NEW OBJECTIVE
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APPLICATION NOTE SERIES

Discovery-to-targeted assays for proteomic fingerprinting of flow-sorted immune cells from healthy human donors

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I. OVERVIEW

Achieving true precision medicine using mass spectrometry requires the ability to rapidly, confidently, and reproducibly develop fit-for-purpose targeted protein assays that can distinguish between healthy and disease states. Prior to committing to large-scale studies on precious samples, we need to establish which protein panels are important for specific biological processes in healthy tissues of certain cell types.

To support the development of fit-for-purpose targeted assays, we aim to establish baseline proteomic profiles of flow-sorted immune cells from healthy donors and design protein "fingerprint" panels for comprehensive cell-type identification. This application note demonstrates a discovery-to-targeted mass spectrometry workflow for developing fit-for-purpose PRM assays that distinguish flow-sorted immune cell populations using cell-type-enriched sentinel proteins.

II. METHODS

Fluorescence-activated cell sorting generates enriched cell populations based on shared surface-marker expression. In this work, we used FACS to isolate immune cells from human blood cones (Figure 1). The resulting sorted populations were at least 98% pure for the intended cell type. Cells were stained with a viability dye and antibodies against CD4, CD8, CD14, and CD19 by the Immune Monitoring and Development Platform (IMDP) at the Ohio State University (OSU).

Column
New Objective PicoChip® 10 cm x 75 µm x 1.9 µm – C18 column

Column Temperature
Heated to 50°C

Mass Spectrometer
Thermo Scientific Stellar™

LC
Thermo Scientific Vanquish™ Neo UHPLC system

Mobile phase A
100% Water + 0.1% formic acid

Mobile phase B
80% Acetonitrile + 0.1% formic acid

Gradient Flow Rate
350 nl/min

Gradient Profile
Linear, 35 min
0–2 min: 2–8% B
2–27 min: 8–28% B
27–30 min: 28–44% B
30–30.25 min: to 98% B
30.25–35 min: 98% B

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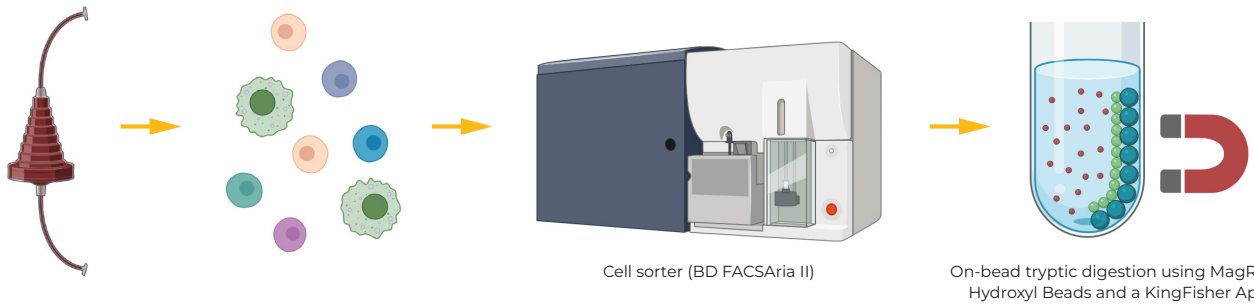

Figure 1: Flow Cytometry cell sorting was used to isolate immune cell populations from blood cones. Blood cones were kindly provided, and cells were flow-sorted by the IMDP at OSU. Once cells were flow sorted, they were prepared for proteomics by lysing in a 5% SDS buffer containing 50 mM TEAB, 2 mM MgCl2, and 1x HALT protease inhibitor. Prior to digestion, proteins were reduced with 20 mM DTT and alkylated with 40 mM IAA; excess IAA was quenched with an additional 20 mM DTT. The samples were then washed and digested using protein aggregation capture (PAC) with MagResyn Biosciences Hydroxyl Beads for 2 hours at 47°C.

To develop fingerprinting assays, we first profiled each cell type individually using a hybrid-quadrupole linear ion trap (Q-LIT) Stellar™ MS coupled to a Vanquish™ Neo UHPLC system. Each cell type was profiled by pooling multiple replicates of CD4+ T cells, CD8+ T cells, CD14+ macrophages/monocytes, and CD19+ B cells (Figure 2). In total, 5 libraries were collected: one for each cell type and an additional library composed of a pooled sample from all cell types.

