment, in line with the title of the manuscript. Additional controls were added.
13. Copyright permission is no longer needed as the experiment was re-done in WT mice (not previously published).

14. Manuscript was revised such that the discussion includes the critical steps, limitations of the technique and clearer significance of the technique.
15. Manuscript was revised such that journal titles are not abbreviated in the reference section.
16. Manuscript was revised such that the materials table is in alphabetical order.

**Reviewer #1:**

Major Concerns: none

Minor Concerns:
1. An alternate method of spleen processing has been added.
2. Greek characters/ symbols were corrected.
3. The names of the populations were revised from "R" to the name of the population.

**Reviewer #2:**

1-All references to VelocImmune®mice and Kappa-on-Heavy were removed from the abstract.
2- Manuscript was revised by deleting "Thus, the size of the B cell compartment should remain unchanged under steady state conditions."
3- Manuscript was revised such that immature and transitional do not sound like synonyms.
4- Manuscript was revised to make it clear that flow cytometry and immunohistochemistry have two non-overlapping purposes.
5- Manuscript was revised to emphasize that the enclosed protocol is for mouse, not human, B cells.
6- The original manuscript (and the re-submission) did list Fc block in the materials section.
7- The names of the populations were revised from "R" to the name of the population.
8- The experiment was reproduced in WT vendor mice rather that genetically engineered mice.
9- Manuscript was revised to include R. R. Hardy gating, nomenclature and references.
10- Manuscript was revised to include all leukocytes in the gate.
11- Manuscript was revised to include viability dye.
12- Manuscript was revised such that Figures 1 and 2 cannot be easily combined into a single figure.

**Reviewer #3:**
Major Concerns: none
Minor Concerns: none

**Reviewer #4:**
Major Concerns:
1. Manuscript was revised to include quantitative analysis of B cell populations.
2. Manuscript was revised to include FMO controls.

Minor Concerns:
1. Manuscript was revised to included published scenarios where B cell development is altered.

2. Manuscript was revised to include the strain background and age of mice

**Reviewer #5:**
Major Concerns: None.

Minor Concerns:
1. Manuscript was revised to make a distinction between phases of animal development and location of B cell differentiation.
2. Manuscript was revised to include R. R. Hardy gating, nomenclature and references.
3. Manuscript was revised to state Fraction F are mature, recirculating B cells.

We hope that our modifications make our manuscript in its current form more suitable for publication in JoVE.

Your truly, on behalf of the authors.

Karoline Meagher, Ph.D.

Faith Harris received her MS in cell biology from Brigham Young University where she studied membrane fluidity in phospholipid bilayers. In 2009 she joined Regeneron and currently holds the position of Lead Research and Development Specialist in the VelocImmune-NEXT technology development group. Her research focuses on evaluating and improving mouse models for antibody generation. Prior to joining Regeneron, she worked at the Boyce Thompson Institute identifying maize photosynthetic mutants and at the J. David Gladstone Institute studying the role of apolipoprotein E in both Alzheimer's and cardiovascular disease.

Karoline A. Meagher (née Hosiawa) is an experienced Immunologist whose work focused on understanding immune regulation in animal models of disease and building improved mouse models to study human antibody effector function. She received her Ph.D. from the University of Western Ontario where she investigated the role of IL-12 and CD80/CD86 costimulatory molecules in cardiac xenotransplantation rejection. She continued her study of immune regulation as a Postdoctoral Fellow at the University of California San Francisco. She then joined a Genomic Technology Development Group at Regeneron Pharmaceuticals in 2009, where she currently holds the position of Senior Staff Scientist. Her work at Regeneron has focused on the development and evaluation of Fc receptor and complement system humanized mouse models, as well as target discovery for biotherapeutic development.

Maggie X. Zhong received her master's degree in biotechnology from Columbia University. Currently, she is a Lead R&D Specialist in a technology development group at Regeneron Pharmaceuticals Inc that focuses on investigation and development of new antibody and cell-based therapies. She is a key player in immunophenotyping of genetically modified small animal models of human immune diseases, development of antibody and antibody-like therapeutic agents, as well as T-cell based therapeutics for immuno-oncology indications. Before joining Regeneron, she worked in the Laboratory of Antimicrobial Immunity at Memorial Sloan-Kettering Cancer Center, where she contributed to the investigation of how the immune system combats infection, specifically the role immune cells such as T lymphocytes play in fighting bacterial infection.

