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Multiparameter Flow Cytometry and Bioanalytics for B Cell Profiling in Systemic Lupus Erythematosus

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Abstract

B lymphocyte involvement in systemic lupus erythematosus has been recognized for several decades, mainly in the context of autoantibody production. Both mouse and human studies reveal that different types of antibody responses, as well as antibody-independent effector functions can be ascribed to distinct subpopulations (subsets) of circulating B cells. Characterizing human B cell subsets can advance the field of autoimmunity even further by establishing B cell signatures associated with disease severity, progression, and response-to-treatment. For this purpose, we have developed specialized B cell reagent panels for multiparameter flow cytometry, and combine their use with advanced bioinformatics strategies that together will likely be advantageous for improving the characterization, prognosis, and for possibly improving treatment regimens of chronic inflammatory diseases such as lupus.

Keywords

B lymphocyte; Autoantibody; 9G4; Transitional B cell; Mature-naïve B cell; Memory B cell; Antibody-secreting cell; B cell subset; Systemic lupus erythematosus; Principal component analysis

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disorder whose clinical complications include skin rashes, joint pain, and in more severe cases, kidney and nervous system pathology (1, 2). Due to the enormous heterogeneity of symptom types, severity, kinetics, and response-to-treatment among patients (3), establishing reliable biological indicators associated with different outcomes (biomarkers) is essential for improving diagnosis, for accuracy of prognosis, and for customizing therapies. Both T and B lymphocyte abnormalities are observed in SLE, including elevated levels of self-reactive antibodies that precede disease onset (4). The importance of B cells in SLE and the phenotypic diversity of human B cell subsets strongly suggest that phenotypic profiles of B cells could be such a biomarker (5). Herein, we review B cell phenotypic diversity in the context of SLE and also describe reliable methods developed by our group to measure B cell changes in human peripheral blood using high-parameter flow cytometry combined with advanced bioinformatics.

1.1. Importance of B Cells in SLE

Antibodies (immunoglobulins, Ig) are expressed on the surface of and secreted by B cells. Each B cell clone expresses an antibody [B cell receptor (BCR), if surface membrane-bound] that is unique in its variable (V) region, and whose constant region is one of nine effector isotypes. Antibodies are designed by each B cell through DNA recombination of Ig V region gene segments, by somatic hypermutation of these assembled V region genes, and

by isotype (class) switching, which changes the Ig constant region to modify its effector function.

During B cell development, Ig DNA rearrangements create a diverse BCR repertoire encoding a wide range of antigen-binding capabilities. Self-reactive B cells are usually deleted or inactivated at developmental checkpoints, which are defective in SLE patients (6–11). Common autoantibody reactivities include nuclear components and plasma membrane inner leaflet phospholipids (12). This reactivity may be attributed to poor clearance of apoptotic cells and debris in SLE patients (13,14), which can allow for exposure to such internal antigens (15,16). Thus, B cell tolerance might be broken by extended exposure of these antigens stimulating self-reactive B cells, which in turn promote inflammation through effector functions such as antigen presentation to T cells, cytokine secretion, and/or pathogenic Ig secretion.

Despite significant technical advances in cell sorting, molecular cloning, and re-expression of Ig-encoding cDNAs as recombinant monoclonal antibodies, the use of anti-idiotypic reagents recognizing self-reactive BCRs allows highly efficient characterization of intact B cells by histology and by flow cytometry. One of these reagents is a rat anti-human monoclonal antibody called 9G4. 9G4 binds to the V region of BCRs or of soluble antibodies (denoted respectively, 9G4⁺ B cells or 9G4⁺ antibodies). Up to 10 % of healthy-human B cells are 9G4⁺, but are largely confined to the antigen-naïve, undifferentiated B cell compartments, maintaining very low-to-undetectable serum 9G4⁺ antibody levels (17). However, SLE patients often have high titers of 9G4⁺ antibody that correlates with disease activity (18–20), with 9G4⁺ IgG levels correlating more strongly with disease (especially nephritis) than 9G4⁺ IgM levels (20).

High-titer autoantibody in SLE has prompted interest in developing biologicals that target B cells (3). Inhibiting the B cell survival factor BAFF/Blys (B cell Activation Factor of the TNF Family/B Lymphocyte Stimulator) or depleting B cells can alleviate symptoms of lupus-associated disease in humans and mice (21–28). These outcomes strongly suggest that B cells actively contribute to disease progression, although the exact mechanisms are not known. Interestingly, both mouse and human data suggest that high-titer autoantibody alone does not account for the B cell-mediated effects (5, 22, 27, 29). In fact, B cells are important for memory T cell accumulation in autoimmune mice, suggesting mechanisms such as antigen presentation and/or cytokine production by the B cells (29).

However, the B cell subsets responsible for each specific function are incompletely understood. Thus, in subsequent sections, we discuss how B cell characterization could be further exploited for understanding this disease and for optimizing clinical benefit (5, 21, 30)

1.2. B Cell Subsets in SLE

In adult bone marrow, nascent B cells begin expressing cell-surface BCR. In a “transitional” stage, the BCR⁺ B cell enters the peripheral circulation and differentiates into a mature-naïve cell that is competent for responding to BCR engagement by antigen. In addition to transitional and mature-naïve B cells, human blood contains 3–4 additional “core” B cell subsets with characteristics of antigen exposure (Ig somatic hypermutation, Ig class switching, and/or markers associated with Ig secretion). Such B cells that are neither naïve nor secreting antibody are generally regarded as “memory” in most nomenclatures (31) (Fig. 1b, c), which are discussed below. All of these basic core CD19⁺ populations can be identified using flow cytometry by various combinations of IgD BCR (more naïve) and CD27 (more differentiated) surface expression (Fig. 1c) (31). Further subsetting these core populations using additional markers is a highly advantageous way to characterize human B cells (31).