Prior to mass spectrometry, a Vanquish Neo separated peptides using a 35-minute linear gradient on a 10 cm PicoChip® column. Ions were generated from a New Objective Universal Flow Station™ (UFS) with an integrated column heater set to 50°C. For acquisition, we collected DIA chromatogram libraries from each pool by injecting 5 gas-phase fractions spanning 400-900 m/z. For library acquisition, MS1 and MS2 rates were set to 67 kDa/s. For the MS1 scan, a Full Scan experiment was designed to measure precursor scans. The maximum ion injection time was set to 100 ms, and the AGC target was set to 30 k ions. For MS2 acquisition, DIA scans used 2 m/z isolation windows within each gas-phase fraction, covering 400–500, 500–600, 600–700, 700–800, or 800–900 m/z. The maximum ion injection time was set to 50 ms, the AGC target was set to 20k ions, and fragmentation was done with HCD at a pressure of 8 mTorr at an NCE of 27. All data was collected in centroid mode. Each library provided an average proteome profile for each cell type, which we used to select peptides for PRM.

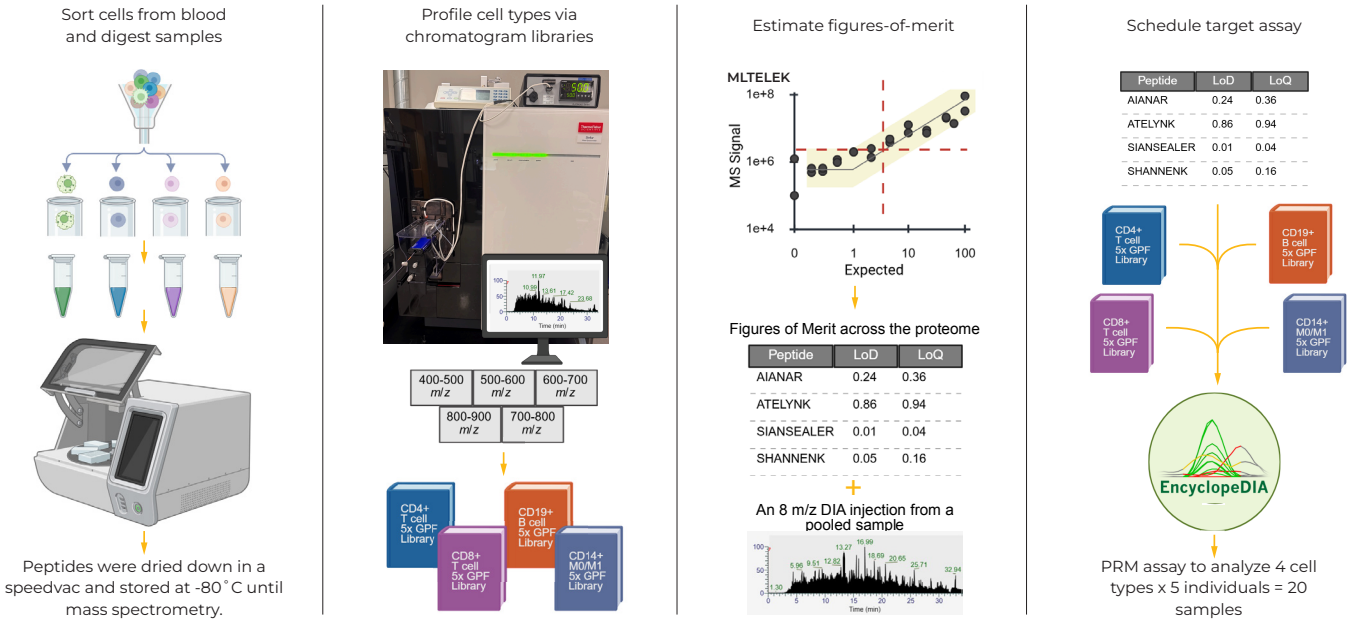

Figure 2: Discovery-to-targeted workflow. Healthy immune cells were isolated from blood cones using antibodies against CD14 (monocyte/macrophage marker), CD19 (B cell marker), CD8 (cytotoxic T cell marker), and CD4 (helper T cell marker).

