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B Cells in Health and Disease – Leveraging Flow Cytometry to Evaluate Disease Phenotype and the Impact of Treatment with Immunomodulatory Therapeutics

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1. Introduction

Flow Cytometers are key devices used to monitor the composition of cells in the blood in the setting of a variety of disease states. Recent advances have produced a range of instruments that range from simple desktop-type devices to multi-laser platforms that allow for high complexity measurements. This variety of instrumentation makes the technology suitable for different budgets, expertise levels and intended uses (research versus diagnostic) with a set of reagents that can effectively be used on any platform so long as the laser line can excite the given fluorochrome and the optics are set up to discriminate emission from the excitation wavelength. Traditional medical applications for flow cytometers include evaluation of CD4 T cell depletion and associated immunophenotypic changes in HIV-infected persons as well as characterization of aberrant cell types used to diagnose hematologic malignancies. More recently, investigators have not only extended immunophenotyping campaigns to other disease settings, but have also taken advantage of fluorescent probes that provide insight into cellular function. For example, it can be inferred that a cell that expresses CD107 on its surface has likely participated in the delivery of cytotoxic granules to a target cell (Michael R. Betts, 2004). Likewise, amine-reactive dyes can be used to track cell division, probes that fluoresce only after enzymatic cleavage can report on caspase activities in apoptosis experiments and intracellular phosphorylation can be measured with specific antibodies and cell permeabilization buffers (phosphoflow) (Maxwell et al., 2009; Krutzik and Nolan, 2006; Wu et al., 2010). The elegance of the flow cytometry platform relies on its simplicity in as much as any combination of fluorescently-conjugated probes can be used to address contemporary hypotheses in cell biology and immunology. It is therefore not surprising that investigators have introduced flow cytometric measurements in biomarker campaigns to study a variety of activities of an immunomodulatory therapeutics), including effects of proximal signaling events as influenced by agonist or antagonistic drugs, or cell immunophenotype as a representative distal pharmacodynamic marker in treated persons.

The breadth of flow cytometric biomarker activities programs by members of our laboratory is quite broad, and we have leveraged our collective expertise to attempt to address contemporary issues in biomarker campaigns that include such assessments. Despite the

availability and precision of measurements performed by flow cytometers, it is important to realize that these measurements are made in the absence of accuracy standards; this is true even in the case of established assays. Thus, the strength of clinical flow cytometry is a function of the approach used for assay set-up and validation. One of the goals of this chapter is to share some of our strategies for the use of immunophenotyping data in the setting of disease and to further discuss the potential limitations of immunophenotyping in settings where correlative functional data may not be available. Even though the technology of flow cytometry is over 30 years old, the applications and ideas of using this platform as biomarker tools are in some ways in their infancy. Questions regarding the reliability of a given measurement, specimen and reagent stability, and methods to improve upon assay performance persist. It is our hope to contribute to maturation of this process and to begin to put forth ideas that could ultimately be used to standardize biomarker measurements in the clinic as executed by the flow cytometry laboratory. We have focused on immunophenotyping of blood from Systemic Lupus Erythematosus (SLE) patients in this chapter, but the principles put forth here are applicable to any disease and cell-type setting. This chapter is divided into four sections – an Overview of B cell development, B cell classification by flow cytometry, Technical Considerations, and B cell flow cytometry in contemporary biomarker campaigns.

2. Overview of B cell development

B cells are a central component of the immune system, not only because they produce one of the most important (and abundant) molecules in human serum – the class-switched high affinity antibody – but also by sensing innate stimuli, processing and presenting antigens to T cells and by producing pro- and anti-inflammatory cytokines. Class-switched antibodies (IgG, IgA, IgE) play critical effector roles as well: directing antibody-dependent cellular cytotoxicity (ADCC), phagocytosis and complement fixation to neutralize pathogens. The terminally differentiated plasma cell, residing primarily in the bone marrow, produces high affinity antibodies, sometimes providing high titer antibodies for as long as 75 years, depending on the antigen (Amanna et al., 2007; Crotty et al., 2003; Wrarmert et al., 2009).

B cells originate from hematopoietic stem cells, starting their journey in the bone marrow (Figure 1). The B cell receptor (BCR) variable region of the heavy chain locus is rearranged to produce a functional heavy chain and spliced together with the μ constant region to produce IgM. The heavy chain is paired with the surrogate light chain forming the pre-BCR. If a productive signal is transmitted, the light chain undergoes rearrangement. Together, the newly paired heavy and light chain undergo selection in the bone marrow: most immature B cells with a high affinity for self proteins undergo apoptosis or the variable region of either the heavy or light chain can be rearranged anew; those with unproductive BCRs undergo 'death by neglect' and the small fraction that signals optimally proceeds down the developmental pathway. B cells that survive this process are now considered immature and begin to transition out of the bone marrow and to the secondary lymphoid organs. Further deletion of the transitional B cell population can occur (Carsetti et al., 1995), and the variable region of either the heavy or light chain can be rearranged further (Toda et al., 2009; Nemazee, 2006). The transitional B cell traffics to the secondary lymphoid organs and differentiates into a mature naïve B cell, now expressing the variable regions of the heavy chain spliced together with the δ constant region to produce IgD (Monroe et al., 2003). A key

difference between the transitional B cell and the mature naïve B cell is the response to antigen – a transitional B cell will undergo apoptosis if the BCR is triggered with a cognate antigen, the naïve B cell will become activated (Monroe et al., 2003). An activated naïve B cell proliferates, internalizes the antigen via the BCR, and processes and presents antigen-derived peptides on MHC class II. With help from a specialized population of CD4+ T follicular helper cells (TFH), the B cell forms a germinal center. Through the course of the germinal center reaction, the B cell proliferates and daughter cells rearrange the BCR locus, resulting in class-switching (from IgM and IgD to either IgG, IgA, or IgE) and introduction of non-germline encoded nucleotides that result in unique BCR specificities. Each new daughter cell tests its BCR for affinity on follicular dendritic cells; those with higher affinity tend to survive. This process is collectively referred to as somatic hypermutation and affinity maturation. A subset of the activated B cells further differentiates into memory B cells and plasma cells. With a higher affinity BCR than those in the naïve pool, memory cells can respond faster and with greater magnitude than their naïve counterparts. Plasma cells are the final stage of B lineage development and travel to the bone marrow (and in some cases the secondary lymphoid organs) where they can produce antibodies for many years.

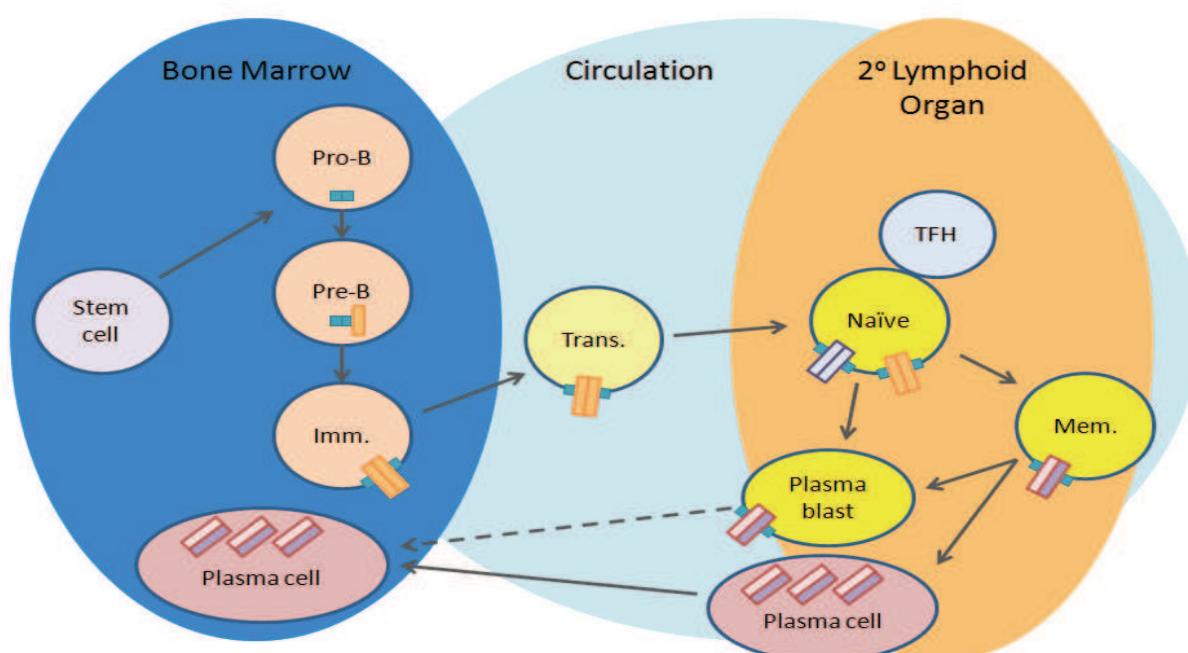


Fig. 1. An overview of B cell development. B cell development initiates in the bone marrow where the B cell receptor (BCR, ) is rearranged and expressed on the surface of immature B cells (Imm. = Immature). The developing BCR pairs with signaling molecules at this early stage (). Cells with appropriate BCR affinity (neither too high nor too low) exit the bone marrow and traffic to the secondary lymphoid organs (2° = Secondary). Here, the transitional B cell completes the maturation process to become a naïve B cell, expressing both IgM () and IgD (). Upon activation by antigen, and with Follicular T cell (TFH) help, the naïve B cell forms a germinal center. The BCR is rearranged further and plasmablasts and memory B cells (Mem. = Memory) are formed, expressing IgG, IgA or IgE (). Plasma cells are the final stage of differentiation, secreting soluble Ig () and homing to the bone marrow. Exceptions to this paradigm are noted in the text

If an optimal survival niche is not found, plasma cells are short lived. The plasma cell is optimally designed to produce large amounts of antibody molecules – somewhat analogous to the manufacturing capabilities of a biotechnology company. It should be noted that, in some cases where multivalent antigens can cross-link the BCR efficiently, T cell help is not required for antibody production, although germinal centers are not typically formed. Throughout this dynamic differentiation process, autoreactive B cells are kept in check by 1) direct deletion through apoptosis, 2) receptor editing of the BCR, and 3) through anergic BCR-driven signals, rendering the B cell unresponsive to stimulation. It is the dysregulation of these tolerance mechanisms that is thought to contribute to the survival of pathogenic autoreactive B cells and possibly result in autoimmunity.