1.2.1. Transitional and Mature-Naïve B Cells—The CD10 marker on human BCR⁺ B cell progenitors continues to be expressed on immature B cells that have acquired the first class of BCR, IgM, as well as on IgM⁺ transitional B cells coexpressing an IgD BCR with an identical V region. In healthy subjects, the frequency of autoreactive B cells is reduced when the BCR reaches the cell surface (6), and then again when CD10 is lost (7). At each step, more of these B cells are self-reactive in SLE patients compared with healthy subjects (7,8). Despite breaching these tolerance checkpoints, SLE patients have decreased numbers of naïve B cells contributing to overall reduced numbers of total CD19⁺ B cells (13, 22, 32, 33). The cause and significance of this reduction is poorly understood.

Given the critical involvement of the transitional-to-mature conversion in determining the SLE B cell repertoire, it is advantageous to define such cells and their subsets in clearer detail. This goal can be achieved using multiparameter flow cytometry that can be efficiently executed in the laboratory with less than 10 cc of blood, and also allows for isolating such subsets for functional analyses. Transitional and mature-naïve B cells are CD27⁺. In addition to CD10, which typically has a poor staining index, the earliest transitional populations coexpress high levels of CD38 and CD24. “T1” transitional cells express slightly higher CD24 and CD38 than “T2” cells, their apparent progeny; however, visualizing this distinction is difficult without overlaying a profile of bone marrow, which lacks T2 B cells. Compared with T1/T2 transitional B cells, mature cells express lower levels of CD38, survive longer in culture, and divide in response to BCR engagement (34). Truly mature naïve B cells can extrude dyes such as Rhodamine 123 using the mitochondrial ABCB1 transporter, which is absent from both transitional and memory B cells (34). This characteristic is especially useful in separating true-mature (mature-naïve) B cells from an additional transitional population (T3). T3 cells express lower levels of CD24 and CD38 than T1/T2, but all three transitional populations fail to express active ABCB1 (35). Thus, without a dye-extrusion step, the T3 population is not distinguished from true-mature B cells. It remains to be definitively determined whether T3 is an obligate precursor to true-mature B cells. Nonetheless, such phenotypic distinctions may be clinically informative, as preferential early reconstitution of transitional B cells correlates with long-term remission in B cell-depleted SLE patients (once treatment is stopped) (30). Thus, thorough characterization of transitional and mature-naïve B cell subsets may contribute to defining more precise prognoses and perhaps predicting response to treatment.

1.2.2. Germinal Center B Cells—Antigen engagement of the BCR on a mature-naïve B cell can stimulate activation and differentiation along any of several pathways that, ideally, help eliminate an invading organism and protect the host. One of these pathways is the germinal center (GC) reaction in secondary lymphoid tissue follicles. Typically, GC reactions require T cell help to promote B cell proliferation, BCR class switching from IgM/IgD to IgG, IgA, or IgE, and also Ig V region gene somatic hypermutation that changes the affinity of the encoded BCR for its cognate antigen (36). Somatic mutations in autoantibodies contribute to their self-reactivity (11,37,38). Antigen-driven selection results in clonal expansion of mainly the highest affinity B cells. In normal individuals with intact peripheral tolerance, these B cells are ideally only reactive with antigens from invading organisms. The surviving cells can differentiate into antibody-secreting plasma cells or into memory B cells (each discussed below) that are ready for a rapid response to a second encounter with an invading organism.

In healthy human tonsil, non-self-reactive B cells are evenly distributed among the B cell populations, but self-reactive 9G4⁺ B cells are confined to the naïve compartment and excluded from GCs (17, 39). Unlike in healthy humans, 9G4⁺ B cells in SLE patients colocalize with proliferating CD38⁺ GC structures (39). These observations strongly suggest that dysregulation of autoreactive B cells at GC differentiation stages contributes to SLE

disease (40), and may explain the ability to detect IgG⁺ 9G4⁺ cells and 9G4⁺ CD138⁺ (plasma) cells in SLE peripheral blood, each of which are rare in healthy controls (39).

1.2.3. CD27⁺ Memory B Cells—B cell memory (specific and rapid recall responses to previously encountered antigens) is often inferred from cells restimulated in vitro and from the ability to transfer such memory to naïve experimental animals (41–43). In humans, most of this activity is attributed to CD27⁺ B cells. Compared with CD27[−] (mostly mature-naïve and transitional) B cells, CD27⁺ B cells are slightly larger (34, 44), are more proliferative in vitro (45–47) and in vivo (48), more efficiently stimulate allogeneic CD4 T cell proliferation (46), and more readily differentiate into antibody-secreting cells (47). GC, memory, and in vitro-activated naïve B cells can all be CD27⁺. However, as GC are generally confined to lymphoid tissues, and few circulating CD27⁺ B cells show evidence of on-going proliferation (34), GC and recently activated B cells unlikely account for a significant portion of the CD27⁺ B cell pool, at least in healthy humans. Additionally, CD27⁺ B cells are barely detectable in natal cord blood, but proportionally increase with age and cumulative antigen exposure (44, 48–50). The few CD27⁺ B cells in the cord blood lack Ig V somatic hypermutations and have been attributed to a newly described innate-like human “B1” B cell equivalent (51).

Adult CD27⁺ B cells either (1) express an IgD/IgM BCR [IgM memory or nonswitched memory (NSM) B cells] or (2) have lost expression of these markers after the irreversible process of antibody class switching (switched memory B cells). CD27⁺ B cells are ~10–30% each IgM⁺ D⁺, IgG⁺, or IgA⁺ (34, 52–54). It is unknown whether these memory B cell subsets derive from common or from distinct differentiation pathways, or even if such pathways are common to all cells in a given pool. Thus, the switched memory B cell pool may contain cells that class-switched prior to and also those that class-switched after acquiring CD27. Similarly, the NSM pool may contain B cells that will remain NSM together with intermediates that will eventually class-switch. Preliminary analysis using high-parameter flow cytometry combined with automated analysis examining all parameters simultaneously (55) suggests that most NSM cells have more in common with other compartments than they have with each other (unpublished). However, most studies to-date have compared all NSM with all switched memory B cells using conventional manual gating methods. Briefly, both compartments have somatically hypermutated Ig V regions with characteristics of antigen selection (11, 36, 54, 56). However, reports disagree on whether NSM have a unique (33, 57, 58) or similar (59) Vh repertoire compared with IgD[−] CD27⁺ B cells. It is unknown whether sorting and/or sequencing strategies account for these different observations.

