proteinsimple
a bio-techne brand

APPLICATION NOTE

WES AND MILO SYNERGIZE TO PROFILE IMMUNE CELL POPULATIONS IN THE TUMOR MICROENVIRONMENT

INTRODUCTION

Immunotherapy has opened the door to a new era in cancer treatment where dramatically successful and durable responses are being reported. Still, the population of patients who respond are a minority, and this highlights the need for a deeper understanding of the immune response to cancer. To that end, accumulating evidence suggests that the heterogeneous makeup of the tumor microenvironment (TME) is a major contributor to the discrepancies in response observed[1].

The TME is an integrated network of cancerous, noncancerous and immune cell types whose interactions drive tumor heterogeneity, metastatic spread and acquired drug resistance. In particular, the infiltration of leukocytes in the TME, including lymphocytes, macrophages and dendritic cells, among others, has been recognized as both an important prognostic factor and a major obstacle to cancer immunotherapy success[2-4]. Broadly speaking, infiltrating CD8+ T lymphocytes are associated with a proinflammatory TME signature and a better therapeutic response. Working out the cellular determinants behind CD8+ T cell recruitment is requisite to retaining that favorable therapeutic response. Then there's the presence of inhibitory immune cell types and subsets, which, in general, work to suppress a tumor-specific immune response—an attractive target in multimodal treatment strategies to come.

Profiling the composition and function of immune cells that exist within the TME is poised to guide an improved response to immunotherapy and may uncover novel therapeutic targets and strategies. But the complexity and heterogeneity of immune cell signatures, combined with a finite sample, make the TME a particularly difficult area of investigation for which advanced tools are required to achieve deeper analyses. In this application note, we'll show you how Wes™ and Milo™ partner to get you critical answers to 1) what type of immune cell populations are present in a sample and then 2) what percentage of cells in that sample make up a specific immune cell subtype. The workflow gives you a population-level and single-cell-level perspective on each sample analyzed, a much faster time to result than the traditional approach you may currently be using and saves on your sample, big-time.

WES AND MILO: AN AFFIRMATIVE ALLIANCE

Simple Western™ assays on Wes are automated, capillary-based immunoassays that solve many of the repetitive and time-consuming processing challenges that come with traditional Western blotting. Transferring your assay is a breeze; you'll get the separation you need, detection of your target protein you can rely on, and truly quantitative data that will enable you to make accurate experimental conclusions. Just add your samples, antibodies and reagents to the prefilled assay plate, pop in Wes's capillary cartridge, load the plate and push start! In just 3 hours, you'll get quantitative, size-based target abundance data on up to 25 samples. With Wes, you can uncover or confirm the immune cell types present in your sample and quantify the abundance of a cell-type-specific marker in a population. And those low abundance protein markers? Wes has you covered. Requiring only 3 µL of sample to get picogram-level sensitivity, Wes will not only reliably detect your targets but save on your precious samples so you can do more with less.

With Single-Cell Westerns™ on Milo, you can drill further into your sample to measure protein expression heterogeneity or quantify what percentage of cells in your sample population is specific for a desired target combination or phenotype. This means you'll likely want to multiplex. Milo has you covered for up to 12 protein targets in each cell, offering views into cell-to-cell variation within the TME. Just load your cell suspension of as few as 10,000 cells onto the scWest chip, lyse the captured cells and Milo will perform an approximately 1 minute, size-based SDS-PAGE separation on each single-cell lysate to reveal distinct and quantitative peaks for each protein of interest. Importantly, Wes and Milo are both open platforms, so you can screen for any cell-type marker so long as you have a Western-validated primary antibody. To save you time, we've already screened and validated antibodies from R&D Systems and Novus Biologicals for both Simple Western and Single-Cell Westerns.

WHY USE WES AND MILO OVER OTHER MULTIPARAMETER METHODS?

Other multiparameter methods may suffice for analyzing heterogeneous TME cell populations, but a Wes and Milo tag-team can get you the cell type identification and characterization information you need with far less time spent at the bench and without compromising assay sensitivity or precision! Plus, you won't need highly trained specialists to run the instruments or continuous maintenance to deal with system blockages, advanced cleaning protocols and necessary laser calibrations. Moreover, with Wes and Milo's size-based separation skills, you'll say goodbye to off-target binding of variants/isoforms that are bound by the same antibody—endemic to, for example, flow cytometry-based methods. Stop hunting for flow-validated antibodies or spending time establishing complex panels and assays. With Wes and Milo, you can get started with simple yet sophisticated cell population-based measurements by running automated Simple Western immunoassays on Wes and then analyze a chosen population further on Milo to gain selective and quantitate single-cell protein expression information irrespective of target localization. Or, if you're sorting cells, use Milo to characterize protein expression in highly enriched FACS-sorted samples where you don't have enough cells in your sample to put back into a flow cytometer; he just needs ~10,000 cells to start!

WORKFLOW OVERVIEW

Human peripheral blood mononuclear cell (PBMC) samples were isolated from the pooled blood of eight donors and differentiated to either dendritic cells (DCs), Natural Killer (NK) cells or Regulatory T (Tregs) cells prior to analysis on Wes and Milo (**Figure 1**).