3. B cell classification by flow cytometry

The flow cytometer is a useful instrument for the study of B cell differentiation, maturation and development. At any moment in time, the cellular composition of our bodies reflects a balance between the input of new cells versus the expansion and death of existing cells. Taken in whole, cells are transported to their tissue sites via the bloodstream and any given sample is a snapshot in time of the constituents of the biologic highway. Cells continuously enter and exit the extravascular space making blood a convenient and minimally invasive sample that captures the diversity of cells as they traverse the body to interact with other cells to mediate their effector functions. With respect to B cells, the antigens displayed on the cell surface are indicative of their developmental stage and may also reflect ongoing pathologic processes. Indeed, the paradigm of B cell classification has evolved to the extent that different B cell types have been awarded descriptive names. However, caution is advised to those that rely solely upon naming convention without functional validation of those immunophenotypic descriptions. As flow cytometers continue to advance in their ability to detect more antigens simultaneously, and as investigators continue to link functional readouts to phenotypic identities the exact definition of a given cell type is subject to change.

For those new to B cell investigations via flow cytometry, it is advisable to start with “anchor” markers and to devise a strategy to establish a B cell gate. For example, CD45 identifies all leukocytes in peripheral blood and can clearly separate this population from debris or dying cells and erythrocytes (Figure 2). The B lineage markers most commonly used to identify B cells in the blood are CD19 and CD20 and additional markers can characterize a variety of distinct subsets. Using this approach at least seven circulating B cell sub-populations can be identified: 1) immature, 2) transitional, 3) mature naïve, 4) non class-switched memory, 5) class-switched memory, 6) CD27- memory, 7) plasmablasts/cells. These B cell populations are identified by markers that are now well established; we can describe them as “pillars” of B cell biology and are expanded in more detail below.

Antigenic pillars of B cell biology – classification and caveats

Two classification systems originated in the early 1990's (Maurer et al., 1990; Maurer et al., 1992; Pascual et al., 1994), both demonstrating discrete populations that could be reliably measured over time. In the classification scheme described by Maurer et al., a combination of CD19, IgD and CD27 provide a means for describing three circulating mature B cell populations where “cB” refers to “circulating B cell”: “cB1 naïve” (IgD+CD27-), “cB2 non-

class switched memory” (IgD+CD27+) and “cB3 class-switched memory” (IgD-CD27+) (Figure 3A). The characterization of a “double negative (DN)” (IgD-CD27-) memory B cell population was introduced later (Wei et al., 2007; Jacobi et al., 2008). This population has been shown to be elevated in some patients with lupus and has become commonplace in the classification system using IgD and CD27. Therefore, DN memory B cells are also included in Figure 3A. This system is still routinely used to monitor changes in peripheral B cell composition in patients with SLE treated with investigational agents (Belouski et al., 2010).

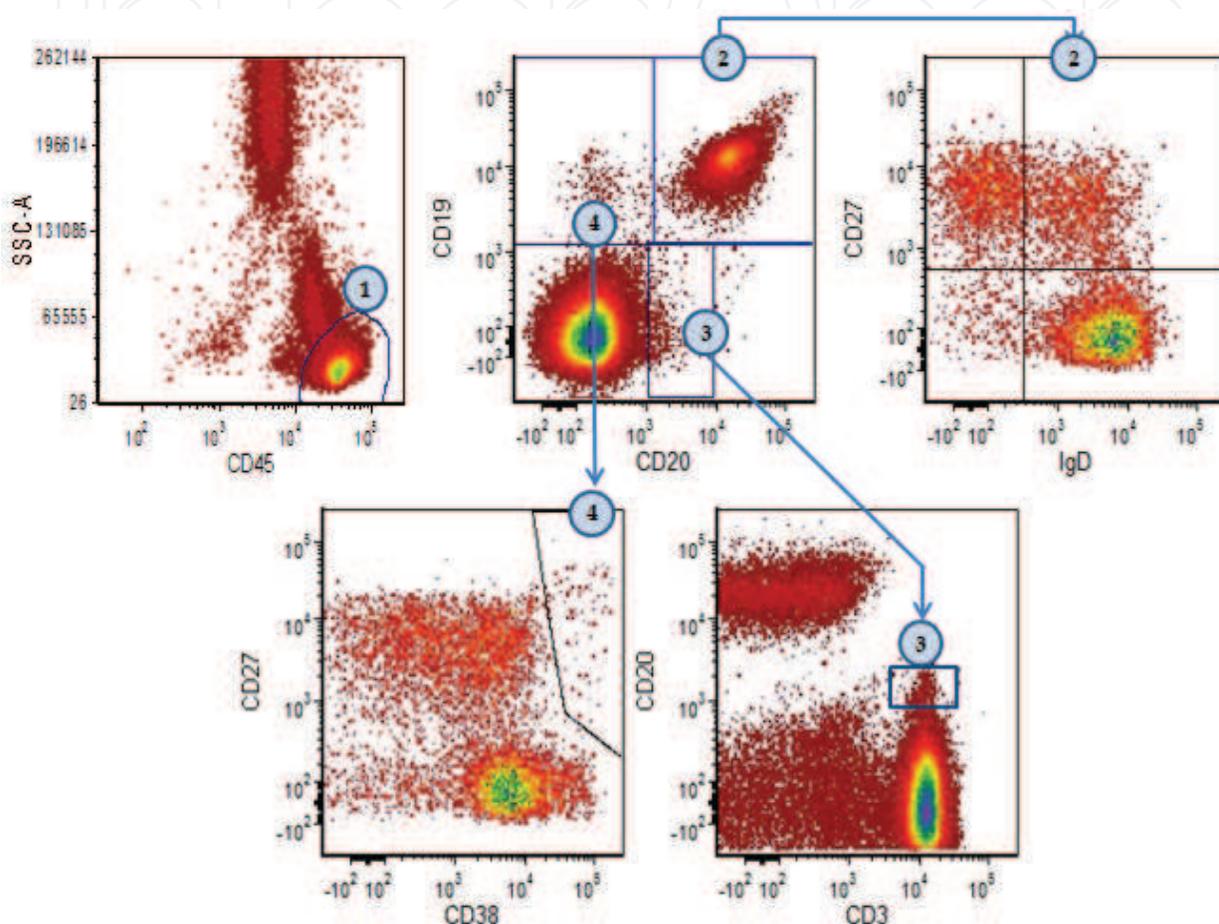


Fig. 2. Establishing the B cell gate. Blood from one healthy donor with recent influenza vaccination is shown to demonstrate B cell lineage gate. CD45 is used to discriminate leukocytes (1) from red blood cells and debris. Co-staining with CD19 and CD20 separates B cells (2) from dim CD20 expressing T cells (3) and CD20 negative plasmablasts/cells (4)

In 1994, Pascual et al. introduced additional markers to further study mature B cells. In the tonsil, B cells were characterized by activation states using CD38, CD23, and CD77 expression in addition to IgD. This resulted in a classification scheme whereby “mature B cells” were binned into categories Bm1 through Bm5 (Figure 3B) (Pascual et al., 1994). However, the focus of this classification system was on B cells involved in germinal centers formed in lymphoid tissue and was not correlated with peripheral blood populations. Mature naïve B cells were classified by surface IgD and CD38 expression (Bm1 naïve: IgD+CD38-, Bm2 naïve/activated: IgD+CD38+intermediate) and somatic mutation status in V_H region genes of the BCR. These populations were shown to have virtually no mutations.

However, it is relevant to note that IgG⁺ cells were depleted prior to gene rearrangement studies. Germinal center founder B cells were identified as Bm3: IgD-CD38⁺⁺CD77⁺ (dark zone centroblasts) and Bm4: IgD-CD38⁺⁺CD77⁻ (light zone centrocytes) and showed elevated proliferation by ki-67 and increased mutational status and class switching to IgG⁺. Memory B cells were identified as Bm5 (IgD-CD38^{+/-}) and demonstrated somatic hypermutation and class switching to IgG⁺. Bohnhorst et al. showed a correlation between tonsil and blood in healthy and primary Sjögren's syndrome donors for most of the Bm subpopulations; Bm1-2 (naïve/activated) and Bm5 (memory: early CD38⁺ and late CD38⁻). Germinal center founder cells identified as Bm3 and Bm4 (IgD⁻/CD38⁺⁺) were not present in Bohnhorst's dataset (Bohnhorst et al., 2001). CD27 was also added to further discriminate memory B cell status. Once again, cells were sorted using cell surface markers (IgD, CD38, CD27) to study the somatic mutation status in V_H region genes of the BCR. These studies demonstrated that the "Bm1" population included both un-mutated BCR (CD27⁻) and mutated BCR (CD27⁺) populations; whereas "Bm2" showed no gene rearrangement, thus bolstering the paradigm that IgD⁺CD38⁺CD27⁻ cells are antigen inexperienced B cells.

