OMIP 076: High-dimensional immunophenotyping of murine T-cell, B-cell, and antibody secreting cell subsets

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1 | PURPOSE AND APPROPRIATE SAMPLE TYPES

This 19-parameter, 18-color flow cytometry panel was designed and optimized to enable the comprehensive and simultaneous immunophenotyping of distinct T-cell, B-cell, and antibody secreting cell (ASC) subsets within murine tissues (Table 1). Cellular populations identified by using this OMIP include two major subsets of B-cells (memory and activated), two ASC subsets (plasma cells and plasmablasts), and seven major subsets of CD4\(^+\) T-cells (naïve, central memory, effector memory, helper, regulatory, follicular helper, and follicular regulatory). Staining was performed on freshly isolated splenocytes from 21-day-old BALB/c mice, however, due to the omission of mouse strain-specific markers, this OMIP can be implemented across a range of murine models where in-depth immunophenotyping of the diverse repertoire of T-cell, B-cell, and ASC populations is required.

2 | BACKGROUND

There is now considerable evidence demonstrating that both prenatal and postnatal exposure to particular classes of microbial stimuli can provide beneficial signals during early life immune development, resulting in the protection against future inflammatory disease [1-3]. The principal target of this beneficial immunostimulation appears to be the innate immune system [4, 5], and the mechanisms driving protection underlay the paradigm of innate immune training, whereby certain classes of microbial stimuli can alter the functional state of innate immune cells, leading to the optimization of immunocompetence [6]. Immune training focuses on the phenotypic and transcriptional profiles of several prototypical innate populations [6, 7], however, the characterization of downstream adaptive responses associated with protection via innate immune training are of critical importance for understanding disease pathogenesis, and the potential for therapeutic mitigation. Due to this gap in our current understanding, the broader protective mechanisms remain incompletely understood. To address this requirement, we have developed and optimized a novel 19-parameter flow cytometry panel to comprehensively and simultaneously characterize distinct T-cell, B-cell, and ASC subsets localized within tissues of BALB/c mice in response to immune training during early life.

The developmental phase of this flow cytometry panel involved the prioritization of T-cell, B-cell, and ASC subsets central to the maintenance of immunological homeostasis, as based on the current literature and forerunner studies. As such, a degree of emphasis was placed on effector, regulatory, and memory subsets within T-cell and B-cell populations. In regard to T-cells, the conversion of peripheral naïve CD4\(^+\) T-cells to effector T (Teff) cells is denoted by upregulation of the activation marker CD25, while concomitant upregulation of both CD25 and intracellular Foxp3 expression is essential for the peripheral induction of regulatory T-cells (Treg) [8], a process previously recognized in the protection against allergic airways inflammation following microbial-derived immunomodulation [9, 10]. Furthermore, the expression of CD44 on Treg has been implicated in promoting enhanced function [11, 12], while inducible costimulator (ICOS\(^+\)) Tregs are recognized to have superior suppressive capacity and interleukin (IL)-10 production compared to ICOS\(^-\) Tregs [13, 14]. Following activation and contraction, CD4\(^+\) T-cells transition toward a...
memory phenotype via the gradual upregulation of CD44 expression in parallel with transient expression of CD62L, driving the establishment of a dynamic repository of central memory (Tcm) and effector memory (Tem) T-cells [15-17]. In addition to establishing peripheral memory, activated CD4+ T-cells have the capacity to upregulate extracellular expression of CXCR5, ICOS, and programmed cell death protein 1 (PD-1) [18, 19], resulting in the generation of a highly specialized population of T follicular helper (Tfh) cells required for the formation of germinal centers within secondary lymphoid organs, while also providing crucial survival signals to support high-affinity B-cells during affinity maturation and proliferation [20, 21]. A separate subset of thymic-derived cells that share homology with the Tfh phenotype in addition to Foxp3 and bimodal CD25 expression, termed follicular regulatory T (TFR) cells, have also been identified, however, this subset has been attributed to the inhibition of Tfh activity and subsequent generation of humoral immunity [22, 23]. The immunophenotypic characterization of B-cell and ASC subsets for this OMIP was centered around the classic expression of CD19 and B220. To maximize the capacity of a 5-laser BD LSFRFortessa™, CD19 (B-cell and ASC subsets) and CD4 (T-cell) antibodies were conjugated to the same fluorochrome, since co-expression is essentially absent in single-cell analysis. Within secondary lymphoid tissues, the antigen-specific activation of B-cells involves the constitutive upregulation of major histocompatibility complex class-II (MHC class II; mouse I-A/I-E) and CD80 expression, in conjunction with the membrane-bound expression of both immunoglobulin (Ig) M and IgD [24-26]. Following antigen-specific activation, B-cells upregulate Synd-1 expression and differentiate into the two major classes of ASC; the rapidly produced and short-lived plasmablasts and the short-lived peripheral plasma cells, both of which have the capacity to secrete IgM [27-30]. A major difference between these two antibody-secreting subsets, however, is the absence of classic mature B-cell markers CD19, B220, and MHC-II on plasma cells [28, 31]. The eventual transition of B-cells toward a memory phenotype results in the loss of Synd-1 expression with parallel upregulation of programmed cell death protein 1 ligand 2 (PD-L2), generating a long-lived secondary lymphoid population expressing IgM +/− IgD that can rapidly differentiate into ASC upon re-stimulation [32-36].

