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OMIP-0XX: High-Dimensional Characterization of Global and Antigen-Specific B cells in Chronic Infection

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Purpose and Appropriate Sample Types

This 24-color flow cytometry panel focuses on characterizing antigen-specific B cells and precise delineation of B cell subsets in chronic infections and is applicable to other chronic diseases such as autoimmunity. The panel was optimized for human cryopreserved peripheral blood mononuclear cells (PBMCs). Markers were chosen to extensively distinguish B cell lineages (CD19, CD20, CD10, CD38, CD24, IgM, IgD, CD27, CD21, CD43, CD5). Inclusion of antigen-specific probes was of high priority in order to assess hepatitis B virus (HBV) antigen-specific B cells for our purposes. These probes can be readily exchanged for other pathogen-specific probes or additional markers for the panel to be tailored to desired research questions beyond HBV. In addition, we included a comprehensive and unique set of functional markers such as chemokine receptors (CXCR3, CXCR5), co-stimulatory molecule (CD86), Fc receptor (CD32), regulatory molecules (BTLA, CD39), and inhibitory markers associated with chronic infections (PD-1, FcRL5, CD11c, CD22) to enable in-depth analysis of global and antigen-specific B cells during chronic infection.

Background

B cells are an important cell type in the prevention and/or control of infections through production of antibodies, professional antigen presentation, and secretion of cytokines¹. During acute HBV infection, B cells are responsible for the production of antibodies against HBV envelope protein, called hepatitis B surface antigen (HBsAg). Induction of anti-HBsAg (anti-HBs) is considered the clinical indication of recovery from an acute HBV infection, and prophylactic induction of anti-HBs is the protective mechanism of the HBV vaccine^{2,3}. B cells also help to maintain control of HBV infection after resolution as shown by HBV reactivation in studies using CD20⁺ depleting rituximab for treatment of hematologic malignancies⁴⁻⁶. Although B cells are known to be an important cell type during HBV infection, it still remains unclear how these functions fail in an acute infection and lead to 5-10% of adults and 90-95% of children developing a chronic infection⁷. For this reason, we

developed the panel described here to provide a comprehensive tool to study global and antigen-specific B cells during HBV infection.

B cells can be defined by their expression of CD19 and CD20 (Figure 1A). We included a dump channel which enables the exclusion of CD3⁺ T cells, CD14⁺ Monocytes and dead cells in order to increase our resolution between CD19⁺CD20^{+/-} B cells and CD19⁻CD20⁻ cell types (Figure 1A). In our panel we observed a population of dye aggregates in some phenotypic markers (CD39 and CD43), and thus excluded these aggregates before further analysis of B cells^{8,9} (Figure 1A).

B cells originate in the bone marrow where they generate a functional B cell receptor (BCR)¹⁰. After leaving the bone marrow, immature transitional B cells further mature into antigen-inexperienced naïve B cells in the periphery¹¹. Upon antigen exposure, naïve B cells are activated and further differentiate either through germinal center (GC) dependent or independent pathways^{12,13}. The fate of a naïve B cell to enter a GC is dependent on its primary BCR affinity for antigen, with the highest affinity cells immediately differentiating into antibody-secreting plasmablasts in a GC independent manner, while lower affinity cells enter the GC and undergo affinity maturation¹⁴. The GC independent process results in the differentiation of short-lived plasmablasts or memory B cells that produce a rapid response with low or medium affinity antibodies against the respective pathogen¹⁵. Cells that enter the GC undergo class-switching and multiple rounds of somatic hypermutation (SHM) that leads to the selection of high-affinity B cell clones. High-affinity GC B cells egress from the GC as either short-lived plasmablasts, long-lived plasma cells or long-lived memory B cells¹⁶. Although the mechanisms underlying post-GC B cell fate decisions need further investigation, it is thought that both temporal factors and BCR affinity may play a role¹².