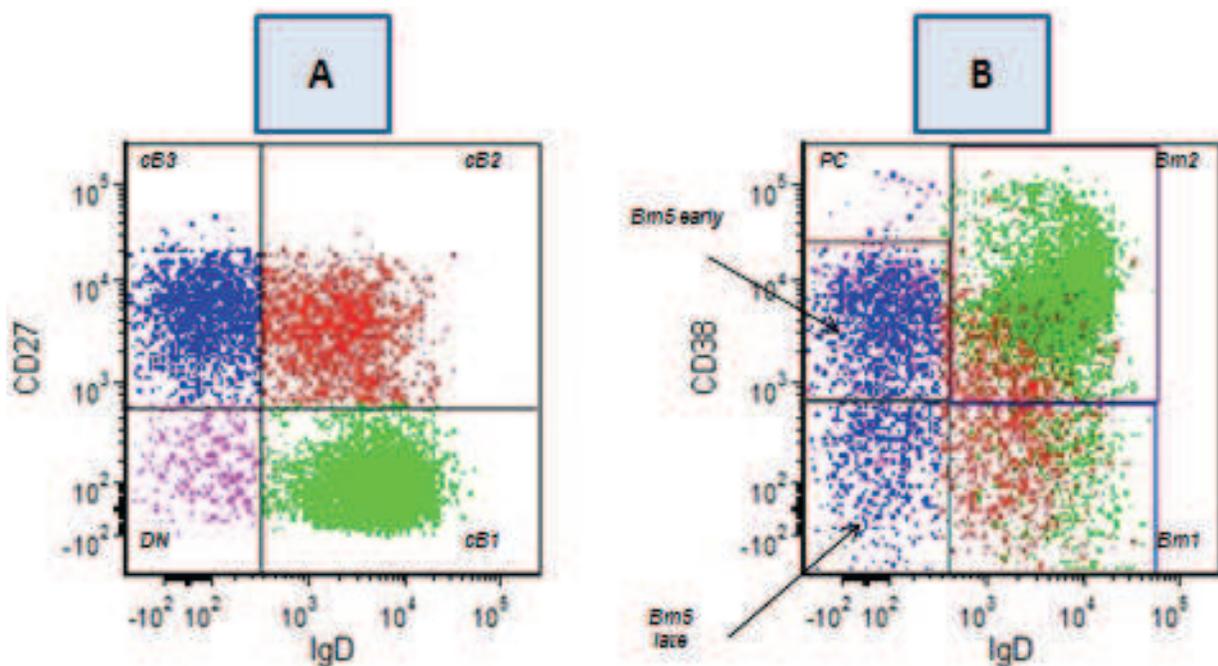


Fig. 3. B cell subset classification. Blood from one healthy donor is shown to demonstrate classification using IgD, CD27, and CD38. B cells are initially gated using CD45⁺ and co-expression of CD19⁺ and CD20⁺. A) cB1-cB3 and DN are defined using IgD and CD27. Mature Naïve B cells (IgD⁺CD27⁻) are designated cB1 (green), non-class switched memory B cells (IgD⁺CD27⁺) are designated cB2 (red), class-switched memory B cells (IgD⁻CD27⁺) are designated cB3 (blue), and CD27⁻ memory B cells (IgD⁻CD27⁻) are designated DN (purple). B) Bm1-Bm5 are defined using IgD and CD38. Naïve B cells (IgD⁺CD38⁻) are designated Bm1, naïve/activated B cells (IgD⁺CD38⁺intermediate) are designated Bm2. Germinal center founder B cells (IgD⁻CD38⁺⁺) in tonsil are defined as Bm3 (CD77⁺) and Bm4 (CD77⁻) (data not shown). In blood, this population (IgD⁻CD38⁺⁺) is comprised of plasma blast/cells and designated PC. Memory B cells are classified as Bm5early (IgD⁻CD38⁺intermediate) and Bm5late (IgD⁻CD38⁻). The color schemes listed in A) are maintained in B) to demonstrate the location of each cB/DN population in the Bm scheme

The cB and Bm classification systems have limitations if used in isolation. For example, naïve B cells have been defined as cB1 or Bm1/2, depending on the investigator. However, with a more comprehensive arsenal of surface markers (Table 1), the heterogeneity of each B cell population becomes evident. As an example of this heterogeneity, Figure 4 demonstrates that the cB1 population also includes transitional CD10+ cells and the Bm1/2 population is muddled by CD10+ transitional cells and IgD+ non-class switched CD27+ memory cells. Indeed, more discrete populations have been described (Wei et al., 2007; Sanz et al., 2008).

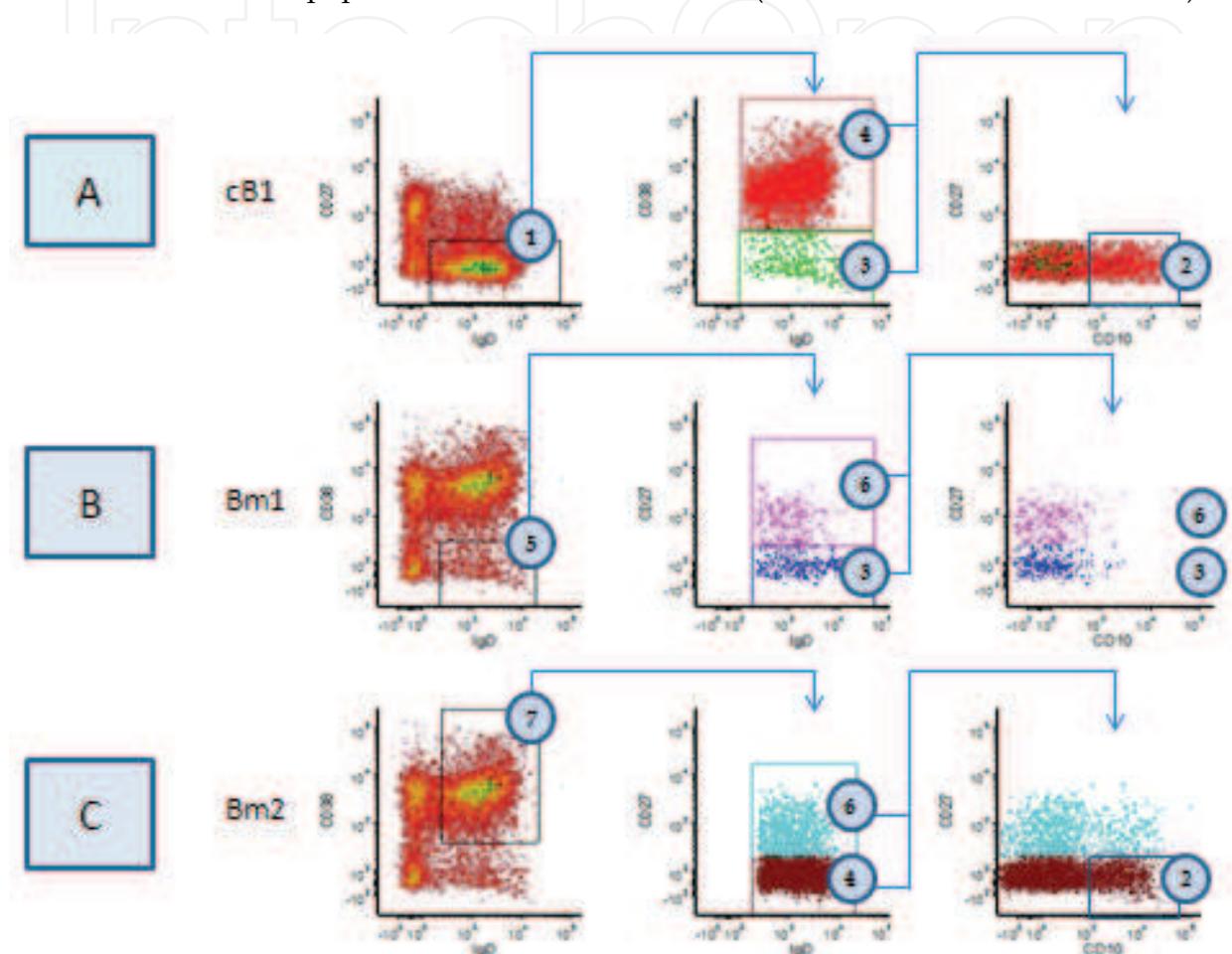


Fig. 4. Limitations of using cB1 and Bm1/2 in isolation to define the naïve B cell population. Blood from one healthy subject with recent influenza vaccination is shown. B cells are initially gated using CD45+ and co-expression of CD19+ and CD20+. IgD, CD27, CD38 are used to capture naïve B cells using both classification systems. Subpopulations are backgated to visualize heterogeneity in reciprocal classification schemes. Additionally, each classification scheme (cB1, Bm1, Bm2) is shown using CD10 and CD27 to identify CD10+CD27- transitional B cells. A) cB1 ① (IgD+CD27-) consists of transitional cells (CD10+CD27-) ② as well as mature naïve (IgD+CD27-CD38-CD10-) B cells ③ and activated naïve (IgD+CD27-CD38+CD10-) B cells ④. B) Bm1 ⑤ (IgD+CD38-) consists of mature naïve (IgD+CD27-CD38-CD10-) B cells ③ and non-class switched memory (IgD+CD27+CD10-) B cells ⑥. C) Bm2 ⑦ (IgD+CD38+) consists of transitional cells (CD10+CD27-) ② as well as activated naïve (IgD+CD27-CD38+CD10-) B cells ④ and non-class switched memory (IgD+CD27+CD10-) B cells ⑥

