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OMIP-047: High-Dimensional Phenotypic Characterization of B Cells

Thomas Liechti,[1*] Huldrych F. Günthard,[1,2] Alexandra Trkola[1*]

[1]Institute of Medical Virology, University of Zurich, Zurich, Switzerland

[2]Division of Infectious Diseases and Hospital Epidemiology, University Hospital Zurich, Zurich, Switzerland

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*Correspondence to: Thomas Liechti; ImmunoTechnology Section, Vaccine Research Center, NIAID, NIH, 40 Convent Drive, Bethesda, MD 20892. E-mail: thomas.liechti@nih.gov and Alexandra Trkola; Institute of Medical Virology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland. E-mail: trkola.alexandra@virology.uzh.ch

Present address of Thomas Liechti: ImmunoTechnology Section, Vaccine Research Center, *NIAID*, National Institutes of Health, Bethesda, Maryland

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Purpose and Appropriate Sample Type

This 16-color, 18-parameter panel was designed to allow a detailed dissection of human B cell subsets and their phenotype in peripheral blood mononuclear cells (PBMC) in healthy donors and in the context of chronic viral diseases such as Human Immunodeficiency Virus 1 (HIV-1) infection. The panel encompasses a range of backbone markers for the accurate definition of common B cell subsets with a focus on memory B cells and a unique collection of phenotypic markers (chemokine receptors, cytokine receptor, B cell receptor isotypes, and proliferation marker) not combined in multicolor flow cytometry B cell phenotyping thus far. This new panel allows highly detailed phenotypic and functional investigations of B cell subsets. The panel was validated using cryopreserved PBMC from healthy and HIV-1 infected donors allowing the retrospective analysis of clinical samples (Table 1).

Background

Chronic viral diseases such as HIV-1 and Hepatitis C virus result in dramatic perturbations of the B cell compartment, most strikingly a shift toward mature and exhausted phenotypes and increased frequencies of immature CD10^{+} transitional B cells (1–3). As a consequence, B cell responses in HIV-1 infection are frequently impaired, resulting in delayed and, in part, insufficient humoral responses to diverse infectious agents and vaccines (4–10). The comprehensive 18-parameter panel described herein will allow in-depth analysis of B cell dynamics with a focus on memory B cells in healthy individuals and during disease in frozen, banked PBMC samples.

B cells can be defined by expression of the canonical marker CD19. We used a dump channel including CD3, CD14, CD16, and a Live/Dead dye to achieve optimal resolution between CD19^{+} and CD19^{-} cell types to allow definition and inclusion of CD19^{dim} plasmablasts into the analysis (Fig. 1A).

CD10 was included in the panel for detection of CD10^{+} transitional B cells (Figs. 1A and B and Supporting Information Fig. S10). While transitional B cells can also be defined by the classical Bm1-Bm5 classification based on CD38 and IgD expression (11), we found that this was less reliable compared to using CD10 expression (11,12).

CD38 was included to define plasmablasts. Using a CD38 specific reagent labeled with the dim fluorochrome Alexa Fluor 700 (AF700) allowed to define plasmablasts readily as these cells express high levels of CD38 (Fig. 1C and Supporting Information Fig. S11) (11). Plasmablasts are defined as CD38^{high}. According to their lower CD38 levels CD10^{+} transitional B cells are referred to as CD38^{+} (Fig. 1B and Supporting Information Fig. S10). As the AF700 conjugated CD38 antibody cannot distinguish between CD38^{-} and CD38^{+} naïve or between CD38^{-} and CD38^{+} switched memory B cells, we refer to these cells as CD38^{-} (11). Integrating CD38 and IgD in the panel allowed a dissection of CD10^{-} mature B cells in IgD^{+} unswitched B cells (IgD^{+}CD38^{-}), memory B cells (IgD^{-}CD38^{-}) and plasmablasts (PB; IgD^{-}CD38^{high}) (Fig. 1C) (11).

Table 1. Summary table for application of OMIP-047.

PURPOSE	B CELLS
Species	Human
Cell types	PBMC
Cross-reference	n.a.