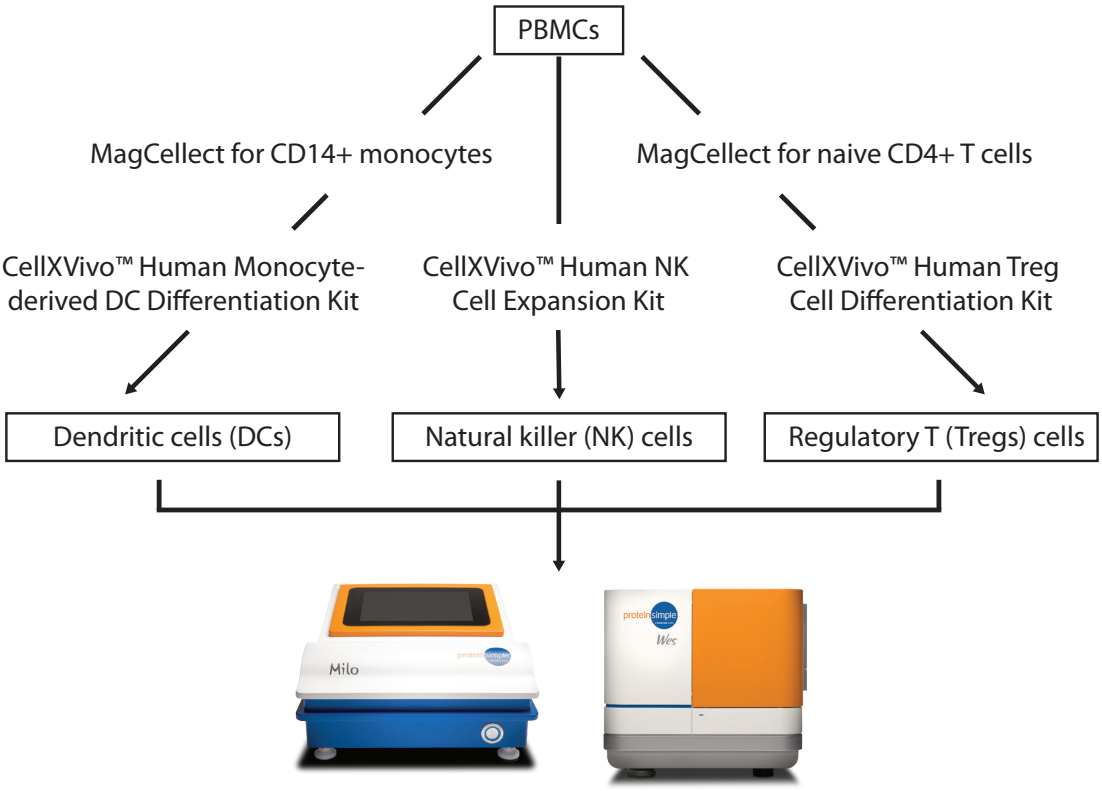

FIGURE 1. Overview of cell type-specific differentiation and identification. A pool of PBMCs was differentiated to DCs (left), NK cells (middle) and Tregs (right). The same sample of differentiated cells was analyzed on Wes and Milo for markers indicative of the differentiated cell type. PBMCs were differentiated to DCs by isolation of CD14+ monocytes, followed by induction and expansion to DCs (left). NK cells were directly expanded from PBMCs (middle). PBMCs were differentiated to Tregs by isolation for naïve CD4+ T cells, followed by induction and expansion to Tregs (right).

DENDRITIC CELL DIFFERENTIATION

To obtain DCs, positive selection for human CD14-expressing monocytes was first performed using the MagCellect™ Human CD14+ Cell Isolation Kit (R&D Systems, MAGH105) by following the recommended protocol found in the product insert. Next, the media and cytokine components within the CellXVivo™ Human Monocyte-derived Differentiation Kit (R&D Systems, CDK004) were used for further differentiation. This kit generates both immature and mature dendritic cells from CD14+ PBMCs under serum-free conditions. DCs obtained are ready for downstream applications.

NATURAL KILLER CELL DIFFERENTIATION

NK cells were expanded from PBMCs using the base media and expander cocktails that come with the CellXVivo™ Human NK Cell Expansion Kit (R&D Systems, CDK015) by following the recommended protocol found in the product insert. This kit is optimized for the expansion of highly cytotoxic CD3-CD56+ NK cells from PBMCs, which are then ready for downstream applications.

REGULATORY T-CELL DIFFERENTIATION

To obtain Tregs, negative selection for human CD4-expressing naïve T cells was first performed using the MagCellect™ Human Naïve CD4+ T Cell Isolation Kit (R&D Systems, MAGH115) by following the recommended protocol found in the product insert. Next, the specially formulated reagents and growth factors within the CellXVivo™ Human Treg Cell Differentiation Kit (R&D Systems, CDK006) were used for further differentiation to FoxP3+CD25+ Tregs. Tregs obtained are ready for downstream applications.

SAMPLE PREP AND RUNNING ON WES AND MILO

For Wes, each cell lysate sample was prepared at a final concentration of 0.5 mg/mL, except for the Treg cell lysate which was prepared at 0.2 mg/mL to detect FoxP3 and 0.04 mg/mL to detect CD25 and CD4. All samples were prepared following the recommended protocol found in the 12-230 kDa Wes Separation Module (ProteinSimple, SM-W003). Finally, samples were denatured and reduced for 5 minutes at 95 °C, then run using the default assay conditions and settings for Wes in Compass for Simple Western software.