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When selecting peptides for targeted assays, we wanted to choose peptides with low limits of detection (LoD) or quantification (LoQ). Because not all peptides provide a linear quantitative proxy for protein abundance, calibration curves were used to identify peptides with suitable figures of merit for targeted assay development. As a result, we want to filter out the remaining ¾ of peptides prior to targeted assay scheduling by estimating figures of merit across immune cell proteomes. Calibration curves require a suitable matrix-matched background to ensure accurate figures of merit. We generated calibration curves by diluting a pool of all cell types into a background of dimethyl-labeled cells. An aliquot of the pooled sample was dimethyl-labeled; all samples underwent a C18 cleanup before testing and were confirmed to have 99% label efficiency.

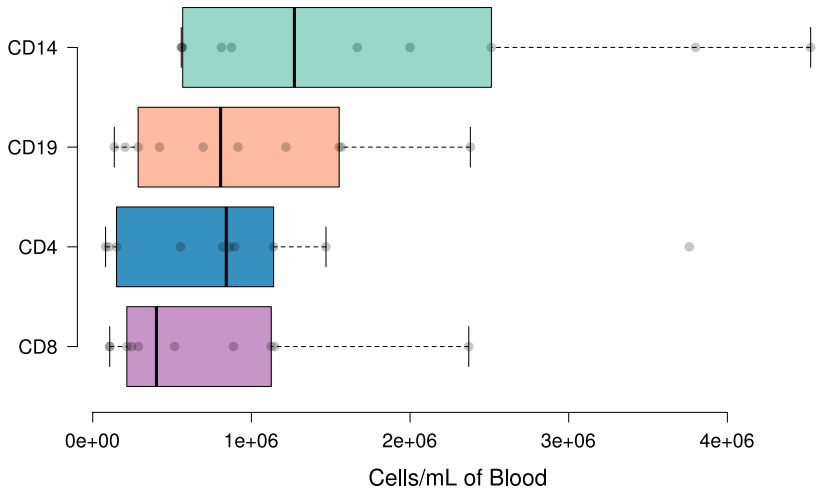

Figure 3: The proportion of cells/mL of blood obtained.

Although CD4+ T cells are generally expected to be the most abundant of these four populations in healthy adult peripheral blood, the CD14+ monocyte/macrophage-enriched fraction produced the highest median sorted-cell recovery in this study. This result highlights an important practical feature of the workflow: cell recovery after blood-cone processing and flow sorting does not necessarily reflect the expected proportions of circulating leukocytes. The increased CD14+ cell recovery provided additional proteomic material from the myeloid fraction, supporting robust detection of myeloid-associated sentinel proteins, including S100A8 and S100A9.

III. RESULTS

Building Targeted Assay

We acquired a matrix-matched calibration curve using DIA with 8 m/z wide isolation windows. The calibration curves were processed in Skyline to calculate figures of merit for peptides detectable in a pooled sample of all cell types. The proteins shown in Figure 4 are A) S10A8 and B) S10A9 proteins (from the S100A8 and S100A9 genes, respectively). These proteins form a heterodimer that mediates immune responses and inflammation in human myeloid-derived cells.

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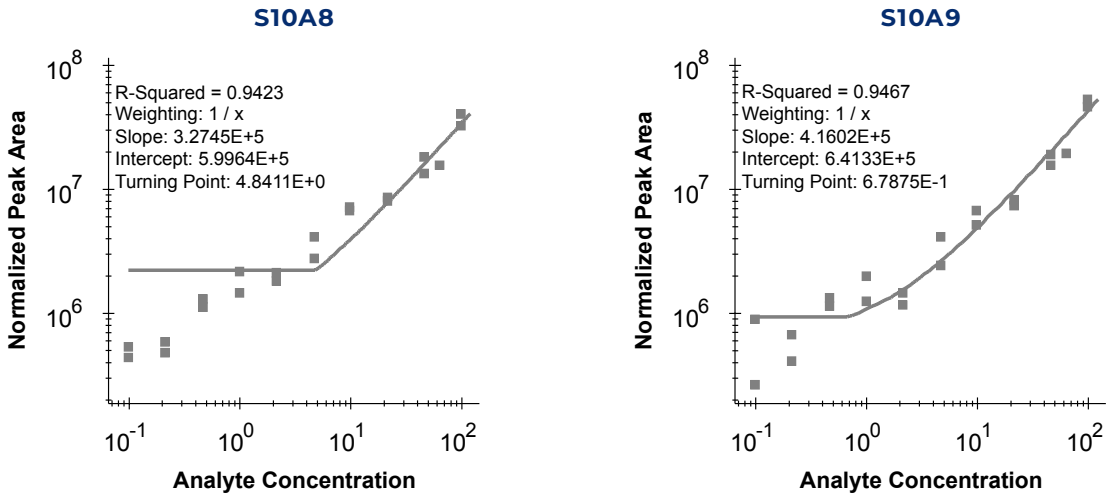