Panel optimization was performed on a BD LSFRFortessa™, with all fluorochrome-conjugated antibodies (Table 2) titrated during the optimization phase (Figure S1). Prior to multicolor extracellular staining, splenocytes were incubated in Fc Block™ (Purified recombinant CD16/32) to inhibit non-antigen-specific binding of fluorochrome-conjugated antibodies to the nonpolymorphic epitope of FcγIII (CD16) and FcγII (CD32) receptors expressed on multiple myeloid populations and B-cells. A representative gating strategy to delineate the T-cell, B-cell, and ASC subsets described above is detailed in Figure 1. Briefly, splenocytes were first gated on side-scatter (SSC) and forward-scatter (FSC) parameters (Figure 1A) to

| Purpose | Comprehensive immunophenotyping of T-cell, B-cell, and ASC subsets |
|---------|---------------------------------------------------------------|
| Species | Mouse                                                        |
| Cell types | Murine tissues containing lymphocyte populations              |
| Cross-reference | OMIP-031, OMIP-032, OMIP-054, OMIP-061                        |

| Specificity | Fluorochrome | Clone | Purpose                      |
|------------|--------------|-------|------------------------------|
| PD-L2 (CD273) | BUV395       | TY25  | Memory B-cells               |
| IgD        | BUV496       | AMS 9.1 | Activated/memory B-cells     |
| CD44       | BUV737       | IM7   | T-cell subsets               |
| ICOS (CD278) | BV421       | 7E.17G9 | T Follicular helper/Treg    |
| PD-1 (CD279) | BV480       | J43   | T Follicular helper cells    |
| Live/Dead  | FVS575       | N/A   | Viable cells                 |
| CD80       | BV650       | 16-10A1 | Activated B-cells           |
| IgM        | BV711       | R6.60.2 | B-cell/ASC subsets           |
| CD4        | BV786       | RM4-5  | CD4+ T-cells                 |
| CD19       | BV786       | 1D3   | B-cell subsets               |
| Synd-1 (CD138) | BB515   | 2B1-2  | Plasmablasts/Plasma cells    |
| TCRβ       | BB700       | H57-597 | Pan T-cells                 |
| Foxp3      | PE          | FJK-16s | Regulatory T-cells          |
| B220 (CD45R) | PE-CF594   | RA3-6B2 | B-cell subsets             |
| CD25       | PE-Cy5      | PC61   | Activated T-cells           |
| CXCR5 (CD185) | PE-Cy7   | 2G8   | T Follicular helper cells    |
| MHC class II (I-A/I-E) | AF647 | M5/114.15.2 | B-cell subsets         |
| CD62L      | APC-R700    | MEL-14 | T-cell subsets               |
| CD45       | APC-Cy7     | 30-F11 | Pan leukocyte                |
FIGURE 1  Overview of 19-parameter gating strategy developed for the characterization of T-cell, B-cell, and ASC subsets within freshly isolated splenocytes from 21-day-old BALB/c mice. $1 \times 10^6$ splenocytes were incubated in Fc Block™, followed by fixable viability stain (FVS) and a 17-parameter extracellular antibody cocktail containing 10% brilliant stain buffer plus (BD biosciences). Intracellular staining was performed following fixation-permeabilization of extracellular stained splenocytes. Data were acquired on a BD LSRFortessa™ (BD Biosciences). (A–C) Removal of cellular debris, doublets, nonviable cells and stromal cells. (D) Primary delineation of TCRβ+/CD19− cells. (E–M) Characterization of (E) plasma cells, (F) plasmablasts, (G) activated B-cells, (H) memory B-cells, (I) naïve, effector memory and central memory T-cells, (J) effector and regulatory T-cells, (K) ICOS+/CD44+ Treg, (L) T follicular helper cells, and (M) follicular regulatory T-cells. All plots are representative of individual samples. Manual gating was determined using fluorescence minus one (FMO) controls where necessary (Figure S4) [Color figure can be viewed at wileyonlinelibrary.com]
remove sample debris, followed by single-cell gating (Figure 1B) to remove doublets. Gating was then performed on viable CD45+ cells (Figure 1C) to remove dead/dying cells and stromal cells from the analysis. The primary T-cell/B-cell/ASC separation involved delineation of TCRβ and CD4/CD19 expression (Figure 1D). Double positive cells were classified as CD4+ T-cells, as CD19+ B-cells and ASC subsets will be present within the TCRβ population (Figure 1D) due to the absence of TCRβ/CD19 co-expression (Figure S2A). An additional TCRβ CD4/CD19+ gate was included to enable the characterization of B220 ‘Synd-1’ MHC class II+ IgM+ plasma cells (PC; Figure 1E). CD19+ B-cells and ASC subsets were then defined as B220+‘Synd-1’ MHC class II+ IgM+ plasmablasts (PB; Figure 1F), B220+Synd-1’ CD80+PD-L2+ MHC class II+ IgM+ IgD+ activated B-cells (Figure 1G) and B220+ Synd-1’ CD80+PD-L2+ ‘Synd-1 MHC class II+IgM+IgD+ memory B-cells (Figure 1H). CD4+ T-cells were defined as CD62L+CD44lo/– naive T-cells (Figure 1I), CD62L+CD44hi TEM (Figure 1J), CD25+Foxp3+ Treg (Figure 1K), CXCR5+ICOS+PD-1+ TFH (Figure 1L) and CXCR5+ICOS+PD-1+CD25+Foxp3+ TFR (Figure 1M).