For Milo, 1 mL of each cell type was seeded onto an scWest chip at a concentration of 100,000 cells/mL and settled for 5–6 minutes. The standard size scWest chip (ProteinSimple, K600) was used for DCs and NK cells, whereas the small scWest chip (ProteinSimple, K500) was used for Tregs. A 10 second lysis time was used for DC and NK types, and 0 second lysis time was used for Tregs. For the molecular weight ranges of the targets analyzed, an electrophoresis run time of 60–70 seconds at 240 V was selected, and UV capture was performed for 240 seconds.

For details on primary and secondary antibodies used on Wes and Milo, please refer to the Appendix of this application note and the validated antibody databases for Simple Western and Single-Cell Westerns.

IMMUNE CELL TYPE IDENTIFICATION AND CHARACTERIZATION

NATURAL KILLER CELLS

NK cells don't rely on antigen specificity to target cancer cells. Hence, boosting their cytotoxicity has emerged as a plausible innate immunity-based therapeutic strategy against various solid tumor types[5]. Still, solid tumor cells are skilled in escaping immune surveillance and spread quickly when coupled with compromised NK cell function. You'll need to monitor both NK cell infiltration and function in the tumor to make meaningful conclusions about their antitumor potential. In **Figure 2**, we demonstrate the synergy between Wes and Milo in this setting. With Wes, we could distinguish between a sample of NK cells and a PBMC sample based on a clear difference in target abundance of the major NK cell marker CD56 (**Figure 2A**)[6]. This was further supported by a lack of CD3 expression in the NK cell sample compared with the PBMC sample (**Figure 2B**)[6].

A deeper analysis of the same samples on a single-cell level with Milo revealed that the measured CD56+/CD3- phenotype of the PBMC sample at a population level is made up of ~22% of NK cells that are of the specific CD56+/CD3- NK cell phenotype (Figure 2C). Interestingly, ~19% of NK cells in the sample were found to be CD3+, despite measured CD3 expression being negligible at a population level, illustrating the power of single-cell resolution measurements at identifying small subsets of cells that may be overlooked at the population level. In the PBMC sample, 2% of cells were measured as CD56+, where 1.1% were CD56+/CD3+ and 0.9% were CD56+/CD3-. This shift of CD56+/CD3- NK cells from 0.9% in PBMCs to ~22% of cells in the NK sample represents a 96% enrichment of NK cells, commensurate to the 88% enrichment observed with flow cytometry (data not shown). This enrichment underpins the upregulation in CD56 expression observed at the population level and highlights the complementarity of information that Wes and Milo can provide. Moreover, the low CD56 expression and high CD3 measured in the PBMC population on Wes accounts for only ~19% of that sample cell population as measured on Milo; ~81% of the PBMC sample was CD56- and CD3-, suggesting numerous other cell subtypes are present (**Figure 2D**). To calculate total cell numbers and determine population percentages in each sample, Histone H3 was used as a loading control. A graphical summary of the sought-after cell phenotypes is represented as percent of the NK cell and PBMC population identified on Milo in **Figure 2E**. These data attest to the importance of combining bulk and single-cell measurement techniques when analyzing heterogenous cell samples and can guide the experimental planning of other assays to gauge NK cell function in the TME.