The panel described here includes markers to distinguish many of the B cell developmental stages, with a focus on identifying antigen-specific memory B cells. Transitional B cells can be reliably determined as CD10⁺ and are gated out to focus analysis on mature B cells¹⁷ (Figure 1A). CD10⁻ mature B cells can be further divided into plasmablasts (CD24⁻CD38^{hi}) and non-plasmablasts based on the expression pattern of CD24 and CD38 (Figure 1B). Plasmablast populations are transient in the periphery and are often upregulated as a result of infection or vaccination¹⁸. Non-plasmablasts comprise unswitched and class-switched B cells based on the expression or lack of IgD and IgM. Class-switched cells that are IgM⁻IgD⁻ are indicative of memory B cells derived from a GC, likely including multiple rounds of somatic hypermutation (SHM), and therefore have increased potential to induce productive antibody-mediated effector functions¹⁶. In contrast, unswitched B cells contain several subsets which can be differentiated by the expression of IgD, IgM and CD27¹⁹. Naïve B cells express both IgM and IgD but lack CD27 (Figure 1B), which is often used as a memory B cell marker. However, in certain circumstances CD27 alone is not a singularly reliable marker for memory B cells²⁰. Furthermore, the IgD⁺CD27⁺ population contains either marginal zone (MZ) B cells (IgM⁺) or IgM⁻IgD⁺ memory B cells (Figure 1B). The development and function of IgD-only memory B cells remains elusive and awaits further clarification^{21,22}. Peripheral MZ B cells are a unique population resembling splenic MZ B cells although their origin has not been definitively clarified²³. MZ B cells are important for glycan-specific antibody responses and patients undergoing splenectomy exhibit reduced

levels of peripheral MZ B cells and show increased susceptibility to bacterial infections²⁴. Finally, unswitched B cells contain a subset of IgD⁻ cells which show high expression of IgM and are defined as IgM-only memory B cells^{19,23} (Figure 1B).

An important goal for this OMIP was to incorporate antigen-specificity into our analysis of memory B cells in HBV infection. To identify HBV-specific B cells, a dual labeling strategy that has been previously published was employed^{25,26}. Briefly, HBsAg conjugated to one of two Dylight fluorophores served as probes to detect B cells expressing BCRs specific to HBsAg. Added at equal concentrations, only dual labeled cells are considered HBV-specific, which increases the overall specificity of the detection method (Figure 1C). Detailed optimization of this staining protocol is described in the online materials and online figure 2. Dual positive staining can be detected in both chronic HBV subjects as well as vaccinated individuals but frequencies of circulating HBsAg-specific B cells are low, as previously described (Figure 1C)^{25,26}. Interestingly, a significant fraction of HBV-specific B cells from chronic subjects were unswitched, and further identified as naïve B cells. Those that were IgD⁺CD27⁺ were mainly marginal zone B cells (Figure 1D). A detailed assessment of HBV-specific B cell phenotypes can be found in Online Figure 6. It is important to note from this analysis that our panel enables rigorous gating strategies in order to ensure precise examination of populations of interest, which in our case are memory B cells. The panel described here can be optimized in the future to incorporate dual-labeling antigen-specific probes for any chronic infection of interest.

Although most markers in this panel are aimed at distinguishing conventional B cell subsets (B2), we also included markers associated with non-classical B1 B cells (CD43 and CD5) (Figure 1E)²⁷⁻³⁰. This B cell subset is considered “innate-like” and produces T cell-independent, polyreactive, low-affinity IgM antibodies²⁷. It has also been shown that human B1 cells play an unexpected role in immunosuppression as a source of anti-inflammatory cytokine IL-10²⁷. Although the exact definition of B1 B cells in humans remains elusive, multiple studies have aimed to address the best gating scheme to define this population, and our panel will contribute to resolve these issues²⁸⁻³⁰. B1 B cells are gated here as CD27⁺CD43⁺ on non-plasmablasts due to the overlapping expression of CD27 and CD43 on plasmablasts, which has been shown to contaminate the putative B1 B cell subset (Figure 1E)^{29,30}. This population is further distinguished by expression of IgM and IgD, with a subset of cells IgM⁺ and/or IgD⁺. B1 B cells can also produce low-affinity IgA, and IgM⁻IgD⁻ cells are likely IgA⁺²⁷. CD5 was originally used as the primary marker to classify human B1 cells similar to mice, but it has become increasingly clear that in humans, CD5 is also expressed on a number of additional B cell subsets including pre-naïve, transitional, and even activated conventional B cells³¹. Therefore, identification of B1 cells here is not based on CD5 expression. However, inclusion of this marker allows for the capability to monitor CD5 expression on B1 cells, which has been suggested to contribute to downregulation in BCR signaling in this population, and appears higher on B1 B cells compared to other B cell and non-B cell subsets in chronic HBV subjects (Figure 1E)³². Examining the dynamics of these cells during chronic infection will provide unprecedented insight into their possible roles in the development of chronic disease.