The NSM pool is thought to consist of adult B1 B cells, regulatory B cells, and splenic marginal zone-like B cells that provide carbohydrate-reactive antibody responses to encapsulated bacteria (38, 50, 51, 60, 61). Recent studies in mice suggest that at least some of the IgM memory pool functions to maintain very long-term memory against protein antigen (62, 63).

Mice and humans with defective GC have a paucity of memory B cells and profoundly decreased levels of class-switched serum antibody (43). These observations led to the dogma that class-switched humoral memory is GC-derived, whereas IgM memory is GC-independent. Indeed, development of NSM B cells with mutated Ig V regions does not require GC formation (64). However, some NSM have mutations in a noncoding region of the Bcl6 gene, which is characteristic of GC participation (59). Although antibody class switching is also characteristic of GC reactions, not all switched cells are necessarily GC-derived. A small proportion of CD27⁺ IgG⁺ cells express the IgG2⁺ subclass (34, 65), which can be induced independently of GC-type (i.e., T cell-derived) stimuli in culture (66).

Some pediatric SLE patients have more IgG3-expressing and fewer IgG2-expressing switched memory B cells compared with healthy controls (65). Given the contribution of IgG2 to anti-polysaccharide responses, this observation may suggest an over-representation of GC-derived memory against protein-type antigens. More notably, clinical relapse of B cell-depleted patients correlates with rapid reconstitution of CD27⁺ memory B cells (30). It is not clear if this effect is due to the functions of the memory B cells or due to pathological circumstances favoring memory over transitional B cell expansion. Interestingly still is the recurring observation that NSM are proportionally reduced in active SLE (32, 67, 68), correlating with higher autoantibody titers (67). Together with the stronger clinical correlation between serum 9G4⁺ IgG compared with 9G4⁺ IgM (20), this result may suggest that some of the IgM memory may play a protective role against pathology otherwise exacerbated by IgG memory. Increasing evidence supports such a dichotomy of protective and pathogenic B cells in cardiovascular disease (69–73).

1.2.4. Double-Negative B Cells—A small proportion of class-switched B cells in healthy subjects do not express CD27 (52, 53). These B cells make up most of the CD19⁺ IgD[−] CD27[−] “double-negative” (DN) compartment, which is typically less than 5 % of the CD19⁺ pool. Most DN B cells in healthy subjects express either IgG or IgA (52). Some evidence suggests that the DN compartment in unchallenged individuals includes B cells that have further differentiated into memory B cells without acquiring CD27. Both CD27⁺ and CD27[−] pools contain B cells that can be readily induced to secrete Ig against tetanus toxin and influenza virus (34), suggesting prior exposure to vaccines. Other than CD27 expression, the DN and CD27⁺ switched memory B cell populations have similar surface phenotypes (34, 53) and have somatically mutated Ig V regions (52, 53). However, Ig V region mutations in DN B cells are less frequent than in switched CD27⁺ memory cells (52, 53). The DN compartment may thus be a mixture of both effector and memory B cells.

To varying degrees, SLE patients have increased frequencies of DN B cells (13, 22, 52) correlating with higher autoantibody titers and with a greater incidence of nephritis (52). The higher frequency of DN cells could result from B cells entering from other compartments, such as the naïve B cell pool becoming activated and losing IgD expression, or from activated cells entering blood from the tissues. Further investigation of the characteristics, origins, and functions of DN B cells in treated and untreated SLE patients, and in newly vaccinated and acutely infected controls will likely contribute to our understanding of B cells in this disease and in B cell activation in general.

1.2.5. Antibody-Secreting Cells—Differentiation into an antibody-secreting cell (ASC) can occur downstream of the appropriate B cell-activating stimuli. ASC in human peripheral blood are typically CD27^{high} CD38^{high}, and nearly all express the intracellular Ki67 antigen, suggesting on-going proliferation (34). ASCs detected by Ig secretion assays or by flow cytometry are very rare in the blood of healthy, unchallenged individuals. However, a rapid and transient ASC response is readily detectable as early as 4 days after booster vaccination (55, 74–76). During acute infection (especially viral), ASCs can be detected as early as day 2 after symptom onset (77). Specificity of the circulating ASCs is unique to antigens of recent exposure (94). Human and mouse studies have identified at least two “subsets” of blood ASC loosely referred to as plasmablasts and plasma cells (78, 79). True, terminally differentiated plasma cells might be enriched in the compartment expressing CD138, which is less than half of the ASC in the blood, but nearly all of the ASC in the bone marrow (78, 79). Compared with CD38^{high} CD138[−] ASC, considered to be plasmablasts, CD138⁺ plasma cells are larger with more cytoplasm, lack surface Ig, and do not seem to be undergoing proliferation (34, 80). As with the various kinds of memory B cells, it is not entirely clear whether some or all plasmablasts and plasma cells have precursor–product relationships, even in elegant mouse reporter systems (78, 79). However, given evidence

that long-lived specific antibody in the serum can be maintained without concurrently circulating memory B cells (42, 81), a model has arisen in which long-lived, CD138⁺ plasma cells in tissues (such as bone marrow, where they are most readily detectable) provide a long-lived Ig source, whereas plasmablasts in the circulation provide an immediate, but transient “boost” to existing serum Ig levels. This model does not favor nor exclude that plasmablasts and memory B cells (from either tissues or circulation) could each progress to the plasma cell stage upon migrating to the bone marrow.