| Marker | Antigen Specificity on B cells* | Function |
|--------|---|---|
| IgM | First heavy chain immunoglobulin isotype expressed by B cells | Eliminates pathogens in the early stages of B cell mediated immunity, often referred to as the “natural antibody” |
| IgD | Second heavy chain immunoglobulin isotype expressed by B cells | Acts as receptor for antigen inexperienced B cells, stimulates basophils to release anti-microbial help |
| IgG | Immunoglobulin isotype expressed B cells after differentiation in the germinal center, secreted by plasma cells | Provides the majority of antibody-based immunity against invading pathogens, provides passive immunity to fetus |
| HLA-DR | Major histocompatibility complex class II, expressed on all mature B cells except non-proliferating plasma cells | Plays key role in antigen presentation |
| CD10 | Expressed on immature and transitional B cells and possibly post germinal center B cells | Important in B cell development |
| CD19 | One of the core components of the BCR expressed early in development, retained throughout maturation process, down-modulated in bone marrow resident plasma cells | Acts as signaling complex throughout life of B cell |
| CD20 | Expressed on B cells from late pro-B cell phase to mature memory cell, down-modulated in plasma blast/cells | Acts as calcium channel in cell membrane and important in B cell activation and proliferation |
| CD22 | Expressed cytoplasmically early in B cell development (late pro-B), surface expression coordinated with IgD, down-modulated in plasma cells | Regulates B cell adhesion and signaling functions |
| CD27 | Expressed on memory B cells and plasma blasts/cells | Involved with memory differentiation, upregulated on plasma blasts |
| CD38 | Expressed on various activated B cell developmental stages | Thought to indicate activation status of cell |
| CD45 | Expressed on all nucleated hematopoietic cells | Essential for antigen receptor signal transduction and lymphocyte development |
| CD138 | Expressed on plasma cells | Important for plasma cell adhesion to bone marrow stromal matrix |

*Please refer to (Neil Barclay et al., 1997) for additional details

Table 1. Description of B cell related markers. Description of the marker’s specificity on B cells only. The marker may be expressed on other cell populations with other functions

The markers most commonly used in our laboratory to analyze peripheral blood B cells are CD45, CD19, CD20, IgD, CD10, CD38, CD27 and CD138. With this strategy, using CD19 and CD20 as our anchor gate, we define the following populations within the IgD positive B cell population: transitional (IgD+CD27-CD38+CD10+), quiescent and activated naïve (IgD+CD27-CD38-/ +CD10-), and non class-switched memory (IgD+CD27+CD38-/ +CD10-).

The IgD negative B cells are comprised of immature (IgD-CD27-CD38+CD10+), class-switched memory (IgD-CD27+CD38-/+ and IgD-CD27-CD38-/+) and plasma blast/cell (CD20-IgD-CD27++CD38++ CD138-/+). These markers may not be appropriate in all situations and a number of caveats should be noted: [1] In clinical situations where a B-cell monoclonal therapy is used, an alternate B lineage marker may be required. For example, CD19 is used in most Rituximab trials since CD20 is the therapeutic target, [2] If CD20 is used in isolation to identify B lineage cells, a T cell marker is recommended as some T cells express low levels of CD20 (Figure 2) (Hultin et al., 1993), [3] Caution should be exercised when using CD20 to establish the initial B cell gate, as plasma cells lose expression of this marker upon terminal differentiation (Figure 2), [4] With regard to naïve B cell populations, while most IgD+ cells in the periphery co-express IgM, and IgD negative populations are assumed to express class-switched immunoglobulins (Klein et al., 1998), rare populations that are exclusively IgM+ or IgD+ have been described (Belouski et al., 2010; Weller et al., 2004), [5] CD38 is continuously expressed on B cells with frequent modulation of fluorescence intensity throughout development and therefore defining CD38^{bright} and CD38^{dim} can be subjective without proper controls and finally, [6] CD19 expression on B cells can be quite dim in patients with SLE, so selecting a bright fluorochrome is important.

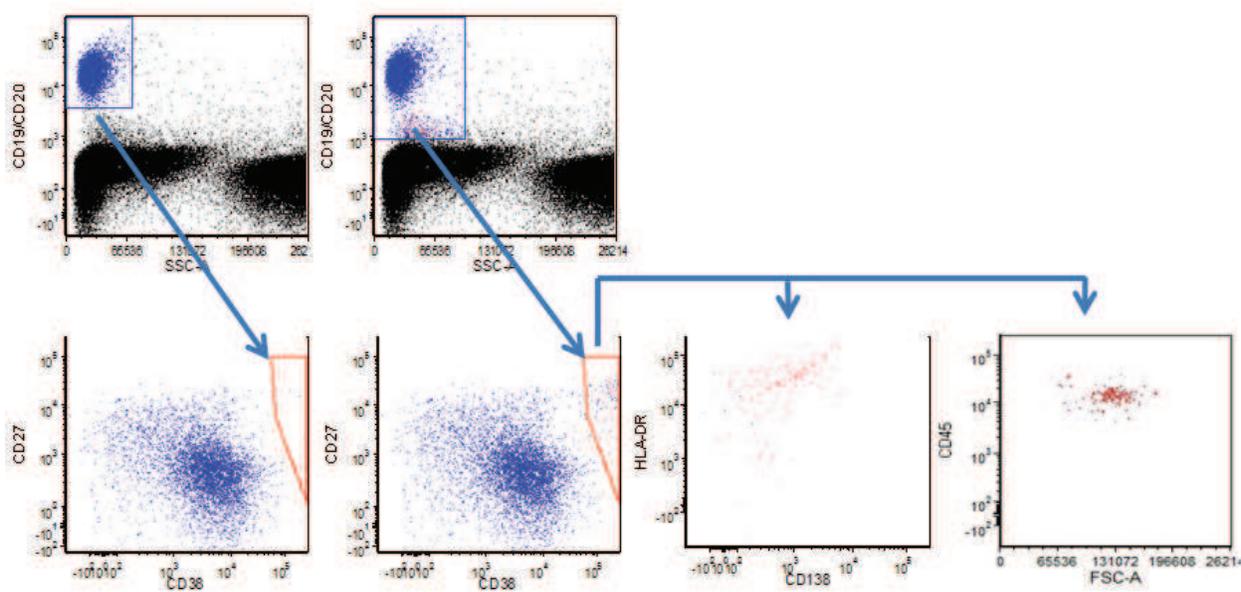


Fig. 5. Impact of gating strategy of B cells. Restricting the B cell lineage anchor gate can result in exclusion of plasma cells. Blood from one healthy subject with recent influenza vaccination is shown. B cells are initially gated using CD45+

There is still considerable debate on the phenotypic classification and nomenclature of antibody secreting cells. Uncertainties in classification most likely reflect the variety of surface antigens that can be modulated on the basis of their maturation and activation state, residence in bone marrow, blood or tissue compartments as well as the relative age of the individual (Caraux et al., 2010). A particular challenge is the differentiation of plasma cell precursors, aka plasmablasts, from the terminally differentiated, non-proliferating plasma cells. Growing evidence suggests that plasmablasts can be distinguished from plasma cells by their expression of MHC class II, elevated chemokine receptor expression (CXCR4,

CXCR3; suggestive of homing to inflamed tissues or bone marrow), and proliferative capacity (as measured by ki-67) (Odendahl et al., 2005; Jacobi et al., 2010b). In healthy subjects plasmablasts appear in the blood as a transient population that arises in response to antigen challenge (Chaussabel et al., 2008) and can be studied in this setting. Although tissue resident plasma cells downmodulate CD45, we are comfortable using CD45 to gate on plasma cells in peripheral blood (Figure 5) (Pellat-Deceunynck and Bataille, 2004; Schneider et al., 1997). Overall, we recommend that each lab carefully evaluate their schema for phenotyping plasmablast and plasma cells and generate data to support their decisions prior to embarking on tests with clinical specimens.

To further understand B cell heterogeneity and for better comparison of data across laboratories, it will be important for investigators to work toward more comparable data sets, and bring together data from many individuals for comparisons. An array of antigens could be summarized into a proteomic array of surface phenotypes and be analyzed in a comparable manner to transcription analysis by microarray. Some investigators are using complex multi-color panels (e.g. 20+ colors) to address these challenges (Lugli et al., 2010b; Lugli et al., 2010a; Gattinoni et al., 2011; Qian et al., 2010), but the impact of fluorescent overlap on the quality of these measurements is still a concern for everyday use, and may be limited to specialized laboratories. One new instrument that holds promise in approaching this kind of global standard is the elemental cytometer (Bendall et al., 2011), capable of analyzing a large array of data (e.g. 50+ parameters) and is not limited by overlapping fluorescence like more traditional flow cytometric platforms.

