A modular, multicolor approach to regulatory T-cell characterization

Understanding Treg heterogeneity using the 12-color BD FACSLyric™ flow cytometer

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Summary

The promise of cell-based therapies for a diverse set of diseases is fueling intense clinical and academic interest in regulatory T cells (Tregs). However, the well-documented heterogeneity of the Treg compartment poses a significant challenge, in terms of understanding which subpopulation would prove to be most efficacious in disease diagnosis and treatment. We have developed a modular flow cytometry approach (8 + 4 colors) to characterize Tregs in a sample using the 3-laser, 12-color BD FACSLyric™ flow cytometer. The 8-color backbone panel was designed such that it could be supplemented with four additional markers whose expression correlates with different aspects of Treg biology, such as homing, function, maturation, proliferation, trafficking and stability, without affecting the resolution of the backbone Treg population.

To demonstrate the utility of the modular approach, we developed two 4-color drop-in panels to analyze the expression of cell surface markers previously shown to correlate with homing and functional properties of Tregs. Addition of the 4-color Treg homing *(drop-in)* panel enabled identification of naive recent thymic emigrant (RTE) Treg cells as well as Th-like subsets. Similarly, addition of the 4-color Treg function *(drop-in)* panel enabled identification of Treg subsets that express high levels of markers previously shown to correlate with immunosuppressive activity. This approach also highlighted donor-specific heterogeneity within the Treg compartment. Furthermore, for any sample investigated via the modular approach, the usage of high-dimensional data analysis approaches facilitated the simultaneous analysis of multiple markers and the visualization of phenotypic relationships between different clusters of cells.

The BD FACSLyric flow cytometer offers high sensitivity, workflow automation and reproducible results across time, users, instruments and sites. By standardizing assays to the same reference settings, assays can be portable across large multisite studies spanning the globe.

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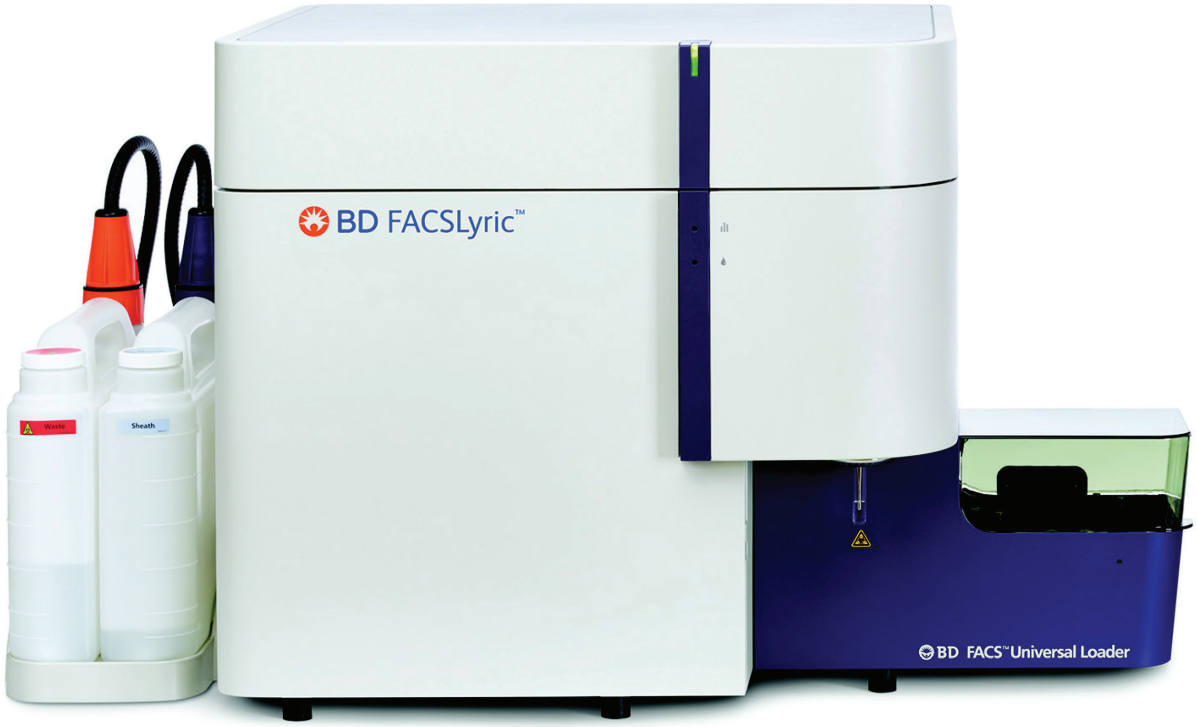
Introduction