ASC detected by functional secretion assays and by flow cytometry are proportionally increased in SLE patients. However, reports differ on whether this effect includes patients with quiescent and those with moderate disease (32, 67, 68, 82) or if it correlates with disease activity (22, 33). The differences are not accounted for by the choice of flow cytometry markers per se, and thus may instead include disparities in patient groups biologically, in clinical assessments, and even in technical strategies including inconsistent resolution and/or event-gating of these rare populations. Biologically, the contribution of antibodies compared with antibody-independent B cell functions might differ among patients or patient groups. B cell depletion and CD40L blockade can each reduce plasmablasts in SLE patients, correlating with symptom reduction (13, 22). However, in only some of these patients do serum autoantibody levels decrease. Thus, plasmablast expansion in SLE could possibly be more of a consequence of systemic inflammation, rather than a cause.

1.2.6. Evolving SLE B Cell Signature—We have thus far discussed the tendency of SLE patients to have proportional increases in CD19⁺ IgD[−] CD27[−] DN B cells (which may become the largest switched memory subset) and in plasmablasts, but decreased proportions of NSM B cells. Previous studies and our own observations suggest that including additional cell-surface markers will provide additional advantages in further characterizing these populations in lupus. As previously reported (31), CD27⁺ resting memory cells in healthy subjects are predominantly B220[−] (Fig. 1d) as the expression of this marker is typically lost during germinal center differentiation. By contrast, B220 expression predominates in IgD[−] CD27[−] cells. This subset is characterized in SLE by the downregulation of CD24, a marker typically expressed by most PBL B cells in general and memory B cells in particular (see Fig. 1d for healthy subject example). Consequently, expanded fractions of B220⁺ CD24[−] cells within the IgD[−] CD27[−] compartment are prominent in lupus patients. Loss of CD21 and upregulation of CD95 have been independently associated with memory B cell activation (83, 84). Accordingly, an indication of memory B cell activation in SLE can be found in both SwMe and DN cells in which the CD21[−] and CD95⁺ fractions are greatly expanded in lupus compared to the healthy controls. By contrast, CXCR3 expression (suggesting migratory potential of activated cells to nonlymphoid, systemic inflamed tissues) is concentrated in the IgD[−] CD27⁺ memory subset. Therefore, the inclusion of CD21, CD95, and CXCR3 in the same reagent panel determines the coexpression of these informative functional markers. Consistent with a resting phenotype, most IgD[−] CD27⁺ memory cells are CD21⁺ CD95[−] in healthy controls (Fig. 1d). A significant fraction of IgD[−] CD27[−] cells, however, lack expression of CD21, a feature consistent with activation. Yet, they lack CD95 expression, indicating that these two markers are not necessarily correlated. Combined, CD95⁺ cells are relatively scarce in healthy memory cells. By contrast, CD95⁺ cells are greatly expanded in both memory subsets of SLE patients. Interestingly, the vast majority of CD95⁺ IgD[−] CD27[−] cells are CD21[−]. CD95⁺ cells within the lupus IgD[−] CD27⁺ memory are almost equally split between CD21⁺ and CD21[−], illustrating again that the expression of these markers is not necessarily reciprocal. Furthermore, the CXCR3⁺ fractions of both IgD[−] CD27⁺ and IgD[−] CD27[−] memory subsets exhibit CD21/CD95 expression patterns that are similar to those of their respective total subsets and are not therefore, particularly enriched in activated cells, at least as defined by these markers.

Given the enormous heterogeneity in SLE disease presentation, and the contributions that B cells make to pathology, further defining subpopulations of these core subsets using the markers described above can establish “signatures” or patterns of changes in B cell populations that may be associated with different clinical outcomes. Such profiling to find B cell signatures could also help prognosis and custom-designed treatments. Essentially, the appropriate modality would ideally eliminate B cells harboring pathogenic properties, while it would enhance the numbers and/or activity of B cells with regulatory properties (5, 21). However, certain signatures may also be predictive of whether more classical therapies are or are not going to be useful in a given individual.

2. Materials

1. Human peripheral blood in a heparinized collection tube (e.g., “green-top” vacutainer; BD Biosciences, Franklin Lakes, NJ).
2. Phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA).
3. BSA (10 % solution; Miltenyi, Auburn, CA).
4. FACS buffer: 0.5 % BSA in PBS.
5. Ficoll-Paque Plus (GE/Amersham, Piscataway, NJ).
6. Freezing media: 10 % DMSO, 90 % fetal bovine serum.
7. Normal mouse serum (NMS; Jackson ImmunoResearch, West Grove, PA).
8. Normal rat serum (NRS; Jackson ImmunoResearch).
9. 5-mL FACS tubes (BD/Falcon, San Jose, CA).
10. 1.5-mL microcentrifuge tube (Costar, Austin, TX).
11. Formaldehyde (10 % UltraPure, methanol-free; Polysciences, Warrington, PA).
12. Fixation buffer: 0.5 % formaldehyde in PBS.
13. Simply Cellular Compensation Standard (Bangs Laboratories, Fishers, IN).
14. ArC Amine-Reactive Compensation Bead Kit (Invitrogen).
15. Rainbow Calibration Peak 6 particles (Spherotech, Lake Forest, IL).
16. LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Molecular Probes/Invitrogen).
17. FITC-IgD (FITC-conjugated mouse anti-human IgD, IA6-2; BD).
18. PE-CXCR3 [PE-conjugated mouse anti-human CXCR3 (CD183), 1C6/CXCR3; BD].
19. PE-A610-CD24 (PE-Alexa610-conjugated mouse anti-human CD24, SN3; Caltag/Invitrogen).
20. PE-Cy5-CD21 (PE-Cy5-conjugated mouse anti-human CD21, B-ly4; BD).
21. PerCP-Cy5.5-CD38 (PerCP-Cy5.5-conjugated mouse anti-human CD38, HIT2; BD).
22. PE-Cy7-B220 (PE-Cy7-conjugated rat anti-mouse CD45/B220, RA3/6B2; BD).
23. Pacific Blue-CD3 (Pacific Blue-conjugated mouse anti-human CD3, SP34-2, BD).
24. Qdot 605-CD27 (Qdot[®] 605-conjugated mouse anti-human CD27, CLB-27/1; Invitrogen).