Dysregulated B cell Phenotypes in SLE

Methods to detect deviation from healthy development patterns have provided information for the diagnosis and monitoring of B cell aberrancies, especially in the field of oncology (Craig and Foon, 2008). Likewise, in autoimmune diseases such as SLE, the cellular composition of the B cell compartment is notably skewed. This dysregulation may provide insight into the steps that contribute to a break in tolerance observed in autoimmune diseases. Some SLE patients have a higher proportion of circulating T follicular helper (TFH) cells (CD4+CXCR5+ ICOS+) and plasmablast/cells (CD19dimCD27+CD38++) compared with healthy individuals (Hutloff et al., 2004; Illei et al., 2010). Increased frequency of TFH cells correlate with anti-dsDNA titer (Simpson et al., 2010) and increased plasma cell numbers correlate with disease activity (Dorner and Lipsky, 2004). The high number of TFH cells could reflect aberrantly high number of germinal centers; this could trigger the development of more plasma cells, and, later-on, pathogenic auto-antibodies. Likewise, on peripheral B cells, CD38 expression can be increased and conversely, CD19 expression decreased. Unusually high numbers of transitional cells have also been reported. Elevation of specific memory cell subsets (CD27-IgD-CD95+) have also been reported by many investigators as well as our own experience (Wei et al., 2007; Jacobi et al., 2008b). Finally, it is worth noting that dysregulation of B cells could potentially lead to high affinity auto-antibodies. For example, SLE patients exhibit an increase in antibody and complement deposition on circulating reticulocytes and platelets, correlating with disease activity (Navratil et al., 2006; Batal et al., 2011). Study of these deviations may provide clues to disease status and the potential efficacy of established or experimental therapeutics.

4. Technical considerations

Flow Cytometry is a powerful analytical tool yet insufficient care in technical considerations can lead to data that is difficult to interpret or worse, data that is misleading or incorrect. When establishing an immunophenotyping assay, all analytical aspects that might contribute to variability must be considered.

Specimen stability

Biological material, regardless of origin, begins to change and degrade once removed from the body. This presents a unique challenge in flow cytometric assays where accurately enumerating and measuring cellular components is dependent on maintaining the integrity of the specimen. Choice of specimen (i.e. whole peripheral blood, isolated and cryopreserved peripheral blood mononuclear cells (PBMCs)), blood collection tube, anti-coagulant, and shipping/storage conditions all play a critical role and should all be considered. Various whole blood stabilization products, such as Cyto-Chex® BCT, TransFix®, and CellSave have become available in the last few years, purporting to provide improved stability of surface marker expression and light scatter properties of lymphocytes and circulating tumor cells in whole blood. These products can be divided into two categories: 1) cell preservative solutions that are added to blood after collection into standard anticoagulant blood collection tubes or 2) direct-draw blood collection tubes that include both anticoagulant and a cell preservative solution. Although these products were initially approved by the FDA for use in extending the stability of blood for CD4 counts in remote laboratory HIV testing and maintaining integrity of fragile circulating tumor cells, there is promise that other surface markers may be stabilized as well. There is growing evidence that the use of blood collection tubes with cell preservative formulation may preserve some surface antigen expression superior to that of blood collected with anticoagulant alone (Plate et al., 2009; Warrino et al., 2005; Davis et al., 2011). However, a cell stabilization formula that truly extends the stability of blood without impacting resolution of dim markers has yet to be brought forward. Peripheral (whole) blood is the specimen of choice in our laboratory because the composition of cells in the unseparated and unfrozen state is most likely to resemble the *in vivo* state of the blood donor (Belouski et al., 2010). Peripheral blood analysis also has the advantage that the cells are exposed to the biologic matrix throughout the assay. This is particularly important in clinical trials because the therapeutic compound is retained in the specimen.

Establishing the antibody panel

The expansion of commercially available monoclonal antibodies conjugated to an ever-increasing list of fluorescent dyes has provided the opportunity for higher complexity multiplexed assays. However, to establish the optimal panel, one must consider: [1] expected antigen density and frequency of the cell population of interest, [2] interaction of reagents within the panel (spectral overlap between fluorochromes), [3] stability and sensitivity to assay conditions (temperature, pH, cell concentration), and [4] the sensitivity limitations of the flow cytometer.

Once the theoretical panel has been constructed, it is good practice to test the features of the cocktail of fluorochrome-conjugated antibodies under the conditions that you will use in

your study. Questions worth answering include: [1] determination of antibody clone(s) and conjugate(s) that correctly identify the population of interest (different clones can generate markedly different staining patterns), [2] The optimal titration of each antibody for its intended purpose (the density of antigen in the target population may exceed that of the healthy range and require a higher antibody concentration to saturate the target), [3] whether compensation controls accurately address spectral overlap for each antibody-fluorochrome, based on expected dynamic range of the data (a fluorescence-minus-one (FMO) matrix experiment (example in Table 2) can provide valuable insight during the development phase and may identify potentially troublesome compensation issues and/or markers with dim or heterogeneous expression), [4] How stable are the fluorochromes in the matrix (some fluorescent dyes are sensitive to pH, fixation and photobleaching (e.g. tandem dyes)?), [5] Whether antibody cocktails with demonstrated stability and extended shelf life (>1 month) can be produced and used. With information about these biochemical components, it is now appropriate to develop the assay.

| | FITC | PE | PerCP | APC | Pacific Blue |
|------------------|------------|-------------|-------------|-------------|--------------|
| Panel | IgD | CD38 | CD45 | CD27 | CD19 |
| FMO FITC | -- | CD38 | CD45 | CD27 | CD19 |
| FMO PE | IgD | -- | CD45 | CD27 | CD19 |
| FMO PerCP | IgD | CD38 | -- | CD27 | CD19 |
| FMO APC | IgD | CD38 | CD45 | -- | CD19 |
| FMO Pacific Blue | IgD | CD38 | CD45 | CD27 | -- |

Table 2. Example of Fluorescence Minus One (FMO) Matrix Experiment. In this 5-color panel, each antibody-fluorochrome is removed from the matrix, one by one to determine spectral overlap issues and establish negative thresholds

Assay development

Numerous assay parameters should be tested. These include the evaluation of: [1]the impact of sample type (e.g. peripheral blood, PBMC), [2] red blood cell lysis (recipe, temperature and timing of lysis), [3] antibody-cell incubation time and temperature, [4] washing and acquisition buffers, and [5] the number of cells needed to acquire meaningful data. This is particularly true when targeting rare cell populations. Many of the cell populations that have been suggested as contributing to chronic inflammation in autoimmune diseases, such as TFH and plasmablast/cells, are quite rare in circulation. Advances in the technology of rare event detection are warranted and enrichment technologies, such as magnetic bead

sorting prior to flow cytometry, may provide an improvement. The impact of enrichment on phenotype and function will need to be characterized for each population of interest.

Setting up and maintaining instrument

A key aspect to generating reliable and accurate data is ensuring that the instrument is properly set up and maintained. There is a wealth of information on how to optimize, validate, and maintain flow cytometers so the scope of this chapter will not include instrumentation specifics (Green et al., 2011). In brief, the instrument must be first properly aligned and characterized. Next, a good routine quality control system must be implemented and adhered to. Some clinical trials persist for years, making it even more important to maintain data integrity and reduce longitudinal variability. Beads with known fluorescent quantities (e.g., MESF, QuantiBRITE) can be used to establish a standard curve by which fluorescent intensity can be converted into semi-quantitative measurements, thus reducing the impact of longitudinal variability (Schwartz et al., 2004; Wang et al., 2008). Ensuring that the flow cytometer has optimal sensitivity for the panel is critical. This is especially true if the expected density of the marker is very dim. Figure 6 is an example of using standardized beads with known fluorescent properties to test these parameters, where Instrument C is inferior in the APC channel, compared with Instruments A and B.

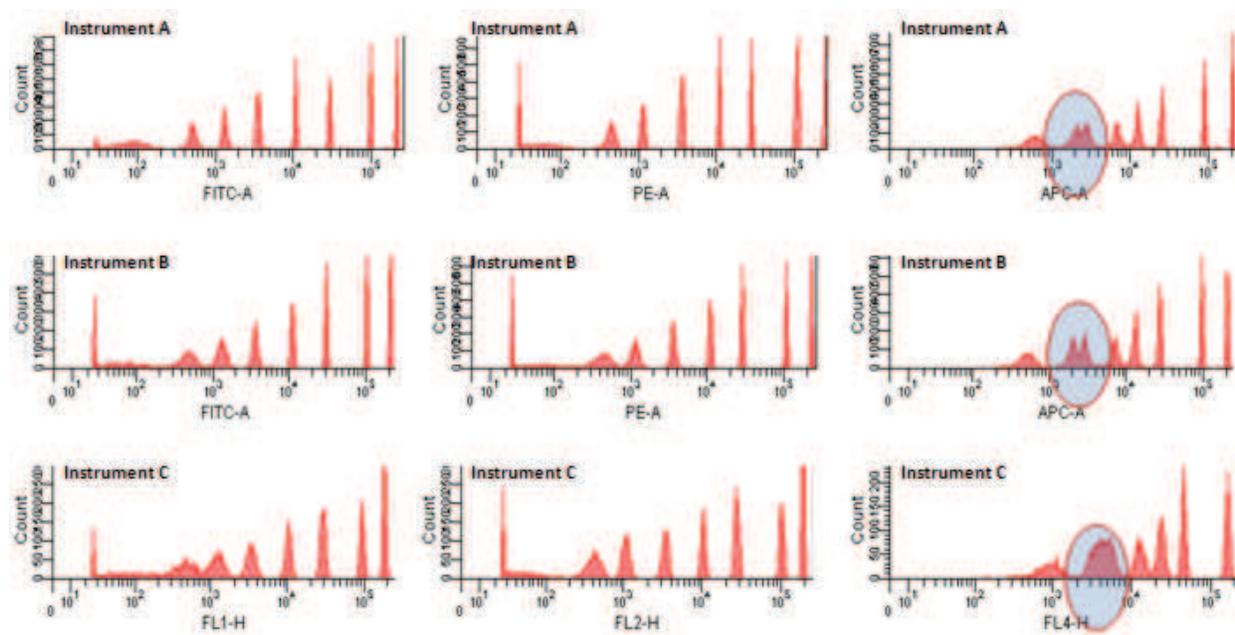


Fig. 6. Sensitivity varies between instruments. SPHERO™ Ultra Rainbow beads were acquired using optimal instrument settings on 3 different flow cytometers. Resolution of the dim peaks in the APC channel on Instrument C is inferior to Instrument A and B