Benjamin J. Daniel is the Director of the Research Flow Cytometry Core at Regeneron. He received his Ph.D. in 2005 from Baylor University where he focused on immunological responses to the pathogen *Toxoplasma gondii*. During his postdoctoral work at the University of Texas Health Sciences Center in San Antonio, his work focused on anti-tumor immunity and the role of regulatory T cells and Myeloid Derived Suppressor Cells in the context of aging. Prior to joining Regeneron, he was research faculty in the Department of Microbiology and Immunology and the Director of the Flow Cytometry Core at the University of Texas Health Science Center in San Antonio.

Mark Eckersdorff is a Project Manager and Operations Associate at Regeneron Pharmaceuticals. He supports the Tech Development group with pipeline tracking, lab management, IT, administrative, and operational functions. Mark received his BS in Neurobiology from the University of Connecticut and his PMP from the Project Management Institute. Mark spent the

first fifteen years of his career working in labs in the fields of DNA repair and VDJ recombination, Aging, and Metabolic Disease before joining the Technology Development Center at Regeneron.

Jesse Green obtained his PhD in Biomedical Sciences from the University of California, San Francisco, where he worked in Jason Cyster's lab to study the regulation of spatial organization and growth homeostasis in germinal center B cells. He then performed his post-doctoral studies in Alexander Rudensky's lab at Memorial Sloan Kettering Cancer Center in New York, where he researched the role of regulatory T cells in wound healing and tumor growth. Now, Jesse works at Regeneron Pharmaceuticals, where he helps to develop new technologies aiding antibody discovery, immune cell engineering, and T cell-driven allergic responses.

Dr. Vera Voronina worked for Regeneron Pharmaceuticals from 2008 until 2020, growing from a Scientist to an Associate Director of a Technology Development group. Her work focused on generation and characterization of mouse lines that produce fully human T cell receptors and antibodies. Prior to joining Regeneron, she completed post-doctoral training in cell biology and signaling at the University of Washington, where she identified mechanisms underlying primary ciliary dyskinesia. Dr. Voronina obtained a PhD jointly from the National Cancer Institute and West Virginia University, and her thesis work focused on elucidation of genetic causes of anophthalmia and microphthalmia.

Chunguang Guo joined the VelocImmune-NEXT technology development group at Regeneron Pharmaceuticals in 2014. Major projects of his team include generation and characterization of mouse models that produce fully human T cell receptors, antibodies, and antibody-like molecules; reagent screening and condition optimization for ex vivo gene therapy and oligonucleotide therapy; as well as molecule screening and regimen development for next generation CAR-T, TCR-T, and antibody therapies. Chunguang Guo obtained his PhD in Molecular Biology and Microbiology from Case Western Reserve University, where his work was to functionally characterize a novel gene in colon cancer development. His postdoctoral training at Harvard Medical School focused on mechanisms of antibody recombination and DNA repair.

Dr. Andre Limnander joined Regeneron Pharmaceuticals in 2014 and is currently Senior Staff Scientist in Immunology and Inflammation. His work focuses on the sources of pathogenic antibody production in autoimmunity and allergy. Prior to joining Regeneron, he worked at the University of California, San Francisco (UCSF) where he characterized molecular mechanisms that control B cell and T cell tolerance. He obtained his PhD at Columbia University in 2005, where he worked on signaling mechanisms enabling leukemic cell transformation.

Lynn Macdonald is Vice President, Research at Regeneron Pharmaceuticals heading the VelocImmune-NEXT technology development group, Neuroscience group, Molecular Profiling & Data Sciences group, DNA core & Automation group. Since joining Regeneron in 1999, she has been a key contributor to Regeneron's ground-breaking VelociSuite of genomics technologies. Specifically, Lynn played a critical role in developing VelociGene®, a novel technology for the modification of the mouse genome, and subsequently worked as part of a

team that used VelociGene® technology to develop the VelocImmune® Mouse. This humanized mouse strain has been used to develop fully human antibodies that can be rapidly translated into treatments for human disease.

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