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OMIP-003: Phenotypic Analysis of Human Memory B Cells

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• **Key terms**
human; B cells; memory; SLE

Purpose and Appropriate Sample Types

This panel was developed to characterize the phenotypic diversity of human memory B cells, with an emphasis on discriminating cell subsets within both the conventional memory population ($CD27^+$) and the more recently described isotype switched (IgD^-) population lacking expression of CD27 (1). It has been tested on fresh and cryopreserved peripheral blood mononuclear cells (PBMC), as well as bone marrow aspirates and tonsillar cells (Table 1). The multicolor panel described herein has been used extensively to analyze large numbers of PBMC samples obtained from healthy controls in steady state and in response to infection (HIV, influenza, respiratory syncytical virus) and vaccination (influenza, tetanus) as well as in hundreds of patients with autoimmune diseases (systemic lupus erythematosus (SLE), rheumatoid arthritis, Sjogren's syndrome, psoriatic arthritis and Type 1 diabetes) and conditions characterized by allogeneic immune responses (renal transplantion and chronic graft versus host disease). This panel is also being applied in a longitudinal study in which 150 SLE patients are to be followed quarterly for a period of 2 years.

Background

Major human B cell subsets are currently defined by pauci-color flow cytometry protocols that typically include IgD, CD27, CD38, and CD24 to classify the major accepted peripheral blood populations (transitional, naïve, memory, and plasmablast subsets). By and large, the expression of other informative markers (including IgM, CD23, CD10, CD21, and CD95, as well as chemokine receptor expression) is assessed by parallel staining of several sample aliquots with different combinations of the aforementioned markers in four to five color schemes. These approaches suffer from multiple shortcomings including: 1) the limited ability of the "defining" markers such as IgD, CD27, and CD38 to properly discriminate major populations; 2) the inability to ascertain the actual co-expression of multiple markers in a single population possibly leading to faulty assumptions of extended phenotypes and, by extension, preventing the discovery of new subpopulations; and 3) the need for larger number of cells to perform multiple stainings, a major practical limitation when dealing with rare samples. Combined, it seems obvious that limited use of available markers not only fails to differentiate multiple populations within the conventional core subsets, but could potentially lead to erroneous attribution of functional properties. To address these limitations, we have developed several multicolor panels to fully characterize human B cells. These multicolor panels share seven so-called anchor markers. Antibodies against CD19 and CD3, along with the Fixable Aqua Dead Cell Stain, allow the unambiguous identification of live $CD19^+CD3^-$ B cells. The inclusion of four developmental markers (IgD, CD24, CD27, and CD38) in the same panel makes it feasible to compare and integrate these different