IgD^{+} unswitched B cells encompass antigen-inexperienced naïve B cells and marginal zone (MZ) B cells (13,14), which can be distinguished by the differential expression of the memory B cell marker CD27 (Fig. 1C). Naïve B cells do not express CD27 but express high levels of IgD, whereas IgD on MZ B cells is lower and paired with

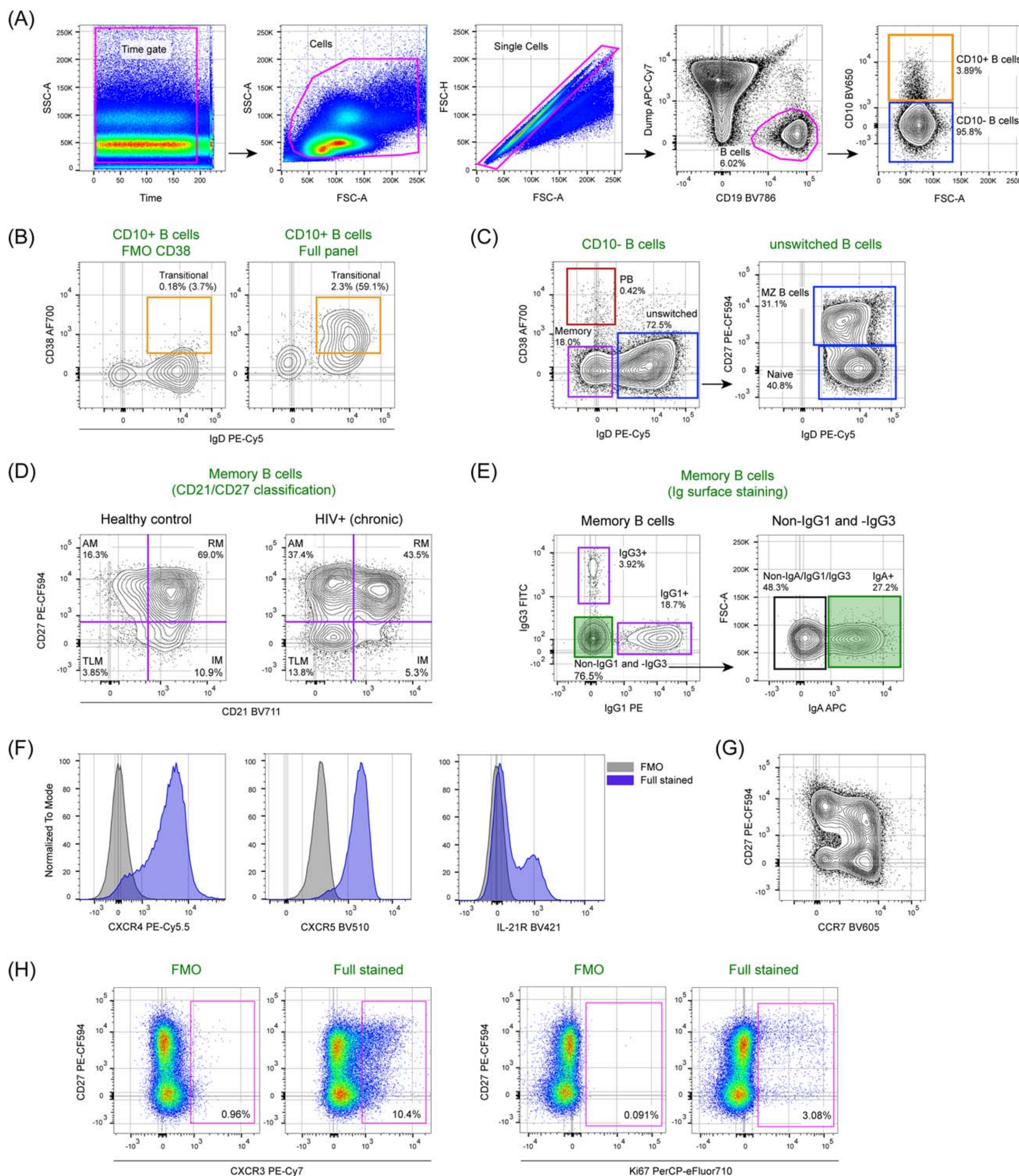

Figure 1.