Tregs play a central role in maintaining self-tolerance and immune homeostasis. Tregs exert their suppressive function through diverse mechanisms, including contact-dependent and cytokine-mediated suppression of effector cells.[1] Impaired function and/or altered frequency of Tregs have been implicated in several conditions, including graft-versus-host disease (GVHD), diabetes *(type 1)*, rheumatoid arthritis, lupus and multiple sclerosis.[2-4] Tregs have also been implicated in the maintenance of immune suppression during tumor progression and chronic infections.[5,6] Therefore, there is growing interest in deeper characterization of Tregs at a single-cell level to unravel new facets of Treg biology *(such as activation, maturation, proliferation and peripheral trafficking)* that could be used to harness the clinical utility of Treg-mediated immunosuppression.

Tregs can be identified based on expression of the transcription factor FoxP3, the highest levels of CD25 expression in the $CD4^+$ T-cell compartment and the low-to-no expression of CD127. Treg heterogeneity in terms of phenotype, function and distribution is widely documented,[7-9] making it critical to characterize these cells in detail for potential clinical applications. Detailed functional characterization of Treg subsets may enable development of more precise, consistent and personalized therapeutic approaches. For example, Tregs can be classified as Th1-, Th2-, Th17- and Th22-like subsets based on shared expression of chemokine receptors, cytokines produced and master transcriptional regulators with the conventional $CD4^+$ T-helper cell lineages.[10,11] During inflammation, the diverse cytokine milieu that drives conventional T-helper cell specialization also drives the polarization of Th-like Tregs that can suppress the specific inflammatory conditions in which they arise.[12] Therefore, in the context of Th1-, Th2- and/or Th-17-driven autoimmune diseases, optimal therapeutic efficacy could be achieved by specifically targeting the cognate Th-like Treg subset(s). For example, the Th1-like subset of Tregs could prove to be more effective, compared to total Tregs, for treatment of Th1-driven diseases, such as type 1 diabetes.[13] Moreover, the ability to monitor alterations in functionally relevant Treg subset(s) for diagnostic and prognostic analysis may provide more useful information.



The previously mentioned importance of Tregs for therapeutic applications, combined with their well-documented heterogeneity, has generated great interest in intense clinical and academic research. Several reports have identified distinct Treg immunophenotypic signatures correlating with different biological function(s), including homing and immunosuppression.[10,11,14-20] Others have combined high parameter mass cytometry with high-dimensional data-analysis approaches to reveal additional heterogeneity within the human Treg compartment, with more than 20 phenotypically distinct subsets of cells identified within the conventional $CD3^+CD4^+CD127^{low/-}CD25^{high}$ Treg population.[21-23]

Here, we report the development and utility of a conventional, modular flow-cytometric approach to explore Treg phenotypic diversity on the 12-color BD FACSLyric flow cytometer. We designed an 8-color backbone panel to identify the Treg population in a sample using well-established Treg markers *(CD3, CD4, CD25, CD127 and FoxP3; Table 1)*. The inclusion of CD45RA in the 8-color backbone panel enabled identification of naïve *($CD45RA^+$)* and effector *($CD45RA^-$)* Treg subsets, while the inclusion of CD15s and CD161 enabled the identification of highly suppressive effector[14] and/or proinflammatory cytokine-secreting[15,16] Treg subsets, respectively. Four channels were left open to allow researchers to assess the expression of additional markers correlating with diverse aspects of Treg biology. To illustrate the utility of this modular approach, we developed drop-in panels to explore two critical facets of Treg biology: homing and function *(Table 1)*. The inclusion of CD183, CD194 and CD196 in the homing drop-in panel enabled identification of Th-like Treg subsets,[10,11] while CD31 helped identify RTE Tregs.[24] The four markers *(PI16, CD39, CD147 and HLA-DR)* in the Treg function panel enabled the identification of previously defined Treg subsets endowed with immunosuppressive activity.[17-20] With extraordinary sensitivity to resolve dim populations, automation to increase workflow efficiency and superior standardization and reproducibility over time and across systems, the BD FACSLyric flow cytometer is an ideal instrument for clinical and academic research.[25,26]

