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Cytometry PART A
Journal of the International Society for Advancement of Cytometry

OMIP-043: Identification of Human Antibody Secreting Cell Subsets

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Received 21 September 2017; Revised 16 November 2017; Accepted 1 December 2017

Additional supporting information may be found in the online version of this article.

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Both authors are employees of MedImmune LLC, and shareholders of AstraZeneca which wholly owns MedImmune.

Published online 29 December 2017 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.23305

• **Key terms**
antibody secreting cells; rare cell analysis; plasma cells; immunoglobulins

Purpose and Appropriate Sample Types

This panel was optimized primarily to determine the frequency and immunophenotype of antibody secreting cells (ASC), historically called plasma B cells [PCs, reviewed in Refs. (1–3)]. The panel can also be used to determine the frequency and phenotype of other B cell subsets including memory B cells and naïve B cells, which occur in various anatomic niches, but particularly in the circulation (Table 1). The panel has been tested on various human tissues from healthy subjects, and has been shown to be applicable to fresh and cryopreserved peripheral blood, spleen, bone marrow, and tonsil cells; we have observed active antibody secretion from thawed cells. We continue to apply the panel to a variety of tissues and donors to build on our understanding of humoral immunity and the cells that contribute to long-lasting vaccine responses. Analysis of rare cells can be difficult, and our aim is to encourage better data in the analysis of ASC.

Background

ASC are differentiated B cells that are the primary contributor to humoral immunity, as they synthesize and secrete highly specific antibodies that bind and neutralize foreign antigens (4). These cells are present in primary and secondary lymphoid tissue as well as the peripheral circulation, and their phenotype is variable depending on their tissue niche (5). ASCs occur at very low frequencies in peripheral blood, and while they are somewhat more abundant in tonsil, spleen, and bone marrow, in all cases they are considered rare cells, making their study a challenge (6). When characterizing these cells using flow cytometry, care must be given to collecting sufficient events that satisfy a desired level of confidence (7). Few published protocols for analysis of ASC have emphasized Poisson statistics for adequate event collection for low percent coefficient of variation (%CV) measurements (8). We targeted a 5% coefficient of variation (CV), which resulted in regularly collecting 400–2,000 events in the ASC gate.

ASC have been identified and described using numerous flow cytometry methods, however, the most important defining factor for positive identification of ASC is the synthesis and secretion of immunoglobulin. ASC also express very high levels of the ectoenzyme CD38 and are positive for the TNF-family receptor CD27 (9). Very high CD38 expression is in fact considered adequate for basic identification of ASC. On-scale display of $CD38^{high}$ events is critical for ASC identification, and can be difficult for the untrained eye; once cytometer settings are established for the CD38 detector, it is useful to increase the number of displayed events to 100,000 during

Table 1. Summary for OMIP-043

PURPOSE	PHENOTYPE OF B CELLS INCLUDING ANTIBODY SECRETING CELLS
Species	Human
Cell type	Fresh or cryopreserved PBMC, bone marrow, spleen, or tonsil cells
Cross reference	None

data collection to ensure all of the brightest CD38^{high} events are fully on-scale. Mature B cells express the phosphoglycoproteins CD19 and CD20, and on differentiation to ASCs, CD20 is downregulated, and while CD19 positive and negative ASC are observed in bone marrow and spleen, the precise ontogeny and functional differences between the two phenotypes are still not fully known. The ratio of CD19+ to CD19- on ASC varies by tissue niche, and was recently described in

Table 2. Reagents used for OMIP-043

SPECIFICITY	CLONE	FLUOROPHORE	PURPOSE
CD20	2H7	BV421	B cell differentiation
CD27	O323	BV786	B cell differentiation
CD19	SJ25C1	APC	B cell differentiation
CD38	HB7	BUV395	Primary identification of ASCs
IgD	IA6-2	PerCP-Cy5.5	B cell maturation
CD3, CD14, CD15, CD193	UCHT1, MΦP9, W6D3, 5E8	BV510	Dump/exclusion
Dead cells	NA	Fixable Blue	Dump/exclusion
IgM	G20–127	FITC	Cytoplasmic Ig
IgG	G18–145	FITC	Cytoplasmic Ig
IgA	pAb	FITC	Cytoplasmic Ig
Ki67	Ki-67	PE	Proliferation status
CD138	B-A38	PE	Alternate identification of ASC
HLA-DR	G46-6	PE	Maturation related