25. APC-CD95 (APC-conjugated mouse anti-human CD95, DX2; BD).
26. APC-Cy7-CD19 (APC-Cy7-conjugated mouse anti-human CD19, SJ25C1; BD).
27. Biotinylated 9G4 (rat anti-human Ig idiotype, Batch#3, 2008; the 9G4 hybridoma was a gift from Dr. Freda S. Stevenson, Southampton, UK).
28. SAv-A680 (streptavidin-Alexa680 conjugate; Invitrogen).
29. LSR II flow cytometer with Blue (488), Red (633), and violet (405) lasers and desktop computer with Diva Software (both from BD).
30. FlowJo (TreeStar, Ashland, OR) and MatLab (MathWorks, Natick, MA) software.

3. Methods

Here, we describe procedures for analyzing B lymphocyte subsets from human peripheral blood mononuclear cells (PBMC). We include methods for preparing, staining, and analysis by flow cytometry followed by bioinformatics analysis. As an example, we focus on our recently developed 12-color memory B cell staining panel (95) analyzed with a Becton-Dickson LSR II instrument. The protocol assumes basic knowledge of operating the instrument. Similar panels have been developed for transitional B cells and plasma cells, and these methods can be amended for other user-developed panels as well. For consistency in large studies, we provide detail on critical steps such as freezing and thawing, with recommended quality assurance/quality control to monitor and regulate consistency (Fig. 2).

3.1. Sample Preparation

3.1.1. Isolation of PBMC

1. Record the original volume of blood in the heparinized blood-collection tube before proceeding. Centrifuge with no brake at $400 \times g$ for 15 min.
2. Remove plasma from the top of the sample, leaving ~0.2 mL of plasma behind. If needed, reserve the plasma at 4 °C.
3. Collect the white blood cells (WBC) from the interface (initially between the plasma and the middle layer of Ficoll; red blood cells will be in the bottom layer) plus ~0.2 mL above and below. Transfer to a clean 50-mL centrifuge tube.
4. Dilute the collected WBC up to the original blood-volume with PBS.
5. Add 15 mL Ficoll-Paque into a separate, clean 50-mL conical tube.
6. Hold the tube as close to horizontal as possible and slowly layer the diluted WBC sample onto the Ficoll-Paque, being careful not to mix layers.
7. Centrifuge at $400 \times g$ for 35 min at 20 °C with brake off.
8. Using a Pasteur pipette and avoiding Ficoll-Paque, collect the mononuclear cell layer at the interface (buffy coat), and transfer to a clean 15-mL conical tube.
9. Fill tube to 15 mL with cold PBS or RPMI, cap the tube, and mix by inverting. Centrifuge for 10 min at $400 \times g$ at 4 °C with high brake.
10. Discard supernatant and resuspend pellet in 10 mL sterile PBS or RPMI. Centrifuge again.
11. Gently dislodge the pellet and repeat steps 9 and 10.
12. Remove supernatant and resuspend in FACS buffer.

13. Count the cells by Trypan blue exclusion.
14. For cells to be stained fresh, transfer 10^7 cells per sample for each multicolor panel into separate FACS tubes. Remaining cells can be frozen.

3.1.2. Freezing and Thawing

Freezing Cells

1. Pellet cells reserved for freezing, discard supernatant, and resuspend in cold freezing medium at 10^7 /mL. Densities less than 5×10^6 /mL will reduce cell recovery.
2. Immediately freeze at -80°C for 24–48 h and then transfer to permanent storage, such as a liquid N_2 freezer (optimal -180°C).

Thawing Cells

1. Before retrieving cells from frozen storage, warm FACS buffer to 37°C .
2. Remove the vial of cells from the freezer and hold in a 37°C water bath while continuously shaking and monitoring the thaw process. Do not submerge the vial and do not allow incubation to proceed after thawing is complete.
3. Once thawed, immediately transfer the cells to a clean 15-mL tube and wash in 10 mL warm FACS buffer.

3.1.3. Staining Cells with the 9G4 Memory B Cell Panel

Stain Compensation Controls

1. Set up twelve, 1.5-mL microfuge tubes and dispense two drops of Simply Cellular Compensation Standard beads into each tube.
2. Reserve one tube as the unstained control. To each remaining tube, add 0.2–2 μg of one of the other antibodies. Gently vortex.
3. Incubate on ice for 30 min in the dark. [Note: for the Alexa680 channel, it is a 2-step staining: First with biotin-CD3 and then with SAV-Alexa680. Stain 30 min for each step with a wash in between.]
4. Wash the beads once with 1 mL FACS buffer. Pellet the beads by centrifugation in a microcentrifuge at $900 \times g$ for 5 min. Resuspend the beads in 200 μL of 0.5 % formaldehyde, and transfer to separate 5-mL FACS tubes.
5. For the Aqua compensation control, gently vortex ArC bead components. Add one drop of Component A (reactive beads) to a clean microfuge tube. Allow beads to sit at room temperature for at least 5 min. Add 1 μL Aqua L/D stain directly to the droplet of the reactive beads and incubate for 30 min.
6. Transfer to a FACS tube with the addition of 3 mL FACS buffer. Centrifuge at $300 \times g$ for 5 min. Add 500 μL FACS buffer to the tube, plus one drop of ArC (negative beads) to the tube.

Stain Blood-Cell Samples (3-Step Staining)

1. Prepare antibody cocktails using FACS buffer in the presence of NMS and NRS (1:20 dilution each). Prepare a cocktail of the fluorescent and biotinylated antibodies sufficient for staining the number of cell samples (100 μL per sample). Prepare also separate 1-sample mixtures of the same cocktail omitting one reagent per cocktail for the fluorescence-minus-one controls.