Data analysis and Interpretation

Many software programs are available for post-acquisition analysis of flow cytometry data files, including instrument associated acquisition software and stand alone third party analysis software. While this flexibility provides the researcher with many tools to customize analysis for a specific purpose, caution is advised when establishing the analysis template. Electronic listmode/FCS files that are imported from various instruments into

third party software may display quite differently based on hardcoded meta-data in the raw data and user preferences set within each software. While the actual electronic files have not changed, variation in data display can significantly impact the final results. Likewise, the technical detail of gating strategy represents another source of variance from standardized definitions because differences in gating can easily result in different data output regarding a given cell type. For example, a stringent CD19/CD20 gate could exclude circulating plasmablast/ cells (Figure 5).

Another component of data analysis entails understanding the reliability of each measurement. Performing validation exercises to establish: [1] the stability of whole blood for intended analytes, [2] assay precision (replicates), and [3] inter- and intra-subject variability is paramount to interpretation of meaningful changes in phenotype or composition after treatment with clinical therapeutics. Although flow cytometric datasets are not inherently different than any other regarding statistical analyses, applications in the setting of early phase clinical trials incorporate cohort sizes that are not always amenable to population-based statistical approaches. We have chosen to highlight one approach to this problem that can prove useful in this setting that is referred to as the coefficient of reliability (CoR) (Taylor et al., 1989b).

The CoR tethers reliability to the consistency of repeat measurements in an individual over a window of time, calibrating a meaningful change after treatment with a therapeutic agent as one compared against each person's baseline measurement. For this analysis scheme to work and reveal a treatment effect in a clinical trial, the within-person variability must be well characterized such that one can call-out a change after treatment that exceeds the intrinsic variability of the assay.

A CoR can be determined by dividing inter-subject variability by the total variability (inter+intra), resulting in a number from 0.00 (least reliable) to 1.00 (most reliable) (Belouski et al., 2010; Taylor et al., 1989a). Using the CoR, one can determine what analytical parameter contributes most variance (Table 3). For example, an analyte that shows very little intra-subject variability but exhibits high variability between subjects can still be considered "reliable". If more variability is seen within repeat measures from the same subject than is observed between subjects on a single draw, the analyte could be considered "unreliable". The reliability of a given measurement can reflect variability that is introduced as a function of specimen stability or specimen processing/analysis or can reflect bona-fide biologic variability. If one takes care to minimize laboratory variability, datasets with high CoR can be attained (Table 3, with discussion below).

We typically begin our investigations by estimating inter-subject variability (donor-to-donor) and intra-subject variability across three repeat blood draws in a group of healthy and/or diseased donors, and use a threshold of 0.64 as a guide to differentiate between a reliable (≥ 0.64) and unreliable (< 0.64) measure; this reflects the original publication from Taylor et al (Taylor et al., 1989b) that examined the CoR for CD4 counts in HIV-infected persons. As an example using data generated in our laboratory (Table 3), we show the CoR of common B cell subsets for nine healthy donors (HD) and five SLE donors with mild disease severity. As shown in the Table, nearly all of the measures as expressed in this analysis exhibited a high CoR, with many approaching a value of 1.00. Such analyses would be promoted for application in a clinical trial, but we caution the reader in assuming that

CoR values such as these are typical (we have failed a variety of assays based on CoR measures that are not shown here).

| Analyte | Parent Gate | HD (N=9) | SLE (N=5) |
|---------------|-------------------|----------|-----------|
| %CD3+ | CD45+ Lymphocytes | 0.87 | 0.97 |
| %CD19+CD20+ | | 0.91 | 0.96 |
| %CD27+CD38++ | B lineage | 0.83 | 0.81 |
| %CD138+CD38++ | | 0.76 | 0.79 |
| IgD+/CD27-% | | 0.96 | 0.96 |
| IgD+/CD27+% | | 0.96 | 0.97 |
| IgD-/CD27+% | | 0.96 | 0.94 |
| IgD-/CD27-% | | 0.66 | 0.94 |

Cell populations <200 events

$$\text{CoR: } \frac{\text{inter-subject variability}}{\text{(inter- + intra-subject variability)}}$$

0.00 least reliable
1.00 most reliable

Table 3. Coefficient of Reliability of B cell subsets

Populations with an average of less than 200 events in Table 3 are highlighted in blue, as low event counts are a common laboratory source of low CoR scores. As depicted in the Table, it can be seen that not all flagged values fail CoR; only the cell types of lowest relative frequency in the blood, i.e., IgD-CD27- B cells in HD appear as (low) outliers in the Table. Interestingly, CoR increased as a function of a disease marked by increased frequencies of IgD-CD27- B cells in SLE (as much as 3X healthy range), that is, the higher CoR in SLE patients most likely reflected the expansion of this cell type in the blood. It is important to perform validation exercises in persons that exhibit a targeted pathology to fully characterize and understand each assay deployed in the clinical setting (Belouski et al., 2010).

Maximizing resources

It is becoming clear that for the medical community to truly leverage the information garnered by such flow cytometric investigations, consensus protocols and proficiency testing will be required. In lieu of that greater goal, it is important for any given investigator to bring forward all details of their immunophenotyping methodologies and to take time to understand the potential differences in reporting that exist between investigators and laboratories. In this regard, the ISAC (International Society for Analytical Cytometry) guidance document “Minimum Information about a Flow Cytometry Experiment” is of great value (Lee et al., 2008). Likewise, it would be inappropriate to discount the impact of resources to the implementation of a flow cytometric program. Instruments are highly technical and provide a service unique to the biomarker portfolio. However, instruments are priced accordingly, and the reagents to detect antigens, especially the ‘cutting edge’

fluorochromes, can be quite expensive. Two products would therefore help to reduce the cost of flow cytometry by allowing batch analysis: [1] lyophilized antibody panels with extended shelf-lives that could detect the “leukocyte proteomic array” of phenotypes and [2] collection tubes formulated to extend the window of time for processing whole blood.

Balancing the needs of the biologist (which is the best population to follow?), the technical considerations of the cytometrist (what is the most precise and accurate way to do the assay?) and resources (is it worth it?) are a few of the challenges encountered in flow cytometry biomarker programs. However, as evidenced in the next section, it is well worth the effort. The flow cytometer can provide essential decision-enabling data that unlocks evidence of therapeutic efficacy, mechanism-of-action and provide a fascinating snapshot into the dynamics of the immune system.

5. B cell flow cytometry in contemporary biomarker campaigns

Flow cytometry undeniably offers great insight into B cell biology in health and disease by enabling researchers with the ability to identify cells and understand their representation in the immune repertoire. The impact of flow cytometry based evaluations becomes even greater in the clinical settings because it allows one to understand the pharmacodynamic effects of a given treatment and when paired with biomarkers testing functional aspects of B cell biology (e.g. the vaccine response). We review some of the more common examples that have emerged from integrated assessment of B cells and B cell subsets during clinical intervention as examples of the value of understanding B cells in the context of therapeutic treatments in the clinic. In particular, we have reported in Table 4 on the phenotype as described by the investigator in each study, heterogeneity notwithstanding, and have summarized the key findings in Table 5. It is within the reach of these efforts to someday use these strategies to measure B cell-related biomarkers for patient selection, or to be leveraged in therapeutic co-development as companion diagnostic assays.