To perform high-dimensional analysis on 21-day-old naïve splenocytes, viable CD45+ cells (Figure 1C) underwent high-resolution FlowSOM clustering to define cell populations, followed by metacoloring for visualization with Uniform Manifold Approximation and Projection (UMAP) [37] using the Cytometry Data Analysis Tool (CATALYST) pipeline [38, 39]. Primary unsupervised analysis was performed to identify CD4+ T-cell and B-cell/ASC clusters based on extracellular receptor co-expression (Figure S3A). CD4+ T-cell (Figure S3B), and B-cell/ASC (Figure S3C) clusters were then isolated for secondary subset analysis.

3 | SIMILARITIES TO OTHER OMIPS

The OMIP described here shares a small degree of marker similarity (TCRβ, CD4, CD44, CD62L, PD-1, CD19, B220) with OMIP-031 [40], OMIP-032 [41], and OMIP-061 [42], which are focused on immunologic checkpoint expression on murine T-cell subsets, the characterization of innate and adaptive populations within the murine mammary gland and murine antigen-presenting cells, respectively. While both OMIP-031 and OMIP-032 characterize TCRβ/CD4+ effector and memory T-cell subsets based on a combination of CD44 and/or CD62L expression, OMIP-032 employs an additional CD19+ gate to delineate B-cells. OMIP-061 utilized B220 to identify B-cells. A distinct difference between these OMIPs and the OMIP described here is that our panel was developed for the sole purpose of comprehensively immunophenotyping T-cell, B-cell, and ASC subsets simultaneously, and we therefore include an additional 12 markers to allow the characterization of two major B-cell, two ASC and seven major T-cell populations within a single sample. The OMIP described here also exhibits minor overlap with OMIP-054 [43], however, our panel was developed to maximize the potential of a 5-laser BD LSRR Fortessa™ in facilities without the capacity to perform mass cytometry.

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AUTHOR CONTRIBUTIONS

Kyle Mincham: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; validation; visualization; writing-original draft; writing-review & editing. Jacob Young: Data curation; formal analysis; investigation; validation; writing-original draft. Deborah Strickland: Conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; writing-original draft; writing-review & editing.

CONFLICT OF INTEREST

The authors declare no conflict of interest exists.

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