2. Pellet the cells reserved for staining at $300 \times g$ for 10 min at 4 °C. Resuspend each pellet with 100 μ L of the appropriate antibody cocktails. Incubate on ice for 30 min in the dark.
3. Wash the cells once with 2.5 mL FACS buffer.
4. Resuspend the cells with 100 μ L SAv-A680 (at 1:500 dilution) on ice for 30 min in the dark.
5. Wash the cells once with 2.5 mL PBS (no BSA).
6. Incubate the cells in 1 mL PBS (no BSA) containing aqua-fluorescent reactive dye (1:1,000) on ice for 30 min in the dark.
7. Wash the cells once with 2.5 mL FACS buffer.
8. Resuspend the cells in 0.5 mL 0.5 % formaldehyde.
9. Run stained cells and controls (single-color beads and FMOs) on a flow cytometer.

3.2. Primary Analysis

Another integral element needed for consistent and reproducible flow results is a standardized strategy for flow data analysis. A rationalized gating strategy can significantly reduce user-to-user variability to ensure consistent interpretation of the data among all operators (e.g., Fig. 1). The following protocol assumes basic knowledge of FlowJo software. Tutorials on getting started with FlowJo are available at the TreeStar Web site (<http://www.flowjo.com/home/basictutorial.html>) and can also be arranged with TreeStar personnel.

3.2.1. Gating Strategy for Memory B Cells

1. Gate on lymphocytes through the FSC-area vs SSC-area (FSC-A/SSC-A) scatter plot (Fig. 1a).
2. Within the lymphoid gate, eliminate cell aggregates by displaying Height/Width for FSC. Gate a region only on events on the optimal-ratio line. Within this gate, repeat the procedure for SSC.
3. Within this region, gate-out dead cells that have taken up the amine-reactive fluorescent dye (Aqua).
4. Gate on CD19⁺ CD3⁻ B cells for analysis.
5. Identify the core B cell subsets with either IgD/CD38 (Fig. 1b), IgD/CD27 (Fig. 1c, *right*), or CD24/CD38 (Fig. 1c, *middle*) plots, and also the 9G4⁺ B cell population, depending on the gating strategy used.
6. Further subset the CD27⁺ and CD27⁻ switched (IgD⁻) memory cells by their differential expression of B220, CD24, CD21, CD95, and CXCR3 (Fig. 1d).
7. Determine the percentages of 9G4⁺ B cells in each core subset (Fig. 1e, *histograms*).
8. Numerical data can be exported from FlowJo by generating a table (keyboard “command+ t” or clicking the grid-shaped icon), and exporting the data as a .txt file (click the grid icon in the new table window, then save-as).

3.3. Secondary Analysis

Once primary analysis (gating) has been performed and crosschecked, the resulting data can be analyzed in numerous ways, depending on the experimental question. Considerations include whether to report the data as mean fluorescent intensity of a particular fluorescent parameter, the percentage of cells within a parent population, as a percentage of all B cells, or as the “absolute” cell number per volume of blood. For cell-number calculations, it is necessary to acquire a clinical blood count (CBC) from an aliquot of whole blood in which total white blood cells and lymphocytes are measured. These procedures can be requested from clinical staff or performed by the analyst using conventional or automated methods, such as a Sysmex XE-2100 instrument. Multiparameter flow analysis invariably generates large quantities of data that need to be managed efficiently in order to derive productive output. For large-scale studies with associated clinical information, it is recommended that the data be structured and housed in a relational database to support flexible interrogation of the data.

In some circumstances, many reagents in the panel are used for narrowing down a particular subset of interest, which is then analyzed for various characteristics and by standard statistical tests with conventional graphing techniques. In other circumstances, a more global profiling approach can be used in which all of the data are considered together to obtain a system-wide view of immune cell populations. Profiling is useful for class-discovery in which natural groupings of samples can be sought (85, 86). Furthermore, it may be possible to relate those groupings to sets of samples with different disease states, disease activities, or clinical manifestations. Univariate approaches on individual subsets fail to reveal how collections of subsets and their relative distributions might contribute to such sample groupings.

3.3.1. Calculating Cell Numbers

1. Calculate B cell numbers with the following formula in which both WBC/mL and % lymphocytes are taken from the CBC, and % B cells among lymphocytes is taken from gating analysis:

$$\frac{\text{WBC}}{\text{mL}} \times \frac{\% \text{lymphocytes}}{100} \times \frac{\% \text{CD19}^+ \text{CD3}^-}{100} = \frac{\# \text{B cells}}{\text{mL blood}}.$$

2. Calculate the core subset (e.g., switched memory) numbers:

$$\frac{\# \text{B cells}}{\text{mL}} \times \frac{\% \text{IgD}^- \text{CD27}^+}{100} = \frac{\# \text{switched memory B cells}}{\text{mL blood}}.$$

3. Calculate finer subpopulations (e.g., in which CD95⁺ was measured as a per cent of the switched memory parent):

$$\frac{\# \text{switched memory B cells}}{\text{mL}} \times \frac{\% \text{CD95}^+}{100} = \frac{\# \text{CD95}^+ \text{switched memory B cells}}{\text{mL blood}}.$$

3.3.2. Clustering and Principal-Component Analysis—The following represent a generic workflow that can be performed by several software packages or by technical computing platforms. This protocol assumes basic knowledge of Matlab software (Mathworks, Natick MA; <http://www.mathworks.com/products/matlab/>).