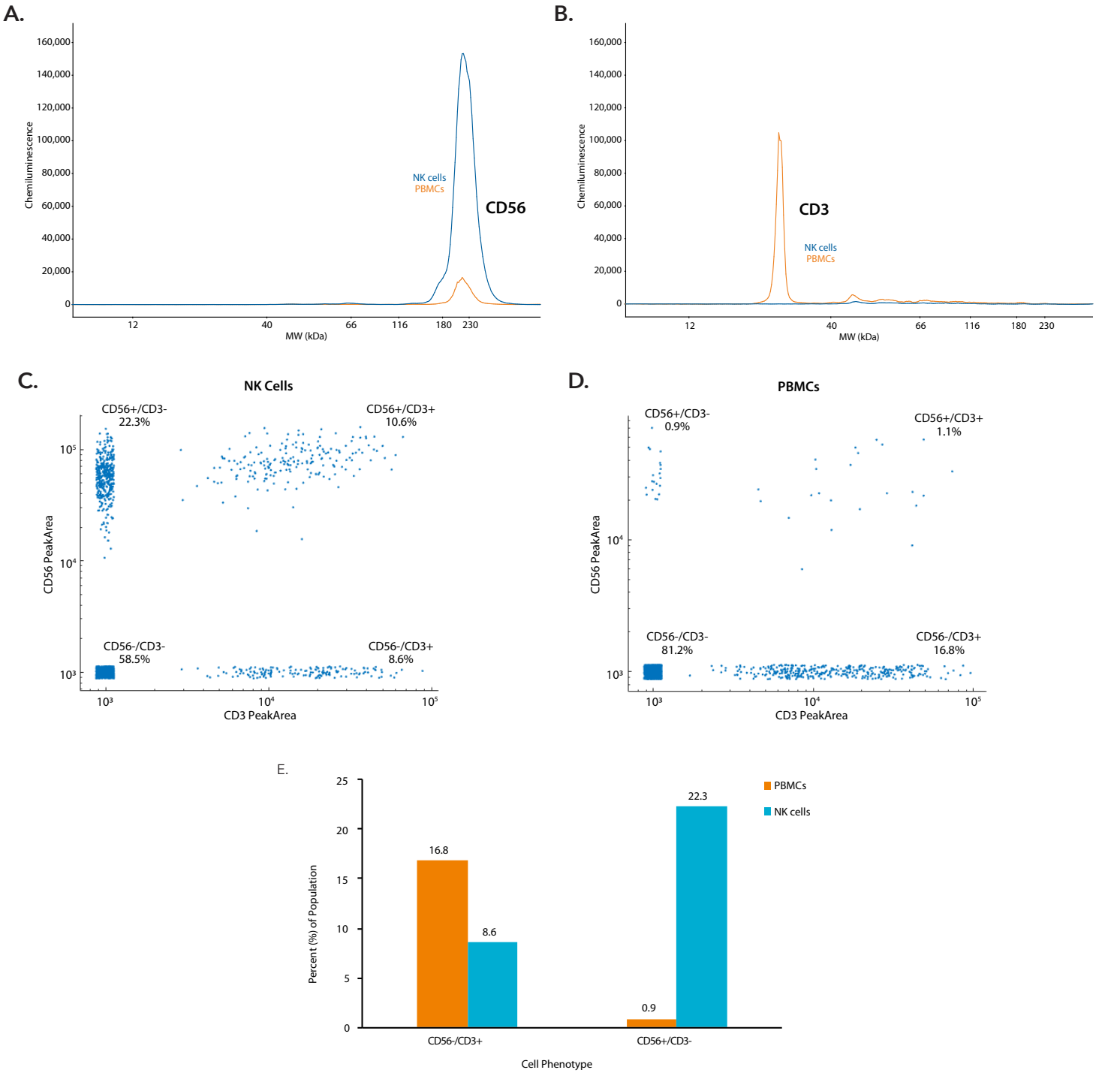

FIGURE 2. Identification and characterization of NK cells on Wes and Milo. On Wes, NK cells yield a stronger CD56 signal compared with PBMCs when 0.5 mg/mL of cell lysate for each sample is loaded (A). Conversely, CD3 is detected in PBMCs but not in NK cells (B). Milo then provides insight into the subpopulations present within samples that underpin an average measurement profile. On Milo, sample analysis shows 22% of NK cells enriched for CD56+/CD3- (C). Whereas 16.8% of PBMCs are enriched for CD56-/CD3+. Interestingly, the largest cell subset revealed within both NK and PBMC samples of 10,000 cells/mL analyzed is of the CD56-/CD3- phenotype (D). The archetypal phenotype for NK cells and PBMCs is shown as a percent of the population analyzed (E). PBMCs, peripheral blood mononuclear cells; NK, natural killer cells

DENDRITIC CELLS

CD8+ T lymphocytes do rely on tumor-associated antigens to eliminate tumor cells. For CD8+ T lymphocytes to elicit a lasting cytotoxic immune response, antigen cross-presentation by professional antigen-presenting cells like DCs is necessary[7]. Still, mechanisms of tumor-induced immunosuppression in the TME compromise DC function and the ability to induce this antitumor response. Strategies that look to repair or enhance DC function represent an opportunity to improve CD8+ T cell-based cancer immunotherapy, making their accurate identification and characterization essential for correlation with increased T cell infiltration[7].

Different DC subsets have varying abilities to process and present antigens. Herein, we looked at a variety of markers belonging to the most commonly studied type, human monocyte-derived DCs[8]. On Wes, we could clearly distinguish mature DC populations from PBMCs based on the relative expression of markers analyzed (**Figure 3**). Specifically, the monocyte marker CD14 is abundantly expressed in CD14+ untreated cells but not DCs (**Figure 3**, left)[9], whereas the DC adhesion molecule, CD209 (**Figure 3**, middle) and the mature DC marker CD83 (**Figure 3**, right) are highly expressed in the DC cell sample but not in the CD14+ untreated cells. CD83, in particular, has emerged as an exciting and promising target whose separation profile (**Figure 3**, right) can be explained by its glycosylation status[10]. On Milo, we took the same monocyte-derived DC sample and further characterized the immunophenotypes present. Our single-cell analysis revealed that 60% and 79% of DCs express the costimulatory molecules CD80 and CD86, respectively, which is indicative of efficient antigen-presenting abilities in this population (**Figure 4**). The DC-specific CD209 and CD83 that were profiled on Wes (**Figure 3**) are present on ~80% and ~45% of the population analyzed, respectively. As before, total cell numbers and population percentages were calculated in each sample using Histone H3 as a loading control. Importantly, while these CD markers can also be detected using traditional flow cytometry techniques, Milo can work with low starting cell numbers (~10,000 versus >100,000). This can be important when working with sparingly available samples, such as those taken from the TME.

As the most potent of antigen-presenting cells that drive cytotoxic T cell-mediated immune responses, understanding the role DCs play in immune regulation within the TME, and enhancing their effector function is an exciting strategy to boost antitumor immunity. With Wes and Milo, you can sequentially determine DC abundance in a tumor and then further define the phenotype to make accurate functional conclusions about the population present.