Figure 1. Characterization of B cell subsets by flow cytometry. **(A)** A Time versus SSC-A gate was set to exclude fluorescence intensity fluctuations due to eventual irregular acquisition of the flow cytometer. Single cells were further defined based on SSC-A and FSC-A and a gate for doublet discrimination based on FSC-H/FSC-A. B cells were defined as Dump-negative (Dump set as CD3, CD14, CD16 positive and dead cells) and CD19-positive cells. A major B cell subset discrimination is based on CD10 and, therefore, B cells were divided into CD10$^-$ and CD10$^+$ subpopulations. **(B)** CD10$^+$ B cells can be further refined into transitional B cells (CD38$^+$IgD$^+$). The gating of CD38$^+$ cells was done based on a FMO control for CD38 AF700 shown in the left panel. **(C)** CD10$^-$ B cells comprise IgD$^+$ unswitched B cells (CD38$^-$IgD$^+$), memory B cells (CD38$^-$IgD$^-$) and plasmablasts (PB; CD38^{high}IgD$^-$), which can be defined based on the surface expression pattern of CD38 and IgD. The unswitched IgD$^+$ fraction can be further divided in naïve (CD27$^-$IgD^{high}) and marginal zone (MZ) B cells (CD27$^+$IgD$^+$) as shown in the right panel. **(D)** Memory B cells derived from the memory B cell gate (CD38$^-$IgD$^-$) in Figure 1C can be further divided in subsets based on their maturation state by expression patterns of CD21 and CD27 as described (3,18,29). In HIV-1 infected patients the distribution is known to be skewed toward activated (AM) and tissue-like (TLM) memory B cells (3,18,29). The analysis of cell samples from HIV-1 infection (Figure 1D, right panel) was thus useful to verify sufficient resolution of CD21 staining. **(E)** Alternatively, memory B cells can be divided in subsets based on their expressed B cell receptor isotype. The panel includes antibodies specific for IgG1, IgG3, and IgA. IgA-expressing switched memory B cells (right panel) are defined from cells within the "Non-IgG1 and -IgG3" gate (left panel, transparent green gate). **(F)** To characterize the potential migratory activity of B cell subsets, we included antibodies for the chemokine receptors CCR7, CXCR3, CXCR4 and CXCR5 in our panel. To measure responsiveness to IL-21, an important cytokine for development of high-affinity antibody responses, IL-21R levels were measured. The resolution of anti-CXCR4, -CXCR5, and –IL-21R antibodies was sufficient as judged by staining differences between the full stained sample (blue) and the corresponding FMO control (grey). **(G)** Resolution of CCR7 proved high so that FMO was not necessary to define positive populations. **(H)** In healthy donors the frequency of CXCR3- and Ki67-expressing B cells is genuinely low necessitating FMO controls. FMO control and fully stained sample are shown as a dot plot in combination with CD27. Both markers show sufficient separation in combination with full staining panel. Data in panels 1A, B, C, and E are from measurements of the same sampling time point of one healthy donor. Data shown in right panel of Figure 1D is from measurements of PBMC from a chronically HIV-1 infected individual. All percentages are related to total Dump$^-$CD19$^+$ B cells except in Figures 1D and E where percentages are related to memory B cells as gated in Figure 1C. Percentages stated in brackets in Figure 1B are related to total CD10$^+$ B cells. [Color figure can be viewed at wileyonlinelibrary.com]

expression of CD27 (13). IgD is a well-established marker to define memory B cells. The majority of memory B cells lose IgD expression and only low frequencies of IgD$^+$ memory B cells in the periphery have been described (15–17).

Analysis of B cell subsets based on CD21 and CD27 expression has been frequently used in the literature, but bears a limitation. Naive B cells with a phenotype of CD21$^+$CD27$^-$ are commonly not distinguished from memory B cells with the same expression profile (5,8,9,18). This OMIP panel defines the naïve B cell population separately based on IgD and CD27 expression. This allows for the exclusion of this subset from the analysis of memory B cells and the definition of CD21$^+$CD27$^-$ intermediate memory (IM) B cells (18). The differential expression of CD21 and CD27 further facilitates the definition of the maturation state of memory B cells: quiescent resting memory (RM, CD21$^+$CD27$^+$), activated

Table 2. Reagents used for OMIP-047.