Pharmacodynamic activity of B cell-directed therapeutics

Strategies that deplete B cells to varying degrees are now commonly applied in the clinic. Initially tested in oncology, Rituximab is a chimeric monoclonal antibody that binds the B cell surface antigen CD20, leading to depletion of this population (reviewed in Boumans et al., 2011; Dorner et al., 2009). Rituximab was first approved in non-Hodgkin’s lymphoma (NHL) and has now been approved in many other indications, including chronic lymphocytic leukemia, rheumatoid arthritis (RA), and two forms of vasculitis. The successful depletion of oncologic B cells in NHL was encouraging enough to trigger the study of Rituximab in autoimmune scenarios. Rituximab proved successful in the treatment of TNF-resistant RA (in combination with methotrexate) and firmly established the B cell as a central player in the *autoimmune* immune system. Investigators also reported success of Rituximab in numerous autoimmune diseases including SLE. Controlled trials of Rituximab in SLE (with and without nephritis), however, failed to meet the primary and secondary efficacy endpoints although a beneficial effect was observed in some ethnic groups (Merrill et al., 2010; Looney, 2010). Rituximab is still used often to treat SLE off-label, and that impact on B cells in SLE still provides interesting insights. Most recently, Rituximab was approved for two forms of vasculitis associated with anti-neutrophil cytoplasmic antibodies (ANCA), Wegener’s Granulomatosis and Microscopic Polyangiitis.

| Marker System or Study | Transitional (T) / Immature (Im) | Naïve | Non class-switched Memory | Class-switched Memory | PI |
|------------------------|--|--|-------------------------------------|---|---------------------|
| cB1-4 | nd | CD19+ IgD+ CD27- | CD19+ IgD+ CD27+ | CD19+ IgD- CD27+ | no |
| Bm1-5 | nd | IgD+ CD27- CD38+/- | IgD-/+ CD27+ CD38+/- | | no |
| Rituximab in SLE | nd | CD19+ CD27- CD38- | CD19+ CD27++ CD38- | | C C C |
| | CD38++IgD+ CD10+CD24+ (Im) | CD19+ IgD+CD38+ | CD19+ IgD+/- CD27+ | | Ig C |
| | nd | CD19+ CD24+ IgD+ CD38- CD10- | nd | CD19+ CD1c+/- IgD- CD27+ IgM +/- | no |
| | CD38 ^{high} CD24 ^{high} IgD- (Im) CD38 ^{high} CD24 ^{high} IgD+ (T)* | CD38 ^{intermediate} CD24 ^{low} IgD+CD27- | IgD+CD27+ | IgD-CD27+ | C C C |
| Belimumab in SLE | CD19+ IgD+ CD27- CD10+ (T) | CD19+ IgD+ CD27- CD10- | CD19+ IgD+ CD27+ | CD19+ IgD- CD27-/+ | C C (P) |
| Atacicept in RA | nd | CD19+ IgD+ CD27- | CD19+ CD27+ CD38- | | C C |
| Tocilizumab in SLE | nd | nd | nd | nd | C C |
| Our system | CD19+CD20+ IgD- CD27- CD38+ CD10+ (Im) CD19+CD20+ IgD+ CD27- CD38+ CD10+ (T) | CD19+ CD20+ IgD+ CD27- CD38+/- CD10- | CD19+ CD20+ IgD+CD27+ CD38+/- CD10- | CD19+ CD20+ IgD-CD27-/+ CD38+/- CD10-/+ | C Ig C (P) |

Table 4. Identification of B cell populations-common marker systems and examples from clinical literature. Phenotypes summarized in this table are not all inclusive. As emerging technologies are developed and implemented, it is likely that characterization schemes will change. *Subsets are further described by investigator



| Mechanism | Example | Transitional | Naïve | Memory | Plasmablast / cell |
|-----------------------|-------------|--------------|-------|--------|--------------------|
| Anti-CD20 (depleting) | Rituximab | nd | ↓ | ↓ | ↔ |
| Anti-BAFF | Belimumab | ↓ | ↓ | ↑ | ↔ |
| TACI-Ig | Atacicept | nd | ↓ | ↑ | ↓ |
| Anti-IL-6R | Tocilizumab | nd | ↔ | ↔ | ↓ |

Table 5. Summary of changes in B cell populations in response to B cell therapeutics. Nomenclature varies for each study and this summary is based on the phenotype as described by the investigator. Please refer to the text for more details. nd = not determined

The effects of Rituximab mediated depletion and repletion of the B cell compartment have been carefully characterized using flow cytometry (Anolik et al., 2004; Anolik et al., 2007). In most subjects, Rituximab leads to the rapid depletion of CD19+ B cells in the peripheral blood (Merrill et al., 2010; Edwards et al., 2004), with fewer than 5 cells/ μ L by two weeks post dose. In a Phase 2 efficacy trial of general SLE, approximately 9.5% of treated subjects did not reach this level of depletion in the peripheral blood. [Removing these subjects from the efficacy analysis did not change the result in SLE.] Interestingly, approximately 26% of subjects had developed anti-therapeutic antibodies (Merrill et al., 2010) at week 52, during the early stages of B cell repletion.

The functional impact of B cell repletion was recently described in a study of neo-antigen (ϕ X174) and recall (tetanus toxoid) responses in Rituximab-treated patients. Peripheral B cell depletion was achieved (fewer than 5 cells/ μ L) in all subjects treated with Rituximab. The Rituximab-treated group had significantly lower anti- ϕ X174 responses compared to placebo during the window of B cell depletion (weeks 6-8) (Pescovitz et al., 2011). However, when re-immunized at weeks 52 and 56, after naïve (CD19+ CD24+ IgD+ CD38-CD10-) but not memory (CD19+ CD1c+/- IgD- CD27+ IgM-/+) B cell repletion had begun, the anti- ϕ X174 response returned to nearly normal levels. The vaccine memory response was tested at 52 weeks and although the memory B cell compartment had not returned to normal levels, all subjects mounted a response, albeit weaker in the Rituximab group. The impact of this study is important to note. The kinetics of Rituximab depletion and repletion of B cells, the half-life of the tetanus titer in healthy individuals (~11 years (Amanna et al., 2007)) and the new knowledge that Rituximab-treated individuals can rebuild their serologic titers once naïve B cell repletion begins will help physicians estimate when to begin re-vaccination on a patient-specific basis.

Resistance to depletion and repletion of B cells may also help identify those patients most likely to benefit from Rituximab or when to re-treat therapeutic responders. In SLE, Rituximab depletes peripheral naïve and memory B cells (CD19+CD27-CD38- and CD19+CD27++CD38-, respectively) and circulating plasmablasts (CD19+ CD20+/- CD27++ CD38++) (Vital et al., 2011). However, the plasmablast level at 26 weeks may predict relapse of the clinical response, as subjects with more than 0.8 plasmablast cells/ μ L were more

likely to relapse than subjects with fewer than 0.8 plasmablast cells/ μ L. In RA, subjects had a more favorable outcome with delayed B cell repletion (both naïve (CD19+IgD+) and memory (CD19+ CD27+) cells) (Teng et al., 2009; Roll et al., 2008) and reduced plasma cells (CD79a+ CD20-) in the synovium (Teng et al., 2009). A second study of RA demonstrated early relapse was characterized by higher non-class switched memory B cells (CD19+ IgD+ CD27+) before therapy (Roll et al., 2008). These studies suggest that the efficacy of Rituximab could be related to pre-treatment levels of unusual memory B cells, plasmablasts or plasma cells. Leveraging these studies, but using transcript analysis, a recent report suggests that plasmablast levels can identify those patients most likely to respond to anti-CD20 depletion therapies (Owczarczyk et al., 2011).

The effects of Belimumab (anti-BAFF) can be differentiated from those of Rituximab, where Belimumab selectively leads to the reduction in naïve (CD19+ IgD+ CD27- CD10-) and transitional (CD19+ IgD+ CD27- CD10+) B cells but leaves the memory (CD19+ CD27+ IgD-) B cell compartment intact (Jacobi et al., 2010a; Jacobi et al., 2010b). Non-class switched memory B cells (CD19+ CD27+ IgD+) cells and the compartment containing plasmablasts and plasma cells (CD19+ IgD- CD27++ CD38++) decreased after much longer exposure to therapy (~1.5 years). The phase III studies of Belimumab used a novel SLE Responder Index (SRI) (Furie et al., 2009) based on Belimumab's clinical phase II experience. With this strategy, Belimumab demonstrated efficacy in SLE and was approved by the FDA in 2011. Further studies of agents targeting BAFF have been initiated. Two phase III studies of Lilly's LY2127399 in patients with SLE are currently recruiting (Clinical Trials identifiers: NCT01196091, NCT01205438) and a phase III study of Anthera's Blisibimod (A-623) is scheduled to study safety and efficacy in SLE (NCT01395745).

The effects of Atacicept treatment (TACI-Ig) are distinct from Rituximab and Belimumab as well. Atacicept is a fusion protein of the extracellular portion of the TACI receptor and the Fc portion of human IgG. The TACI receptor binds BAFF and APRIL, two proteins with numerous functions (Davidson, 2010), including providing homeostatic survival signals to naïve B cells (BAFF) and plasma cells (APRIL) (Mackay and Schneider, 2008). Atacicept reduces the level of circulating naïve B cells (CD19+ IgD+ CD27-) and plasma cells (CD19^{dim} CD38^{bright}). Memory B cells (CD19+CD27+CD38-) exhibit a transient increase in the peripheral blood (van Vollenhoven et al., 2011). Atacicept additionally reduces total immunoglobulin levels (IgM, IgG and IgA) (Dall'Era et al., 2007; van Vollenhoven et al., 2011). In RA, Atacicept did not meet the primary endpoint in two studies - in subjects with inadequate responses to methotrexate (van Vollenhoven et al., 2011) and subjects with inadequate response to TNF antagonist therapy (Genovese et al., 2011). By impacting a broad spectrum of B cell subsets, BAFF/APRIL blockade may deplete long lived tissue resident plasma cells that produce pathogenic autoantibodies; however, BAFF/APRIL blockade may carry a greater infectious risk due to the depletion of protective antibody titers as well.

Finally, we discuss a molecule that significantly decreases the frequency of circulating plasma cells, Tocilizumab (anti-IL-6R). IL-6 has numerous roles in immune regulation, hematopoiesis, inflammation and oncogenesis (reviewed in (Kishimoto, 2010)) as well as providing survival signals to plasmablasts and plasma cells. Tocilizumab was efficacious in RA patients in several Phase 3 trials and has been approved in the US for patients who have failed TNF-blockers (Yazici et al., 2011) and in small studies of other autoimmune disorders (Kishimoto, 2010). In an open-label study of SLE subjects, Tocilizumab led to a significant

decrease in circulating plasma cells (CD19^{low} IgD⁻ CD38⁺⁺⁺), a decrease in serum IgG levels and a promising clinical response (Illei et al., 2010). There were no other changes in peripheral T or B cells.