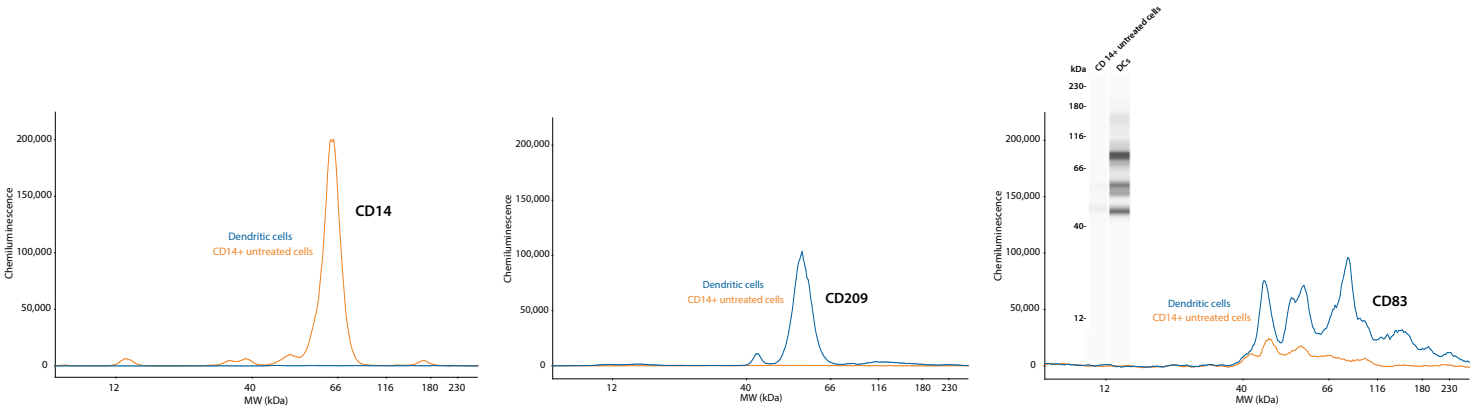

FIGURE 3. Monocyte-derived DC marker expression analysis on Wes. CD14 positive monocytes were enriched from PBMCs and differentiated into mature DCs. DCs were profiled according to the absence of CD14 expression and presence of CD83 and CD209 when 0.5 mg/mL of each cell sample lysate was loaded. The electropherograms show that CD209 (middle) and CD83 (right) are enhanced in DCs compared with the CD14+ monocyte cell sample control. Conversely, and as expected, CD14 is expressed in the monocyte sample, but not in the differentiated DC population analyzed (left). *PBMCs*, peripheral blood mononuclear cells; *DCs*, dendritic cells

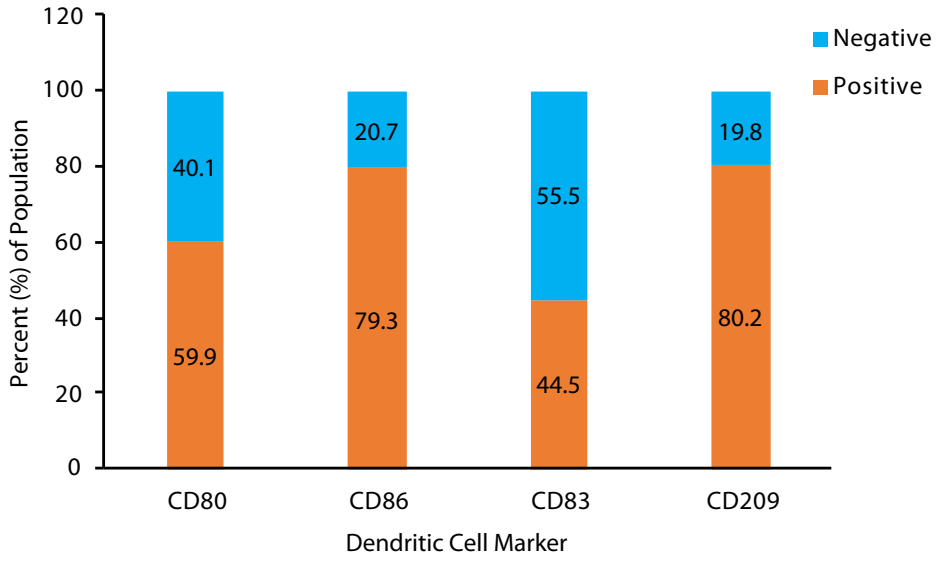

FIGURE 4. Characterization of monocyte-derived DCs on Milo. Bar graph defining the subpopulations of DCs that are positive for CD80, CD86, CD83 and CD209. Histone H3 was used as a loading control to determine the total number of cells in the population. *DCs*, dendritic cells

REGULATORY T CELLS

It's well-known that Tregs suppress antitumor immune responses and are a big player in the creation of the immunosuppressive or tolerant zone that is the TME[10]. As such, multimodal approaches that include the depletion of Tregs are being explored to enhancethe immune response within the TME further. Although they are composed of diverse subsets, Tregs present in immune infiltrates of various cancer types have been extensively characterized[11,12]. And now you can accurately profile them using the streamlined workflow of the Wes and Milo tag-team.