Figure 4: Matrix-matched calibration curves to obtain figures-of-merit on sentinel proteins, S10A8 and S10A9.

Cell Type Differentiation

After calculating figures-of-merit, targeted assays were scheduled using parallel reaction monitoring (PRM). In total, the assay targeted 266 peptides from 135 proteins related to macrophage, T cell, or B cell function.

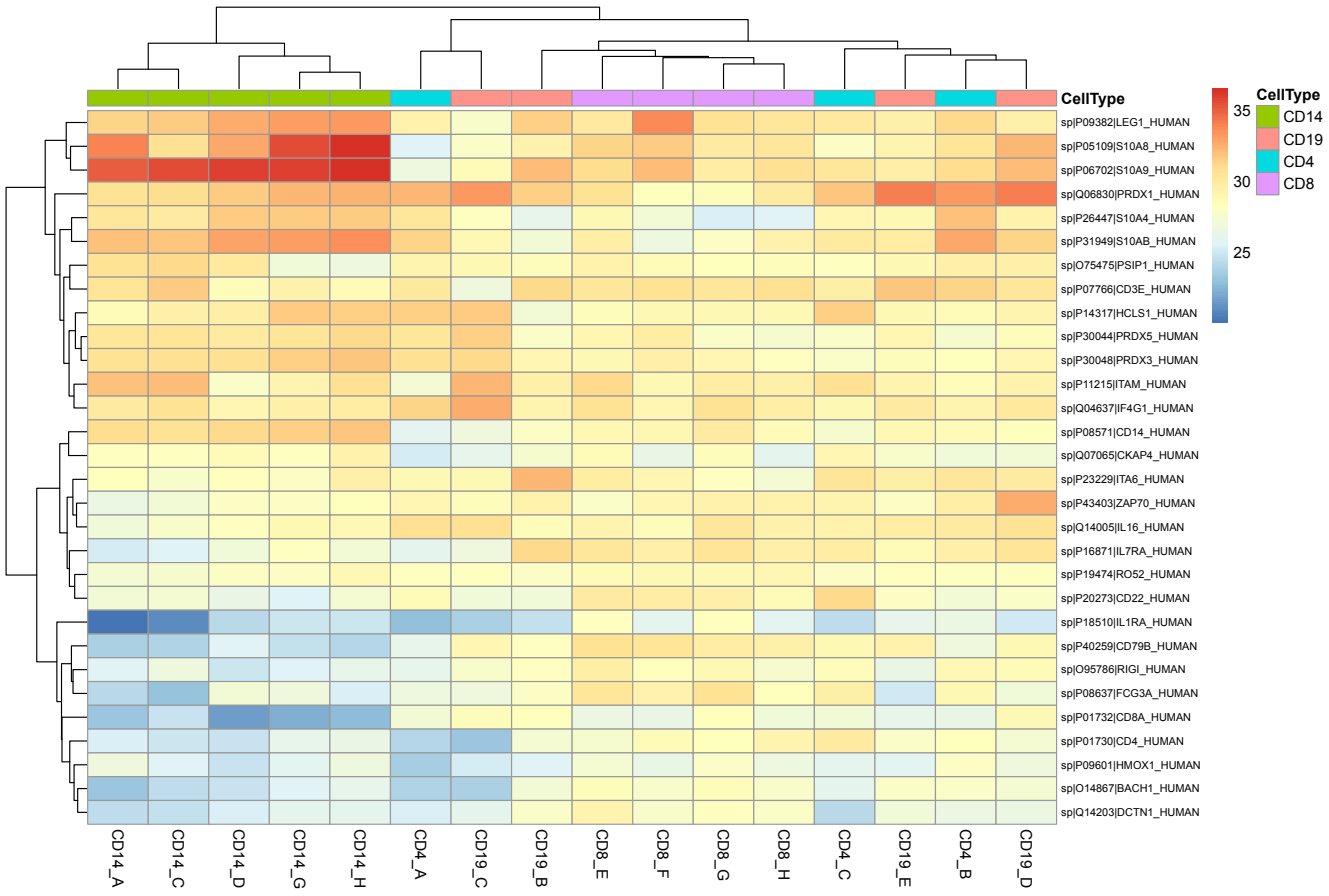

Figure 5: Hierarchical clustering of targeted PRM measurements across flow-sorted immune cell samples. Sentinel proteins show cell-type-associated abundance patterns across CD14+, CD19+, CD4+, and CD8+ populations.

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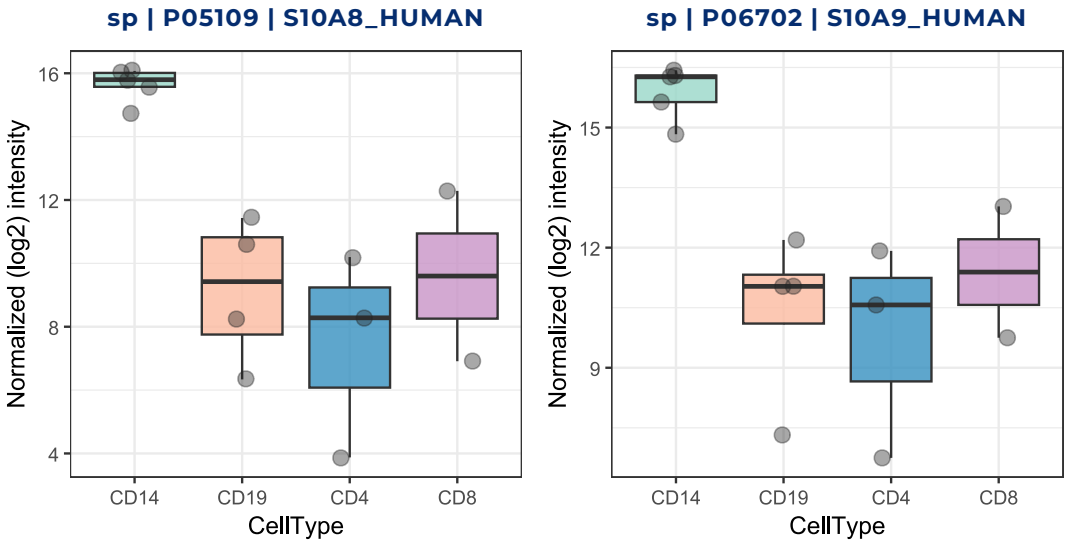

Figure 6: S100A9 and S100A8 protein expression across individual replicates measured with a PRM assay.

The sorted cell recovery quantities shown in **Figure 3** provided a sample-specific context for interpreting the targeted PRM expression data in **Figure 6**. Although recovered cell counts showed significant donor-to-donor variability, each sorted population yielded sufficient proteomic material for comprehensive downstream analysis. Notably, the increased recovery observed in the CD14+ monocyte-enriched fraction facilitated the robust evaluation of myeloid-associated sentinel proteins. In alignment with these recovery data, **Figure 6** demonstrates markedly elevated signals for S100A8 and S100A9 within CD14+ samples compared to lymphoid lineages. These findings confirm that the workflow successfully produces viable sorted-cell input while preserving the cell-type-specific protein signatures necessary for measurement by targeted mass spectrometry.

IV. CONCLUSION AND FUTURE PROSPECTS

We generated a peptide atlas from DIA chromatogram libraries collected across human CD19+ B cells, CD14+ monocytes/macrophages, CD4+ T cells, and CD8+ T cells isolated from healthy donor blood cones. By pairing these libraries with peptide-level sensitivity measurements from matrix-matched calibration curves, this workflow enables the rational selection of peptides for fit-for-purpose targeted assays.