More traditional therapies like cyclophosphamide may result in B cell depletion to a degree not as fully appreciated in the past. For example, cyclophosphamide induced significant B cell depletion in a trial compared with Rituximab in ANCA-associated vasculitis (Stone et al., 2010).

Modulation of B cell function

CD22 is an important signaling molecule for the homeostasis of early B lineage cells (Tedder et al., 2005). CD22 is expressed on developing pro- and pre- B cells as well as naïve B cells and is lost after activation; i.e. memory B cells, plasmablasts and plasma cells do not express CD22 (Dorken et al., 1986; Tedder et al., 2005). Epratuzumab is an anti-CD22 monoclonal antibody that modulates B cell function although the mechanism of action *in vivo* is unclear. *In vitro*, Epratuzumab leads to the rapid internalization of the CD22/antibody complex, resulting in significant CD22 phosphorylation (Carnahan et al., 2003) as well as a change in adhesion molecule cell surface expression and migration (Daridon et al., 2010). Epratuzumab is not thought to mediate ADCC or complement-dependent cytotoxicity (CDC) *in vivo*. In SLE, however, Epratuzumab leads to a significant reduction in CD27-negative B cells (primarily naïve and transitional populations) (Dorner et al., 2006; Jacobi et al., 2008a). The recent hypothesis that Epratuzumab alters B cell migration is compelling (Daridon et al., 2010) and may shed light on the mechanism of action, but has yet to be tested in the clinic. Epratuzumab has shown promising results in combination with Rituximab in oncology (Grant, 2010); SLE trials were terminated early due to insufficient drug supply. An interim report of the data suggested that Epratuzumab was effective (Wallace, 2010), however this study was not powered to detect statistical differences between treatment groups. A new set of phase III studies in severe general SLE (NCT01262365 and NCT01261793) will provide a clearer picture soon.

Additional molecules such as those targeting JAK3 kinase, mTOR and Syk (Changelian et al., 2008; Fernandez and Perl, 2010; Weinblatt et al., 2008) have been shown preliminarily to modulate B cell function in autoimmune subjects. Ongoing trials continue to evaluate the effectiveness and safety of these agents in various autoimmune disorders.

Pharmacodynamic activity of indirect B cell therapeutics

Therapeutic blockade of T cell help also removes essential B cell survival signals. In such circumstances a “T cell therapeutic” may also be considered a “B cell therapeutic”; or an *indirect* B cell target. A number of molecular interactions are required to form the germinal center reaction and to produce high affinity antibodies. Costimulation through CD28:CD80, CD28:CD86 or ICOS:ICOSL is required for naïve T cell activation. T cell help to B cells requires CD40:CD40L interactions as well as ICOS:ICOSL and IL-21 signaling. Therapeutic blockade of each of these signaling nodes could reduce the generation of high affinity autoreactive antibodies.

Abatacept is a fusion protein of the extracellular domain of CTLA4 and the Fc portion of human IgG. CTLA4 binds CD80 and CD86 with higher affinity than CD28, thus making a fusion protein a good therapeutic candidate for blocking early steps in T cell activation.

A controlled trial of Abatacept in SLE (in patients with arthritis, serositis, or discoid lupus) did not reach the primary endpoints, however a post-hoc analysis demonstrated a significant benefit in certain SLE subgroups and issues with the study design, including the relatively high dose of glucocorticoids mandated during the trial, may have obscured the trial results (Lateef and Petri, 2010). Abatacept increases the proportion of monocytes in RA subjects (Bonelli, 2010) and baseline numbers of CD28+ T cells may predict remission (Scarsi et al., 2011).

Another approach is to block signaling in established, autoreactive germinal centers. BG9588, a monoclonal anti-CD40L antibody, showed initial clinical success in renal SLE (Boumpas et al., 2003). Safety concerns led to the discontinuation of this program due to thrombotic events due to platelet CD40L expression (Buchner et al., 2003; Henn et al., 2001). A second generation anti-CD40L antibody (IDEC-131) showed initial benefit in SLE subjects, however in a large phase II IDEC-131 study failed to show efficacy over the placebo group. Additional clinical study of IDEC-131 was initiated in other autoimmune settings but halted after a thromboembolic event in a Crohn's trial (Sidiropoulos and Boumpas, 2004). Of interest to the flow cytometry expert, in a study of four renal SLE patients, BG9588 in combination with prednisone was capable of modulating effector B cell populations and return components of the dysregulated phenotype to normal. Highly activated naïve and memory B cells (expressing CD38++) and intracellular Ig+ plasma cells (CD19+ CD38++) were reduced following two treatments of BG9588 (Grammer et al., 2003), although they returned to high baseline levels after treatment. This in combination with a reduction in proteinuria and anti-dsDNA antibodies (Grammer et al., 2003; Sidiropoulos and Boumpas, 2004) suggested that CD40:CD40L interactions contribute to the generation of autoreactive plasmablast and plasma cell populations and that perhaps other, safer, therapeutics that target the GC reaction could provide benefit in SLE.

Recent advances in T cell immunology have identified a specialized subset of CD4 T cells that provide help to B cells attempting to form a germinal center. This population has been called T follicular helper cells (TFH) (Crotty, 2011). Naïve CD4 T cells become activated in the T cell zone of the secondary lymphoid organ. The inducible costimulator, ICOS, is expressed, and ICOS engagement drives the upregulation of CXCR5 and Bcl6, migration toward the B cell zone and differentiation into a TFH (Choi et al., 2011). Phenotypically, TFH are characterized by their high expression of CXCR5, ICOS and PD-1, and location (if possible). Patients with an ICOS-null mutation do not develop TFH and have significantly reduced serum IgG concentrations (Bossaller et al., 2006; Warnatz et al., 2006; Grimbacher et al., 2003).

MEDI-570 is a monoclonal antibody that depletes ICOS-bearing T cells and is currently being tested in a phase I study of SLE (NCT01127321). AMG 557 is a monoclonal antibody that binds ICOSL and blocks the ICOS:ICOSL interaction; AMG 557 is currently being studied in SLE (NCT00774943), Subacute Cutaneous Lupus Erythematosus (NCT01389895), Psoriasis (NCT01493518), and SLE with Lupus Arthritis. NN8828 is a monoclonal antibody that binds the cytokine IL-21, which has many effects including the prolongation of the germinal center reaction; NN8828 is being studied in RA (NCT01208506). It will be intriguing to see these data come forth over the next few years – does safe blockade of the GC reaction with anti-ICOS, anti-ICOSL or anti-IL-21 lead to similar effects on the immunophenotype as anti-CD40L?

Patient selection

One of the goals of personalized medicine is to identify biomarkers for patient selection. An elegant example of this is the recent description of a peripheral blood plasmablast biomarker to identify non-response to anti-CD20 depleting therapy in RA (Owczarczyk et al., 2011). Many studies had shown that the level of plasmablasts (or pre-plasma cells) were high in non-responders (Boumans et al., 2011). Behrens et al. demonstrated that two mRNA biomarkers of plasmablast levels in peripheral blood ($IgJ^{hi}FCRL5^{lo}$) identify a group of one in five RA subjects who are not likely to respond to anti-CD20 depletion therapy. Peripheral blood B cells were identified flow cytometrically (CD19+) and whole blood RNA samples were assayed for CD20 mRNA expression by RT-qPCR. Levels of the FCRL5 transcript in whole blood correlated with the proportion of naïve B cells while the IgJ transcript was anti-correlated with the levels of naïve and memory B cells. Therefore, the $IgJ^{hi}FCRL5^{lo}$ whole blood transcript could identify subjects with high levels of plasmablasts and plasma cells and low levels of naïve cells.

For other therapeutic programs, data is more preliminary. For example, baseline numbers of CD28+ T cells have been shown, in a small study, to predict remission of RA treated with Abatacept (Scarsi et al., 2011). One thing that is clear is that flow cytometry has utility in patient selection in its own right but can also enable utilization of other platforms towards this goal.

6. Conclusion

In summary, the clinical flow cytometer has provided decision-enabling data in the monitoring of therapeutic impact (e.g., B cell depletion and repletion, normalization to the healthy phenotype), and is beginning to be used to identify biomarkers for patient selection. We hope to help clarify the context of flow cytometry in clinical development and provide some of our insight into successful implementation of immunophenotyping biomarker programs. However, to those new to B cell investigations via flow cytometry, caution should be utilized - it should be acknowledged that “*anyone can get dots*” on the flow cytometer but that it is up to the investigators to truly understand their assay. With this rigor, it will be possible to generate meaningful biomarker data and help guide decision-making on the next generation of B cell therapeutics.

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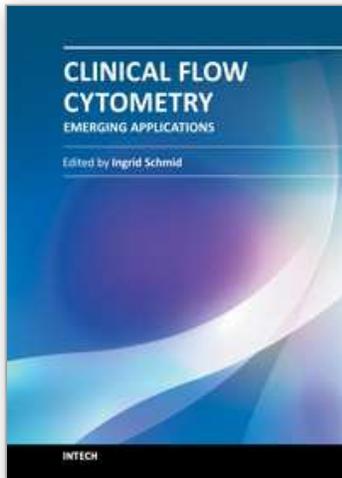
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