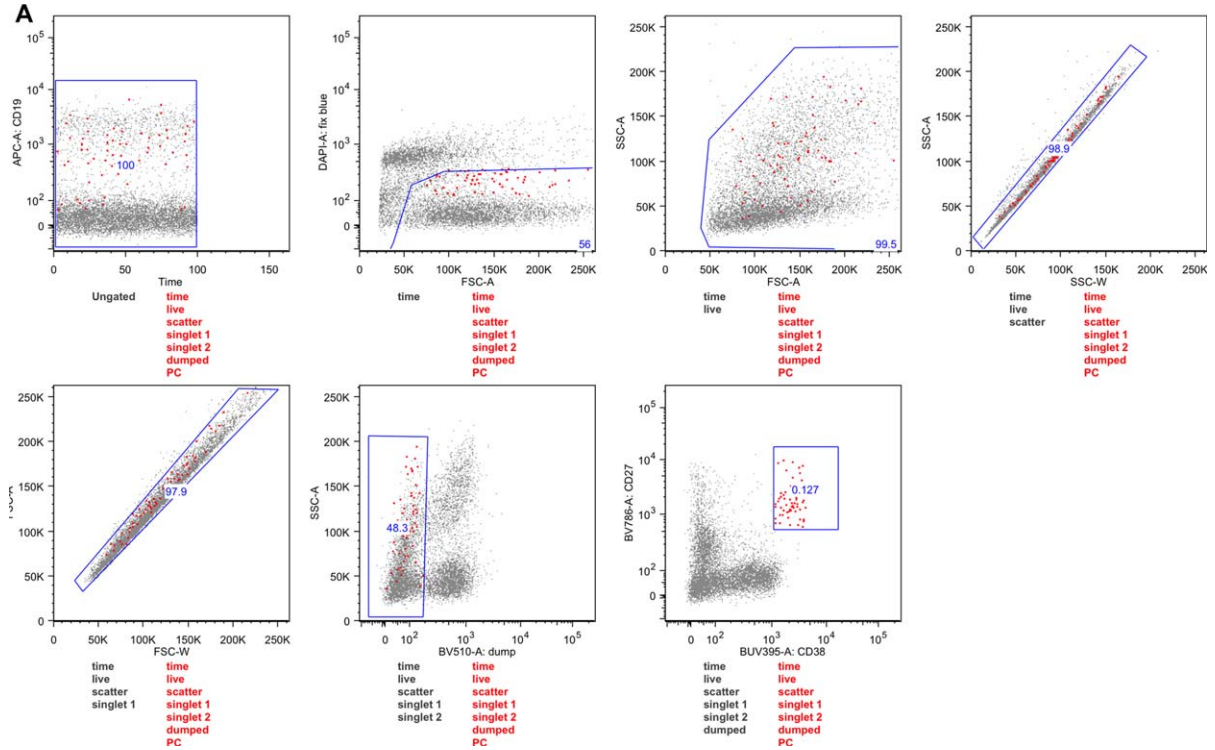

Figure 1. **A**. Gating scheme; example shown is from bone marrow. A key methodology we employ to ensure complete visualization of ASC is to start with the final cells of interest (CD38high/CD27+) and to then work backward through the gate hierarchy to ensure that these cells are within the gates, including light scatter, viability, and singlet regions. Importantly, ASC contain more cytoplasm due to their active production of antibody, and thus do not cluster with a typical low-side scatter "lymphocyte" gate. **B**. Immunophenotype and Ig detection among ASC in human PBMC, bone marrow, spleen, and tonsil. Note particularly the tissue-specific variation of CD19, Ki67, and HLA-DR among CD38high ASC. ASC are colored red, and non-ASC are blue. **C**. Representative plots of other B cell subsets may be identified; examples shown are from peripheral blood.