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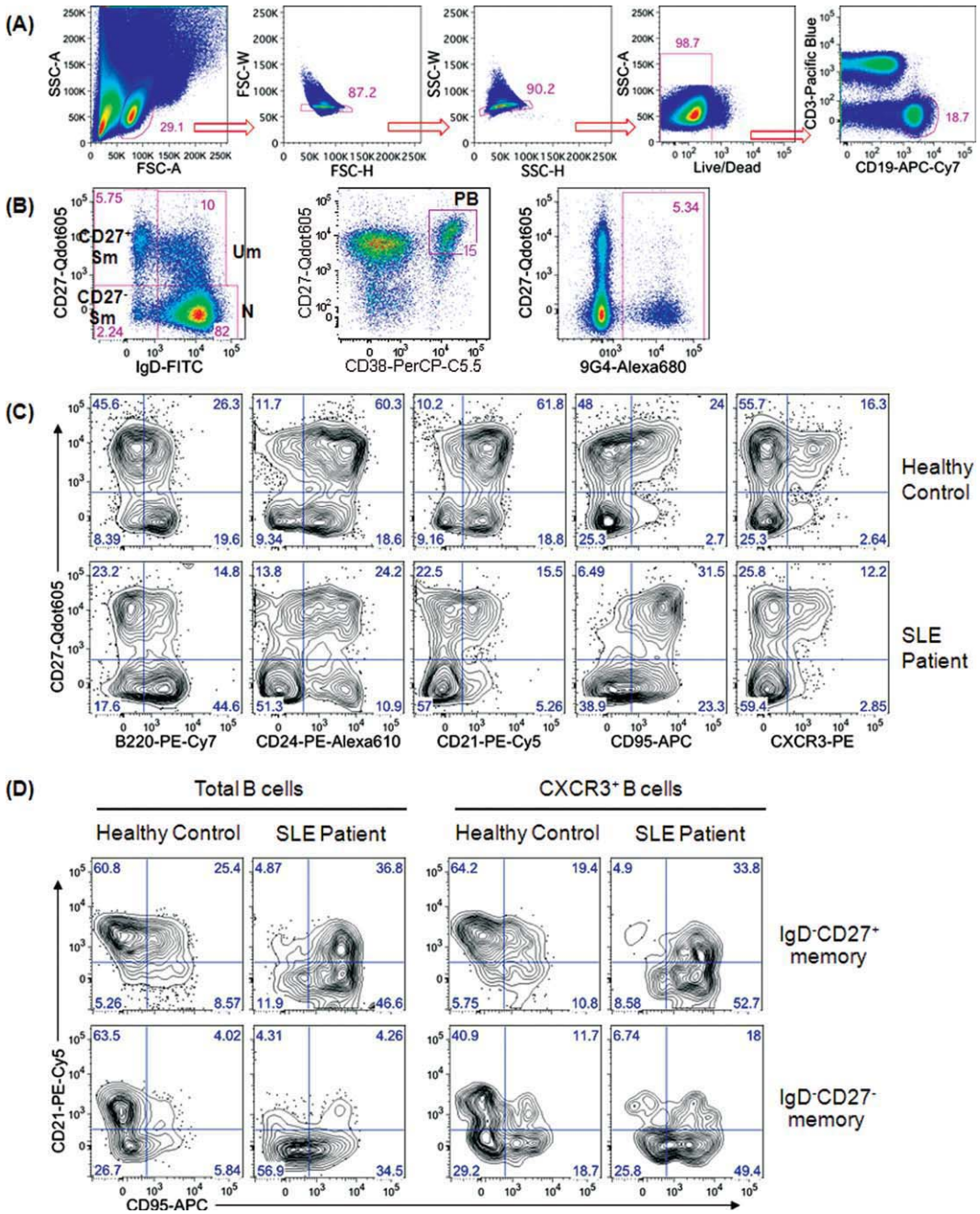