In **Figure 5**, we validate the use of Wes for the detection of the most well-understood immunosuppressive Treg cell markers, namely CD4, CD25 and FoxP3. CD4+ T cells were isolated from PBMCs and differentiated to obtain a Treg cell population that was assessed for the expression of CD25 and FoxP3. CD4 expression was observed in both the originating PBMC population and differentiated Treg cell sample, albeit with an obviously higher abundance in Tregs (**Figure 5**, left)[13]. CD25, another cell surface marker and a component of the IL-2 receptor is detected in Tregs but not PBMCs (**Figure 5**, middle)[13]. Moreover, the intracellular transcription factor, FoxP3, often dubbed "the master regulator" of Treg development and function is clearly present in only the Treg population (**Figure 5**, right)[13]. These data confirm the use of Wes for the analysis of Treg cell populations, where the ability to distinguish between cell-type profiles based on target abundance can help you to direct and prioritize experiments that further dive into both Treg function and the underlying molecular mechanism(s) responsible.

Taking a deeper dive into the same Treg cell sample with Milo reveals that a large portion of the differentiated Treg population is CD4+ (85%) and CD25+ (95%), while only 34% are positive for FoxP3 (Figure 6A). This is important as Tregs are often further divided into subsets based on activation and differentiation (otherwise determined by flow cytometry), where FoxP3^{high} and FoxP3^{low} are associated with either effector or resting status[10].

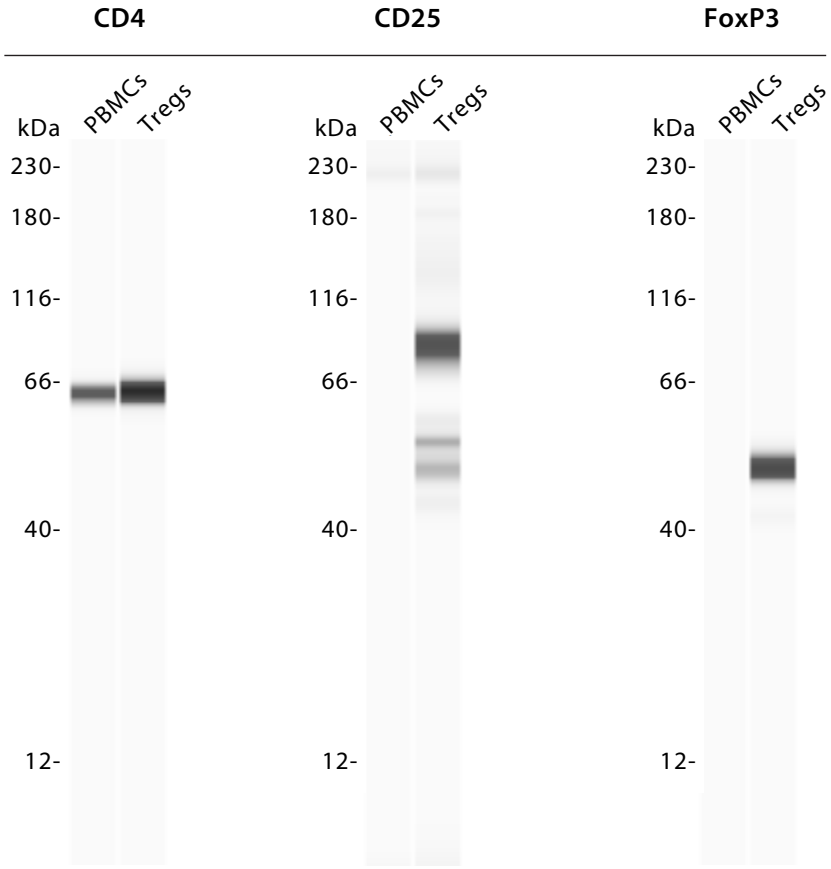

FIGURE 5. Treg marker expression analysis on Wes. CD4+ T cells were enriched from PBMCs and differentiated into Tregs, 0.2 mg/mL of each cell sample lysate was loaded for analysis of FoxP3 and 0.04 mg/mL for analysis of CD25 and CD4. CD4 expression is detected in PBMCs, but much more so postenrichment in the Treg population (left). Tregs are characterized by the presence of FoxP3 and CD25 markers, which were not detected in PBMCs (middle, right). *PBMCs*, peripheral blood mononuclear cells

Milo simplifies flow cytometry workflows by providing the assay flexibility you need to detect those hard-to-get-to intracellular proteins, like FoxP3, enabling simultaneous detection of surface markers and intracellular markers without requiring fixation and permeabilization. As shown in the FoxP3 and CD4 scatterplot (**Figure 6B**), Milo can effectively uncover subpopulations of cells within a sample containing the same cell type. Moreover, Milo's quantitative measurements, and ability to threshold to differentiate between two populations in a sample of the same cell type, also account for the FoxP3high and FoxP3low level of heterogeneity characteristic of Tregs (**Figure 6C**). For more on Milo's ability to threshold to resolve distinct populations based on differences in protein expression levels, check out this application note and available literature14. Herein, we can conclude that the Treg population differentiated is of the resting status based on the low number of cells positive for FoxP3. This seems logical as the source of Tregs, in this case, is the peripheral blood of apparently healthy donors, rather than a tumor. In sum, these data establish proof-of-principle and validate the application of Wes and Milo in the TME setting.