1. Construct a data matrix by arranging the cell-subset frequencies (or numbers, MFIs, etc.) for each sample as a row in a sample-by-feature matrix. Each row is then annotated by a sample ID (which can be linked to a variety of clinical, demographic, or other data) and each column is annotated by a cell subset name.
2. It may be beneficial to log-transform data, especially if some of the cell subsets are rare. To determine whether transformation is warranted, independently perform a QQ (Quantile–Quantile) plot on each data column (the values associated with a single cell subset across all samples) as well as the log of a data column to see whether log transformation results in a distribution that is closer to normal (Gaussian). It is common for log transformation of a subset to result in a more Gaussian distribution. The Matlab command is “qqplot.”
3. It is possible that there are both typically abundant and typically rare B cell subsets in the profile whose frequencies can be orders of magnitude different. This situation can cause the clustering to be dominated by abundant cell subsets (if Euclidean distance measures are used). To standardize the scale for cell subset expression, each column can be normalized by subtracting the mean from each value, then dividing the result by the standard deviation, so that the abundance (frequency) measure for each subset will have a mean of 0 and a standard deviation of 1.
4. Cluster the samples using the “pdist” command to find all the pairwise distances (specifying a distance measure such as Euclidean distance, Pearson correlation, or Spearman correlation) followed by “linkage” to construct the dendrogram (specifying a hierarchical clustering linkage method). To render, use the “dendrogram” command. Alternatively, see the “clusterdata” command.
5. Render the heat map (with the reordered samples and/or subsets) using the “imagesc” command. To align the sample and subset dendrogram to the heat map, see the Matlab documentation on multiple axes on a single figure. If showing normalized data in a heat map, it is preferable to use a black-centered color-map (e.g., either red–black–green or cyan–black–yellow) so that black corresponds to the mean (0). Alternatively, if showing nonnormalized data, a non-black-centered color-map (e.g., “jet” in Matlab) is useful.
6. To perform PCA, use the “princomp” command on the transformed sample-by-feature matrix. This command will display a matrix of the same dimensionality, but where each column is a principal component (typically the first two or three are considered). It will also return a square matrix from which the principal-component loadings can be extracted.

4. Notes

1. It is essential to minimize the time between blood collection in the clinic and running stained samples on the flow cytometer. The Ficoll-Paque should be at room temperature prior to and during the centrifugation. It is important not to “overload” the Ficoll, keeping it one part Ficoll-Paque per two parts blood/ PBS. Apart from the Ficoll and the cell thawing steps, samples should be kept cold (4 °C or on “wet ice”), especially if they need to be shipped to another facility. In this case, it is important to invest in a carrier service with a minimal delivery time. Depending on the reagent panel used and the cells of interest, time constraints can be partially circumvented by cryopreserving the cells. Cryopreservation also allows excess cells not needed for fresh staining to be reserved for future staining with other panels. Additionally, frozen cells can be shipped on dry ice and may thus provide an option for improving consistency if samples are collected at multiple clinical

sites, but stained and analyzed at only one central facility. Even with the most careful freezing and thawing techniques, however, cryopreservation will always result in cell loss and may affect certain populations more than others. The plasmablasts/plasma cells are one such population. When cryopreservation is used, it is important to minimize the time between blood sample collection in the clinic and the freezing step. When freezing and thawing, it is critical to minimize the time that nonfrozen cells are exposed to the freezing media containing DMSO, which is toxic.

2. Fluorescent reagents should be kept cold and out of light. These conditions are especially critical for tandem dyes, in which two fluorochromes are linked (e.g., PE-Cy7). This linkage is highly sensitive to oxidation, which can be catalyzed by both light and increased temperature. The result of a degraded tandem dye is that the reagent now fluoresces similarly as one of the parent dyes, and not of the conjugate. Thus, completely erroneous results can be acquired without careful control of this parameter, which can be monitored with the single-color control samples. It is also important to run fluorescence-minus-one (FMO) controls (panel lacking one reagent per FMO control sample) on a regular basis. FMO controls are used to determine the lower-limits of a positive stain when gating. Another critical component of a flow cytometry experiment is to include a consistency control, which is a single biological sample that has been aliquotted and frozen for staining with each run. For human B cell analysis, it is highly advantageous to establish such a control from patients with hemachromatosis, as their blood is highly enriched for B lymphocytes, typically without excessive perturbances to known B cell subsets. This control allows for careful monitoring of changes in instrument performance that are not readily detected with the bead preparations.
3. Some 9G4⁺ antibodies can bind to B220 (87). Thus, patients with high titers of 9G4⁺ antibodies in the circulation can have a “painting” effect in which B220⁺ (especially naïve) B cells are bound by the rat anti-idiotypic 9G4 reagent, even if that B cell’s BCR is not truly 9G4⁺ (i.e., usually encoded by the Vh4–34 gene segment). This effect is apparent when an unusually large percentage (<50 %) of total or of naïve B cells stain with the 9G4 reagent. To circumvent this issue, incubate the cells in serum-containing media at 37 °C for 1 h, then wash prior to staining with the 9G4 memory B cell panel (87).
4. The quality and consistency of multiparameter flow data is affected by each step in the operational process. Artifacts can be avoided by addressing several technical challenges associated with these processes. Foremost is proper and reproducible flow cytometer setup. Users and/or core facilities should follow manufacturer’s (e.g., BD Biosciences) guidelines to establish the “Baseline PMT Gains” for each LSRII or other cytometer with its particular configuration of lasers and filters using Rainbow Calibration Peak 2 Particles (Spherotech, RCP-30-5A-2). These baseline voltages are then used as starting-points to fine-tune the voltage settings to achieve optimal detection and resolution of each fluorescent reagent used in each staining panel. The resulting settings are then saved as “application-specific settings,” and used in all subsequent experiments with the same reagent panel.
5. Once the “application-specific settings” are established for the flow cytometer, the mid-range Rainbow Calibration Peak 6 Particles (RCP-30-5A-6) are used to determine target channel values for each detector. The Peak 6 Particles are also run before each subsequent experiment to check instrument performance and to ensure that the particles fall into the target channel values for the same voltage settings. These target channel values can also be used to convert the application-specific settings determined on one instrument to voltage settings on another instrument

with the same laser and filter configuration. With a properly set-up and regularly calibrated instrument, experimental results can be meaningfully compared and compiled even if they were collected during a lengthy study, by different operators, and from different instruments. Such an instrument standardization process can also facilitate collaborations among different laboratories and institutions.