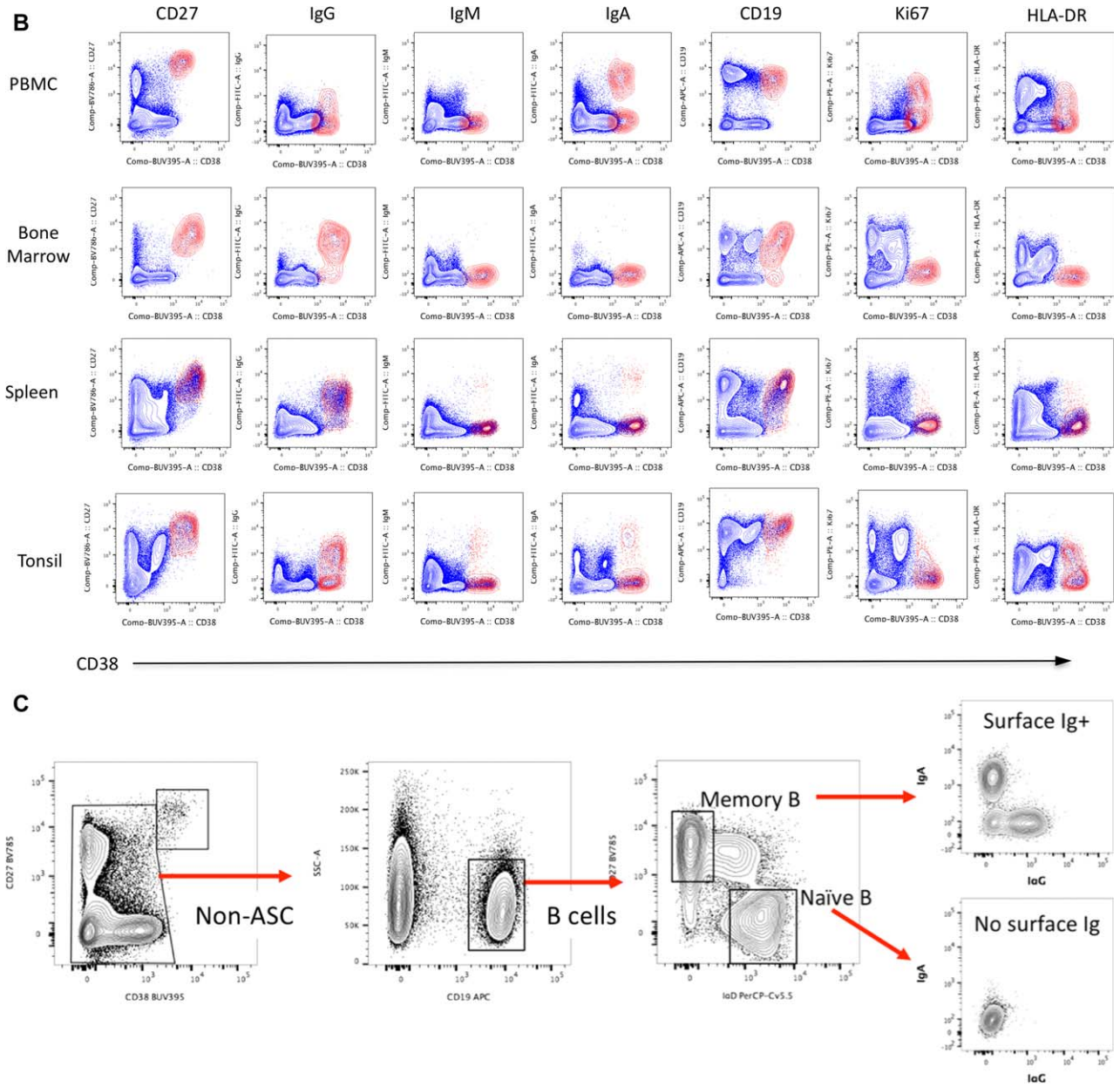

Figure 1. (Continued)

detail elsewhere by us and by others (10,11, our data *in press*). CD19 is of particular interest, as historically the most long-lived ASC residing in bone marrow were thought to be CD19 negative, but recent work has indeed demonstrated that long-term vaccine responses are also maintained among a possibly circulating CD19 positive pool. In developing the panel, care was given to devise a scheme that also identifies non-ASC B cells, and surface IgD is a useful marker for identification of naïve, preclass switched B cells, and CD27 is useful for identification of memory B cells. Thus, the core of the immunophenotyping panel is CD19, CD20, CD27, CD38, and IgD, with CD3, CD14, CD15, and CD193 (CCR3) as "dump" reagents to gate out non-B cells (Table 2). Employing a dump gate simplifies analysis, aids in resolution of rare ASC, and effectively reduces the denominator when calculating the proportion of ASC, thus, reducing the size of the haystack in which the needles hide. The panel was designed with rigorous attention to clone-fluor combinations, particularly for the primary ASC-identifying antibodies, such that each reagent was titrated to yield optimal stain index, while minimizing spread of background of unstained events (12,13). Importantly, cytoplasmic IgG, IgM, and IgA are included. The panel has been validated by using nonfixed, nonpermeablized cells in cell-sorting experiments that demonstrate that secreted IgG, IgM,

and IgA are detectable by ELISpot in proportions similar to those determined with cytoplasmic Ig detection (our data, *in press*). Among naïve B cells, no surface immunoglobulin is detectable, while among memory B cells, all three isotypes are detectable, with only a single isotype expressed by each individual cell (Fig. 1C).

We chose to use the fluorophore phycoerythrin (PE) as an "open" fluor space for exploratory antigens that may be added to the panel, and we also routinely include the proliferation-related marker Ki67 conjugated to PE. We show the quiescence of ASC cells, except in peripheral blood and a small proportion in the tonsil. CD138, a historically traditional marker for ASC may also be added in the PE channel, however, due to the variability in CD138 expression between tissues, non-uniform expression on ASC, and the high selectivity of CD38, CD138 is no longer considered essential for ASC. HLA-DR, which may be present on plasmablasts (14), may also be substituted in the PE channel. The panel allows for deep subsetting of ASC by CD19± then by HLA-DR± using the $CD38^{high}$/CD27+ scheme.

Similarity to Published OMIPs

The present OMIP shares some antigens with OMIP-003, Phenotypic Analysis of Human memory B Cells (15). However, the present OMIP utilizes newly-available fluors rather than QDOTs, and more specifically is intended for specific identification of newly described antibody secreting B cell subsets. Please refer to Supporting Information where we present evaluation of alternate clone-fluor combinations tested in development. In our approach, we do not pregate on CD19+ cells, therefore, we can evaluate ASC regardless of their CD19 expression, and we add immunoglobulin detection.

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