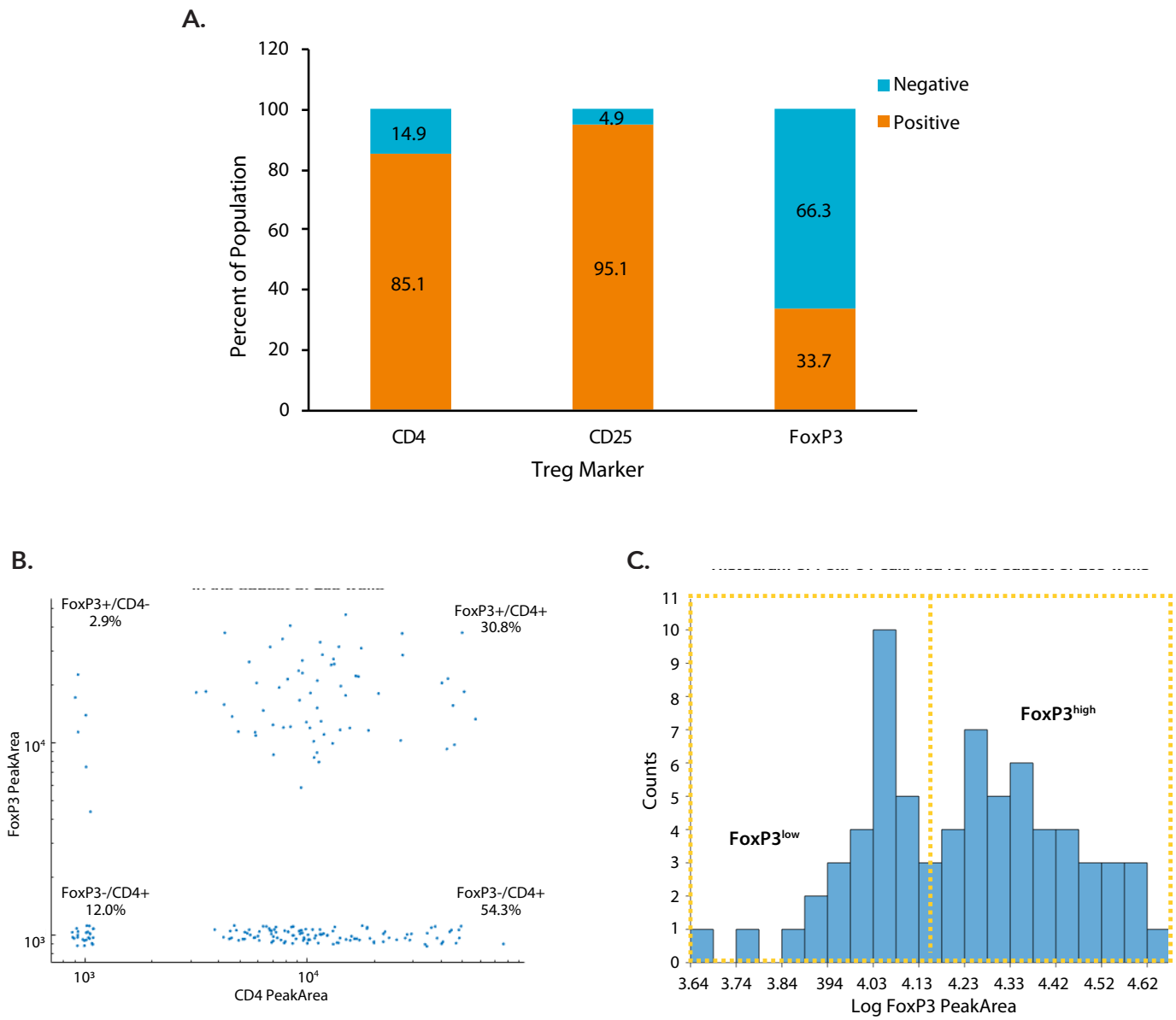

FIGURE 6. Characterization of CD4+ PBMC-derived Tregs on Milo. Bar graphs illustrating the quantification of cell subpopulations present within our Treg sample according to CD4, CD25 and FoxP3 (**A**). Two-dimensional scatter plot of the subpopulations present based on the presence or absence of FoxP3 and CD4 (**B**). Histogram showing classification of Treg populations based on intracellular FoxP3 expression (**C**). *PBMCs*, peripheral blood mononuclear cells; *Tregs*, regulatory T cells

CONCLUSION

Translating immune TME biology into viable immunotherapies requires advanced technologies to decipher the complexity and diversity of the cell types present. The profiling and characterization of different immune cell types in the TME is essential to our understanding of metastatic spread, the immune response to cancer, the discovery of therapeutic targets and influence on immunotherapeutic strategies to come—all areas of intense investigation. In this application note, we've demonstrated how cell differentiation solutions from R&D Systems enable the partnership between Wes and Milo to provide you with the high-resolution view of cell composition and function you need to guide your research efforts in achieving the aforementioned goals. No matter your approach when tackling the immune TME, Wes and Milo will lead you to your next discovery.

REFERENCES

1. Microenvironmental regulation of therapeutic response in cancer, F Klemm and JA Joyce, *Trends Cell Biology*, 2015; 25:198–213.