Figure 1. Classification of core human B cell subsets and phenotypic characterization of memory B cells in healthy subjects and patients with systemic lupus erythematosus. **(A)** Lymphocytes gated through the FSC-A vs SSC-A plot were further interrogated by the ratios of height to width in forward scatter and side scatter, as well as their ability to uptake the amine-reactive Aqua fluorescent dye to gate out cell aggregates and dead cells, respectively. Live $CD19^+CD3^-$ B cells were then selected for analysis as shown in B. **(B)** The customarily used IgD/CD27 scheme classifies peripheral blood B cells into four core subsets (left panel): naïve (N: IgD^+CD27^-); unswitched memory (Um: IgD^+CD27^+); IgD^-CD27^+ switched memory ($CD27^+$ Sm); IgD^-CD27^- switched memory B cells ($CD27^-$ Sm). IgD^-CD27^{++} plasmablasts (PB) are a rare population in steady-state healthy subjects and can be better discriminated as $CD27^{++}CD38^{++}$ cells in the IgD^- fraction of a healthy subject who, in this example, received HPV vaccination 11 days before the analysis (middle panel). The rightmost panel shows that autoreactive $9G4^+$ B cells concentrate within the naïve compartment in healthy subjects (3). The comparisons among different B cell classification schemes made possible by this panel is discussed and illustrated in Supporting Information Figure 3. **(C)** Comparisons of IgD^- B cells between a healthy subject and a SLE patient illustrate the value of multicolor panels to reveal informative phenotypic differences in health and disease. As previously reported (2), B220 expression is lost during GC differentiation and thus $CD27^+$ resting memory cells are predominantly $B220^-$. In contrast, IgD^-CD27^- cells, which as in this example may be the dominant memory population in active SLE, are predominantly $B220^+$. This expanded subset is also characterized in SLE by the down-regulation of CD24 and CD21, markers expressed by the majority of PBL B cells in general and $CD27^+$ memory B cells in particular. Loss of CD21 and up-regulation of CD95 have been independently associated with memory B cell activation (4,5). Accordingly, expansions of activated switched memory subsets ($CD27^+$ and $CD27^-$) are evident in active SLE. In contrast, CXCR3 expression (suggestive of migratory potential of activated cells to Th1 areas of systemic inflamed tissues) is concentrated in the IgD^-CD27^+ memory subset. **(D)** The inclusion of CD21, CD95, and CXCR3 in the same panel allows informative co-localization of these markers. In contrast with $CD27^+$ resting memory cells in the healthy control, a significant fraction of IgD^-CD27^- cells lack expression of CD21, a feature consistent with activation. Yet, the lack of CD95 expression indicates that these two markers are not necessarily correlated. In SLE, $CD95^+$ cells are greatly expanded in both memory subsets and the vast majority of $IgD^-CD27^-CD95^+$ cells are also $CD21^-$. In contrast, $CD95^+$ 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. The $CXCR3^+$ fractions of both IgD^-CD27^+ and IgD^-CD27^- memory subsets exhibit CD21/CD95 expression patterns that reflect the differences generally observed between healthy subjects and SLE patients. As illustrated in this example, the expression of CD95 in $IgD^-CD27^-CXCR3^+$ cells is increased in SLE. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 1. Summary table for OMIP-003

Purpose	Phenotyping memory B cells
Species	Human
Cell type	Fresh or cryopreserved PBMC, bone marrow, and tonsil mononuclear cells
Cross reference	None

classification schemes and provide precise identifications of the core human B cell subpopulations (2).

In addition to these anchor markers, each panel is extended with specific markers for further discrimination of memory, transitional/naïve B cells and plasma cell subsets, respectively. Thus, the incorporation of CD21, CD95, CD45/B220, and CXCR3 in the memory panel (Table 2), as described in this OMIP, provides information regarding the activation status and homing potential of both the $CD27^+$ switched memory and $CD27^-$ switched memory B cells (Figure 1). The addition of the rat anti-human Ig idiotype 9G4 antibody completes a 12-color human memory B cell panel, and provides a useful measure of autoreactivity through the identification of B cells expressing autoantibodies encoded by the VH4-34 variable region gene (3).

Similarity to Published OMIPs

None to date.

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Table 2. Reagents used for OMIP-003

SPECIFICITY	CLONE	FLUOROCHROME	PURPOSE
IgD	IA6-2	FITC	Differentiation
CXCR3	1C6/CXCR3	PE	Homing
CD24	SN3	PE-Alexa610	Differentiation, epithelial adhesion
CD21	B-ly4	PE-Cy5	Activation, BCR co-receptor for antigen-bound C3d
CD38	HIT2	PerCP-Cy5.5	Differentiation
CD45/B220	RA3/6B2	PE-Cy7	Differentiation
CD3	SP-34-2	Pacific Blue	Exclusion
Dead cells		Aqua (Pacific Orange)	Exclusion
CD27	CLB-27/1	Qdot605	Differentiation, receptor for CD70 on activated T cells
CD95	DX2	APC	Activation; pro-apoptotic in the presence of activated CD95L T cells
VH4-34-encoded idiotype	9G4	Biotin	Autoreactive B cells
Biotin	Streptavidin	Alexa680	
CD19	SJ25C1	APC-Cy7	Lineage

FITC, fluorescein isothiocyanate; PE, R-phycoerythrin; Cy, cyanine; PerCP, peridinin-chlorophyll protein; Aqua, LIVE/DEAD fixable aqua dead cell stain; Qdot, quantum dot; APC, allophycocyanin.