A key challenge in comparing proteins across sorted immune cell populations is maintaining reproducible peptide measurement across distinct biological matrices. Because matrix composition can affect retention time, peak shape, and quantitative performance, reliable chromatography is essential for valid cell-type comparisons. In this study, the Universal Flow Station coupled with PicoChip columns provided stable retention times and low backpressure throughout the experiment. The median peak widths (± inner quartile range) for the CD14, CD19, CD4, CD8, and Pooled cell proteomes 21±11.4 s, 18.6±9.6 s, 18.6±9.9 s, 27.0±13.5 s, and 20.4±12.6 s (**Figure 7**). This chromatographic stability supported targeted measurement of sentinel proteins across samples from multiple human donors.

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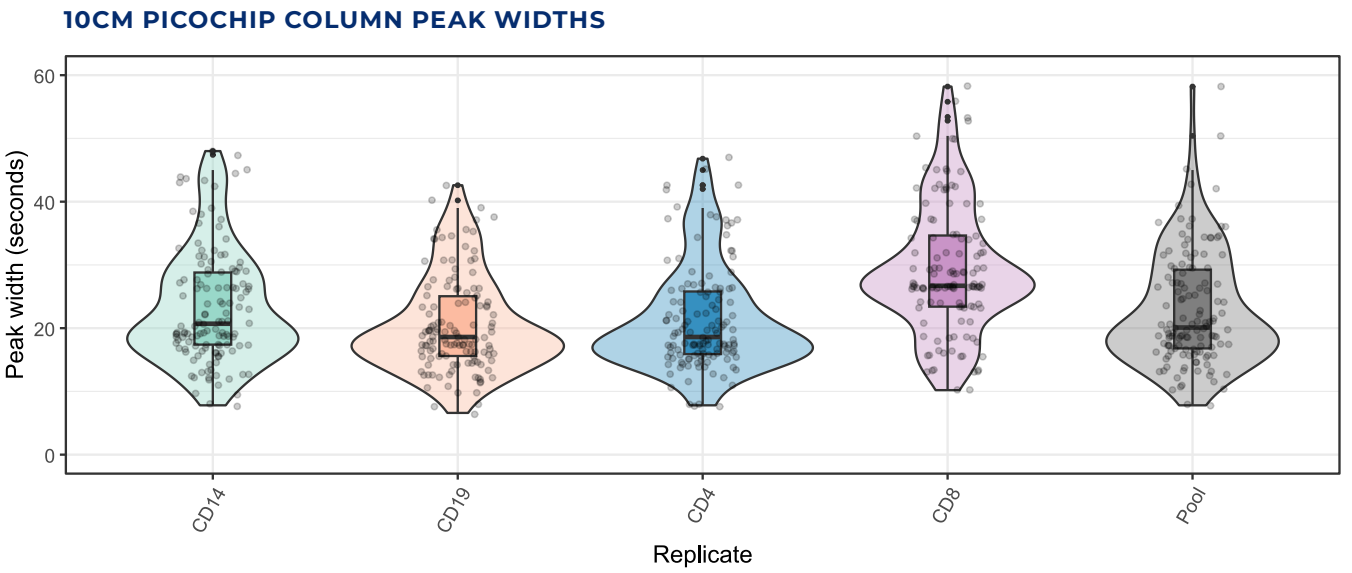

Figure 7: Column peak width across biological matrices using a PicoChip C18 column (10 cm length, 75 µm inner diameter).

Together, these results demonstrate a discovery-to-targeted workflow for developing immune cell fingerprinting assays from limited, flow-sorted sample material. Future work will focus on refining the PRM assay to maximize the number of sentinel proteins included, automating calibration-curve processing and figure-of-merit estimation, and extending the workflow to larger clinical cohorts. Although this study used 35-minute gradients, further optimization using a trap-and-elute configuration on the Vanquish Neo could reduce injection times while preserving chromatographic performance. Once optimized, this approach could support scalable targeted proteomic analysis of immune cell populations across larger sample sets.

V. REFERENCES AND ACKNOWLEDGEMENTS

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