6. Depending on the quality and distribution of each fluorescent parameter in various two-dimensional plots, the axis scales may need to be transformed (88) so that events at extremely low or negative fluorescent intensities are clearly visible, and thus accurately gated (e.g., note the events less than 0 on the CD38 scale in Fig. 1b, c). We recommend bi-exponential transformation. In Flowjo v9.2, “Platform” in the pull-down menu has an option for “bi-exponential transformation.” Start with “custom transformation” and the default settings (0 for negative region, 4.5 for log scale) in the IgD/CD27 plot (Fig. 1c). If the results are not satisfactory (e.g., CD27), then “manual transformation” can be used to modify the settings on the CD27 channel. After that, visually inspect whether you need to transform any other channels. These settings can be adjusted for the optimal display of each parameter. After the transformation process, it is important to go back to check the parental gates, such as live/dead and CD19 gates, which may shift upon transformation. FSC and SSC will not be affected by transformation.
7. Profiling-based visualization is enabled by structuring data into a two-dimensional matrix, where each row is a sample and each column represents a feature (or “variable”; in this case, cell subsets). Data are then displayed as a heat-map by assigning the numeric values (e.g., % of CD19⁺) to color in this matrix. The samples, and independently, the features can then be clustered, resulting in the rows and columns being rearranged so that those most like each other are grouped closely. Dendrograms can be displayed adjacent to the rows and columns to indicate the nature of the clustering, namely, whether the samples (or features) are grouped into distinct clusters or whether they represent more of a continuum of diversity. This approach has been widely used for representing gene expression data (89).
8. Principal-component analysis (PCA), can be used to map the original variables (cell subsets) into a set of transformed variables that are uncorrelated with each other. Because many cell subsets in a profile may covary across samples (this is especially true in the extreme case where subsets are arithmetically related, such as “9G4⁺ and 9G4[−] frequencies within the naïve parent population”), those subsets do not provide additional information regarding class separation of the samples. Most of the variation in the entire data set is typically accounted for by the first few components (Fig. 3a, b). Thus, PCA is a dimension-reduction technique where the variation in the data is explained by fewer informative variables (90–92). By performing PCA on the original sample-by-feature matrix, one can plot the samples in two- or three-dimensional space, which may be revealing if some attribute of the sample is coded by color or symbol (Fig. 3c) (93). Note that the first few principal-components highlight aspects of the data that maximally contribute to the overall variance, which is not necessarily the consequence of some biological or clinically relevant factor, but could instead be influenced by process noise or experimental artifact. Clustering and PCA are both unsupervised in that they are solely based on the data readout, not the class-membership of the samples. They can be useful for visualizing trends among many subsets in many subjects. The underlying data structure is well-suited for a variety of data-mining approaches, which, despite the exploratory nature, are ideally utilized based on established experimental questions.

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Fig. 1.

Healthy human PB B cell subsets and phenotypic characterization of memory B cells. **(a)** Sequential gating strategy for (*left to right*) lymphocytes, singlets based on FSC, singlets based on SSC, viable cells that do not take up amine-reactive Aqua-fluorescent dye (“Live/Dead-negative”), CD3⁻CD19⁺ B cells, **(b)** The Bm (mature B cell) nomenclature, first developed to classify tonsil B cells by IgD and CD38 expression, is commonly used in PBL analysis. This approach fails to separate IgD⁺CD27⁺ nonswitched memory cells, which fall into the Bm1 and Bm2 gates together with naive cells (Bm1 and Bm2). Transitional 1 and 2 (T1/2) are included with Bm2’ (pre-GC). Furthermore, isotype-switched CD27⁺ cells are not distinguished from CD27⁻ memory cells within the Bm5 population (IgD CD38^{+/+}). **(c)** The more commonly used IgD/CD27 classification scheme identifies four core B cell subsets in human peripheral blood: IgD⁺CD27⁺ naive B cells (N), IgD⁺CD27⁺ nonswitched memory B cells (NSM), IgD⁻CD27⁺ switched memory cells (CD27⁺ SwMe), and IgD⁻CD27⁻ double-negative B cells (DN). In this scheme, transitional T1/2 colocalize with naive cells in the CD27 vs IgD plot, but can be identified based on their CD24⁺⁺CD38⁺⁺ phenotype (*middle panel*). Likewise, plasmablasts (PB) are embedded in the IgD⁻CD27⁺ fraction and can be accurately identified by first gating on IgD⁻ fraction followed by gating on the CD27⁺⁺CD38⁺⁺ region as depicted in the third panel. **(d)** The differential expression patterns of the surface markers in the IgD⁻CD27⁺ (*blue histograms*) and IgD⁻CD27⁻ (*green histograms*) memory B cells were overlaid on the IgD⁺CD27⁻ naive B cells (*filled red histograms*). **(e)** Most autoreactive 9G4⁺ B cells are substantially CD27⁻ and thus underrepresented within the CD27⁺ memory compartment as depicted in the bivariate plot of the total CD19⁺ B cells (*right*). Histograms further demonstrate that among the core subsets (gated as in **c**), the transitional and naive B cells exhibit the highest fraction of 9G4⁺ cells.

Fig. 2.
General work-flow for phenotypic B cell profiling.

Fig. 3.

PCA applied to summarized human B cell phenotyping data. Twenty PBMC samples were selected from our lupus cross-sectional study (*in preparation*) as follows: ten healthy controls were selected at random. Ten flaring lupus patients were chosen, including two severely flaring patients, three moderately flaring patients and five additional mildly flaring patients that were selected randomly from a larger pool of available samples. Primary analysis (gating as per Fig. 1) of these samples resulted in 40 cell subsets (the original variables). (a) Pareto plot showing the contribution to the overall variance by the first ten (out of 40) principal components (PCs). The *line indicates* the cumulative contribution of the PCs to the overall variance of the data from the samples. Most of the variance is captured by the first few components. (b) Contributions (loadings) of each original variable to the first principal component. Each *horizontal line* corresponds to one of the original cell subsets. Similar plots can be generated for other principal components as well. The loading (x axis) is the coefficient of an original variable for the principal component (with values from -1 to 1); larger coefficients indicate a larger contribution of that subset to the principal component in question. (c) Score plot of 40 B cell subsets reduced to the first two principal components, representing an informative summary of the phenotypic profiles. Frequency data were first log-transformed and then normalized. *Open circles* are healthy controls (HC) and *asterisks* are flaring lupus patients (SLE). Note how the two groups of samples segregate based on their phenotypic profiles (mainly along Principal Component 2, y axis).