Functional subsets of conventional class-switched and unswitched memory B cells can be further refined using CD21 and CD27. In the field of chronic infections, CD21 and CD27 alone are often used to distinguish naïve and memory B cell subsets. However, this analysis leads to complications with the CD21⁺CD27⁻ population, which is typically described as naïve B cells and excluded from memory analysis, but also contains a subpopulation of memory B cells known as intermediated memory (IM)³³. In addition, naïve B cells have been shown to downregulate CD21 in autoimmune diseases and chronic infections, which may lead to contamination of the atypical memory phenotype (CD21⁻CD27⁻)³⁴⁻³⁶. Here, we further characterize the maturation state of switched memory B cells to examine intermediate memory (IM, CD21⁺CD27⁻), resting memory (RM, CD21⁺CD27⁺), activated memory (AM, CD21⁻CD27⁺), and atypical memory (AtM, CD21⁻CD27⁻) subsets (Figure 1F). This classification of memory B cell subsets is commonly used to characterize memory B cells in chronic infections including malaria, TB, HIV, HCV and HBV^{25,26,37,38}. Chronic subjects have been shown to exhibit elevated levels of global as well as antigen-specific AtM B cells compared to healthy controls, and these AtM B cells appear to be less functional than other memory subsets³⁷. Figure 1F shows our observation of a similar trend with the increased frequency of AtM B cells identified from the class-switched IgM⁻IgD⁻ population in chronic HBV subjects compared to a healthy control.

A leading hypothesis as to the cause of chronic infections is that prolonged exposure to antigen leads to functional impairment of adaptive immune cells and ultimate persistence of infection³⁹. T cell exhaustion has been studied in depth in chronic infections, and only recently has the idea of B cell exhaustion also been suggested. To address this possibility, our panel includes multiple markers to assess the functional capacity of global and HBV-specific B cells during HBV infection.

Chemokine receptors are important surface proteins that guide trafficking of immune cells in response to chemokine expression gradients⁴⁰. In order to assess the capability of B cells to traffic to different sites during infection, we included analysis of CXCR5 and CXCR3 in this panel (Figure 1G). CXCR5⁺ cells traffic to the lymph node in response to expression of CXCL13 produced by follicular dendritic cells in the GC, and once there, encounter cognate antigen and mount a productive immune response against a pathogen. CXCR3⁺ cells, in contrast, traffic to sites of inflammation in response to CXCL9-11 secreted by monocytes and fibroblasts at inflamed tissue sites^{41,42}. It is unclear whether trafficking of HBV-specific B cells to sites of inflammation such as the liver is necessary for a productive response against HBV, but upregulation in CXCL9-11 has been shown in the liver and is associated with CXCR3⁺ hepatic infiltrates in HCV infection^{43,44}.

The costimulatory activation marker CD86 was included in the panel in order to determine the activation status of B cells during infection (Figure 1G). CD86 is constitutively expressed on B cells but is upregulated upon B cell activation and will help determine the functional status of global as well as HBV-specific B cells within this panel⁴⁵.

Regulatory molecules BTLA and CD39 were included in the panel to examine the inhibitory status of HBV-specific and global B cell subsets during HBV infection (Figure 1G). BTLA is expressed on B cells and upon binding its ligand recruits negative regulator SHP-1 that

decreases downstream BCR signaling⁴⁶. CD39 is an ectoenzyme that works in concert with CD73 to produce inhibitory ADO and IL-10, and CD39 expression on B cells is associated with a B regulatory (Breg) phenotype⁴⁷.

As mentioned above, an increase in frequency of circulating B cells deemed atypical memory B cells (AtM) has been shown in many chronic infections including HIV, malaria, TB, and viral hepatitis. AtM B cells are characterized by increased expression of a number of exhaustion markers, including, but not limited to, PD-1, FcRL5, CD11c, CD22 and CD32, all of which have been included in this panel³⁷ (Figure 1G-I). As shown in Figure 1I, these exhaustion markers are upregulated on AtM B cells compared with other memory subsets in chronic HBV subjects. Through incorporation of many exhaustion markers, this panel allows for comprehensive analysis of the dynamics of global and antigen-specific AtM B cell phenotypes throughout the course of chronic infections.