SPECIFICITY	CLONE	FLUOROCHROME	PURPOSE
CD3	SK7	APC-Cy7	Dump
CD14	HCD14	APC-Cy7	Dump
CD16	3G8	APC-Cy7	Dump
Dead cells	–	Near-infrared	Dump
CD19	SJ25C1	Brilliant violet 786	Lineage
CD10	HI10a	BV650	B cell subsets
IgD	IA6-2	PE-Cy5 *(in-house conjugation)*	B cell subsets
CD38	HIT2	AF700	B cell subsets
CD21	B-ly4	BV711	B cell subsets/Exhaustion
CD27	M-T271	PE-CF594	Differentiation, Memory
IgG1	HP6001	PE	IgG1 class-switched B cells
IgG3	Polyclonal Sheep IgG	FITC	IgG3 class-switched B cells
IgA	Polyclonal goat IgG	APC	IgA class-switched B cells
CCR7	G043H7	BV605	Migration pattern
CXCR3	G025H7	PE-Cy7	Migration pattern
CXCR4	12G5	PE-Cy5.5 *(in-house conjugation)*	Migration pattern
CXCR5	RF8B2	BV510	Migration pattern
IL-21R	2G1-K12	BV421	Cytokine receptor
Ki67	20Raj1	PerCP-eFluor710	Proliferation marker

memory (AM, CD21$^-$CD27$^+$) and exhausted tissue-like memory (TLM, CD21$^-$CD27$^-$) B cells (Fig. 1D) (3,18). To obtain a refined dissection of phenotypic differences amongst the memory B cell subsets our staining panel monitors IgA, IgG1, and IgG3 B cell receptor isotypes (Fig. 1E). We included in our panel IgG1 and IgG3 as these specificities were of interest in the context of the research question we addressed when developing the panel, where we were aimed to monitor HIV-1 Env IgG1 and IgG3 responses linked with the development of neutralization breadth. Depending on the research topic addressed the addition of IgM to also define IgM-only memory B cells may be of interest. For an easy adaptation of the panel to incorporate IgM or other markers we recommend markers in the FITC and PE channel, which in the present setting of our panel are occupied by IgG1 and IgG3. These markers can, however, be readily swopped to other markers (e.g., pan IgG or IgM) as needed since PE and FITC labeled reagents are among the most widely available reagents.

Chemokine receptors CCR7, CXCR3, CXCR4, and CXCR5 are measured herein to provide insight into the potential tissue destination and migratory profiles of the diverse B cell subsets, (Figs. 1F–H). CXCR4, the chemokine receptor for CXCL12, is important for homing of B cells to bone marrow and lymphoid organs, including the spleen (19,20). In B cell follicles, follicular dendritic cells secrete high levels of CXCL13, which attract CXCR5-expressing B cells. CCR7 plays an important role guiding cells to the T cell areas in secondary lymph organs. CXCR3-expressing B cells are attracted to inflamed tissues by CXCL9 and CXCL10 produced at sites with ongoing immune activation.

An additional marker included in our panel is IL-21R (Fig. 1F). IL-21, mainly secreted by follicular helper T cells in the GC, supports somatic hypermutation (SHM) and isotype switching through signaling of the IL-21R and is crucial for the development of high-affinity antibody responses (21–25). Including IL-21R in the B cell panel enables analysis of the dynamics of IL-21R expression. This allows one to determine whether chronic viral infections manipulate the responsiveness of B cells to SHM and isotype class switching through regulation of IL-21R (22,23,26).

Examination of the proliferation marker Ki67 identifies recently activated B cells and makes it possible to define their phenotype and dynamics during ongoing immune responses (Fig. 1H) (27).

Combining classical lineage markers with a range of phenotypic parameters (Table 2) our comprehensive 18-parameter B cell panel provides the possibility for a highly detailed characterization of the B cell compartment in healthy individuals, as well as in response to infections or in autoimmune diseases.

Similarity to Published OMIPs

This panel can be used for in-depth phenotyping of B cells in peripheral blood samples. The panel differs from the panel described by Wei et al. [OMIP-003 (28)] which focused on memory B cells. In contrast, the panel described here allows the analysis of most known B cell subsets and in addition includes phenotypic assessment of chemokine receptors, IL-21R, B cell receptor isotypes and the proliferation marker Ki-67.

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