2. HYPE or HOPE: The prognostic value of infiltrating immune cells in cancer, TA Barnes and E Amir E, *British Journal of Cancer*, 2017; 117:451–60.

3. Understanding the tumor immune microenvironment (TIME) for effective therapy, M Binnewies, EW Roberts, K Kersten, V Chan,DF Fearon, M Merad, LM Coussens, DI Gabrilovich, S Ostrand-Rosenberg, CC Hedrick, RH Vonderheide, MJ Pittet, RK Jain, W Zou, TK Howcroft, EC Woodhouse, RA Weinberg and MF Krummel, *Nature Medicine*, 2018; 24:541–50.

4. Regulatory T cells in the tumor microenvironment and cancer progression: Role and therapeutic targeting, B Chaudhary B and E Elkord, *Vaccines*, 2016; 4(3):E28.

5. Overcoming resistance to Natural Killer cell based immunotherapies for solid tumors, G Nayyar, Y Chu and MS Cairo, *Frontiers in Oncology*, 2019; 9:51.

6. CD56bright Natural Killer (NK) cells: An important NK cell subset, A Poli, T Michel, M Thérésine, E Andrès, F Hentges and J Zimmer, *Immunology*, 2009; 126:458–65.

7. Dendritic cells and CD8 T cell immunity in the tumor micro-environment, F Chunmei and J Aimin, *Frontiers in Immunology*, 2018; 9:3059.

8. Antigen cross-presentation by dendritic cell subsets: one general or all sergeants? S Nierkens, J Tel, E Janssen and GJ Adema,*Trends in Immunology*, 2013; 34:361–70.

9.Differentiation of human monocytes into CD14 negative accessory cells: do dendritic cells derive from the monocytic lineage?JH Peters, J Ruppert, RK Gieseler, HM Najar and H Xu, *Pathobiology*, 1991; 59:122–6.

10. Dendritic cell CD83: A therapeutic target or innocent bystander? CM Prazma and TF Tedder, *Immunology Letters*, 2008; 115:1–8.

11. Regulatory T cells in the tumor microenvironment and cancer progression: Role and therapeutic targeting, B Chaudhary and E Elkord, *Vaccines*, 2016; 4:E28.

12. Regulatory T cells in cancer immunotherapy, H Nishikawa and S Sakaguchi, *Current Opinion in Immunology*, 2014; 27:1–7.

13. Phenotypic alterations, clinical impact and therapeutic potential of regulatory T cells in cancer, B Chaudhary, M Abd Al Samid,zBK al-RamadiA and E Elkord, *Expert Opinion on Biological Therapy*, 2014; 14:931–45.

14. Regulatory T Cells and Foxp3, AY Rudensky, *Immunology Review*, 2011; 241:260–68.

15. Profiling human breast epithelial cells using single cell RNA sequencing identifies cell diversity, QH Nguyen, N Pervolarakis, K Blake, D Ma, RT Davis, N James, AT Phung, E Willey, R Kumar, E Jabart, I Driver, J Rock, A Goga, SA Khan, DA Lawson, Z Werb and K Kessenbrock, *Nature Communications*, 2018; 9:2028.

APPENDIX

Table A1. Antibodies used on Milo in this application note. Antibodies were diluted in Antibody Diluent 2 except when Goat or Sheep primary antibodies were used in the antibody cocktail. In the latter case, all antibodies were diluted in Milk-Free Antibody Diluent.

PRIMARY ANTIBODIES			
ANTIBODY	**VENDOR**	**PRODUCT NO.**	**WORKING CONCENTRATION**
Goat Polyclonal Anti-CD4	R&D Systems	AF-379-NA	100 µg/mL
Goat Polyclonal Anti-CD25	R&D Systems	AF-223-NA	100 µg/mL
Goat Polyclonal Anti-CD56	R&D Systems	AF2408	100 µg/mL
Goat Polyclonal Anti-CD80	R&D Systems	AF140	100 µg/mL
Goat Polyclonal Anti-CD83	R&D Systems	AF2044	100 µg/mL
Goat Polyclonal Anti-CD86	R&D Systems	AF-141-NA	100 µg/mL
Sheep Polyclonal Anti-CD209	R&D Systems	AF161	100 µg/mL
Goat Polyclonal Anti-FoxP3	R&D Systems	BAF3240	100 µg/mL
Rabbit Polyclonal Anti-Histone H3	Novus Biologicals	NB500-171	50 µg/mL
Mouse Monoclonal Anti-Histone H3	Novus Biologicals	NBP2-36468	50 µg/mL
Rabbit Monoclonal Anti- CD3	ThermoFisher	MA5-14524	1:10

WHERE SCIENCE
INTERSECTS INNOVATION™

bio-techne®

bio-techne.com

R&D SYSTEMS | NOVUS BIOLOGICALS | TOCRIS | protein simple | ACD | exosome dx

Global info@bio-techne.com bio-techne.com/find-us/distributors TEL +1 612 379 2956 North America TEL 800 343 7475
Europe | Middle East | Africa TEL +44 (0)1235 529449 China info.cn@bio-techne.com TEL +86 (21) 52380373

FL_Application Note_PL7-0076 Rev A