In summary, this OMIP describes a comprehensive 24-color flow cytometry panel to study global and antigen-specific B cells, their subsets, and phenotypes in humans with chronic infections. Our B cell panel enables B cell analysis at unprecedented depth and holds promise to uncover new patterns and dynamics of B cell subsets and phenotypes that are either beneficial or detrimental in the progression of chronic infections. The added ability to incorporate dual-labeling antigen-specific probes increases the power to make relevant pathogen-specific conclusions regarding B cell functions in chronic infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1 :

Gating strategy for the 24-color panel to assess B cells in HBV infection.

A) Gating of B cells from viable CD3⁺CD14⁻ cells in PBMC is depicted. Dye aggregates were removed from analysis. Mature B cells were defined as CD10⁺ B cells.

B) CD10⁺ mature B cells can be divided into CD24⁺CD38^{hi} plasmablasts and non-plasmablasts (upper left plot). Non-plasmablasts can be further characterized based on their surface expression of Ig classes IgM and IgD. IgM/IgD double negative cells are defined as class-switched memory B cells. Surface expression of IgM, IgD, or in combination characterizes unswitched B cells (upper right plot). Unswitched B cells can be further delineated into naïve B cells (IgD⁺CD27⁺), IgM-only memory B cells (IgD⁺CD27⁺) and IgD⁺CD27⁺ cells (lower left plot). The latter population contains IgM⁺IgD⁺CD27⁺ marginal zone B cells and IgM⁺IgD⁺CD27⁺ IgD-only memory B cells (lower right plot).

C) HBsAg double positive cells within unswitched (first column) and class-switched (second column) subsets are shown for a healthy HBV vaccinated subject (top row) and chronic HBV patients (bottom row). Double positive cells provide increased specificity to determine HBsAg-specific B cells.

D) HBsAg-specific B cells derived from unswitched B cells were overlaid on total unswitched B cells from the chronic HBV patients. A subset of HBV-specific cells were

naïve B cells which can be removed from further analysis. The majority of unswitched CD27⁺ HBsAg-specific B cells represent marginal zone B cells.

E) The definition of B1 cells in humans remains enigmatic^{27,28} and our panel will shed light on a refined definition of non-classical B1 cells. Gating of CD27⁺CD43⁺ non-classical B1 B cells within non-plasmablast cells is shown for chronic HBV patients (upper left plot). Since plasmablasts have overlapping expression of CD27 and CD43, B1 B cells are gated on non-plasmablasts to avoid inaccurate delineation of plasmablasts as B1 B cells (lower left plot). B1 cells can be divided into unswitched (IgM⁺ and/or IgD⁺) or IgM⁻IgD⁻ switched cells. The latter may represent IgA⁺ B1 B cells but has not been examined in depth in humans^{47,48} (upper right plot). CD5 expression appears higher on B1 B cells compared with non-B cells, plasmablasts, and total B cells (lower right plot).

F) Class-switched B cells can be further classified based on their expression of CD21 and CD27. Here, class-switched memory B cells are defined as follows: CD21⁺CD27⁻ intermediate memory (IM), CD21⁺CD27⁺ classic resting memory (RM), CD21⁻CD27⁺ activated memory (AM), and CD21⁻CD27⁻ atypical memory (AtM).

G) Expression pattern of phenotypic markers on viable CD3⁺CD14⁻ cells is shown. The marker CD19 is used on the y-axis to visualize unique expression patterns of phenotypic markers on B cells.

H) FMO control for CD22 shows sufficient separation of CD22⁺ B cells from non-B cells despite dim staining of CD22 BUV805. One chronic HBV subject was stained with the full panel as well as CD22 FMO.

I) Expression levels of phenotypic markers associated with chronic infections were examined on the four memory B cell subsets, IM, RM, AM and AtM to show increased expression of all markers on AtM B cells in chronic HBV infection.

PBMCs from 3 chronic HBV patients were concatenated for analysis shown in Figure 1A-1C (bottom row), 1D, 1E, 1G and 1I. In Figure 1C (top row) PBMCs from a healthy vaccinated subject AC51 (D189) were used (see online figure 2 for details on this subject). In Figure 1F, PBMC from 3 chronic HBV patients were concatenated and downsampled to compare equal cell numbers as healthy vaccinated subject AC51 (D189). In Figure 1H, one chronic HBV patient was used to test CD22 FMO.