Methods

Peripheral blood mononuclear cells (PBMCs) were prepared from fresh whole blood from three healthy donors. The PBMCs were stained in the presence of BD Pharmingen™ Stain Buffer (BSA) and BD Horizon™ Brilliant Stain Buffer with the antibodies listed in Table 1A–C. For intracellular staining of FoxP3, BD Pharmingen™ Transcription Factor (TF) Buffer Set was used according to kit recommendations. Samples were acquired on a 12-color BD FACSLyric flow cytometer and analyzed using BD FACSuite™ software. Lyse/wash assay settings were used and single-color stained PBMCs were used as compensation to update spillover values. The easy-to-use BD FACSLyric flow cytometer offers built-in automated and standardized instrument setup and generates a spillover matrix good for up to 60 days, eliminating the daily run of compensation controls.[25,26]

A **8-color backbone panel**

	Fluorochrome	Target antigens
1	BV421	CD25
2	BV510	CD15s
3	FITC	CD4
4	PE	CD161
5	BB700	CD127
6	PE-Cy™7	CD45RA
7	Alexa Fluor® 647	FoxP3
8	APC-H7	CD3

B **4-color homing drop-in panel**

	Fluorochrome	Target antigens
1	BV605	CD31
2	BV711	CD183 *(CXCR3)*
3	BV786	CD194 *(CCR4)*
4	APC-R700	CD196 *(CCR6)*

C **4-color function drop-in panel**

	Fluorochrome	Target antigens
1	BV605	PI16
2	BV711	CD39
3	BV786	CD147
4	APC-R700	HLA-DR

Table 1. Twelve-color modular flow cytometry Treg panel
The modular flow cytometry approach includes an 8-color backbone panel for Treg identification *(A)*, and two drop-in 4-color panels *(B and C)* for characterization of markers known to correlate with Treg homing and function.

Results

Resolution of 8-color Treg backbone panel is not affected by the addition of drop-in panels

The successful development of a modular flow cytometry assay relies on the design of a backbone panel that can be expanded by the addition of drop-ins without loss of resolution. To achieve this goal, the fundamental principles of panel design *(antigen relative expression and coexpression, and fluorochrome brightness and fluorescence spillover)* must be followed while designing the backbone panel. Also, the strategic choice of fluorochromes for the drop-in markers must minimize the impact of their addition on the resolution of the backbone panel.

To identify fluorochromes that should be paired with the drop-in markers, fluorescence spread was analyzed *(data not shown)*. Fluorochromes with relatively low spillover into the detectors used for the backbone panel were identified *(BV605, BV711, BV786 and APC-R700)* and paired with the drop-in markers based on antigen density and relative expression *(in both the drop-in panels)*. The 8-color backbone panel enabled clear identification of Tregs as $CD3^+CD4^+CD127^{low/-}$ $CD25^{high}FoxP3^+$ cells (***Figure 1***, *top row)*. In addition, CD45RA, CD15s and CD161 in the backbone panel allowed discrimination of previously defined functionally suppressive effector *(esTreg, $CD45RA^-CD15s^+$)*[14] and proinflammatory cytokine secreting suppressive *(CD45RA⁻CD161⁺)*[15,16] Tregs within the heterogeneous $CD45RA^-$ effector Treg (eTreg) population. $CD45RA^+CD15s^-$ naïve Tregs and $CD45RA^-CD15s^-$ nonsuppressive cells were also detected in the sample.[14] Importantly, addition of the 4-color Treg homing or function drop-in panel(s) had minimal effect on the resolution of the major Treg subsets (***Figure 1***, *top row vs middle and bottom rows)*. Furthermore, the addition of the drop-in panel(s) did not impact the relative quantification of the different Treg subsets (***Figure 1***, *all rows; see population statistics in dot plots)*. Collectively, this data demonstrates that our panel-design approach provides the flexibility of supplementing the 8-color Treg backbone panel with any 4-color drop-in panel of interest without the need to redesign the backbone panel.

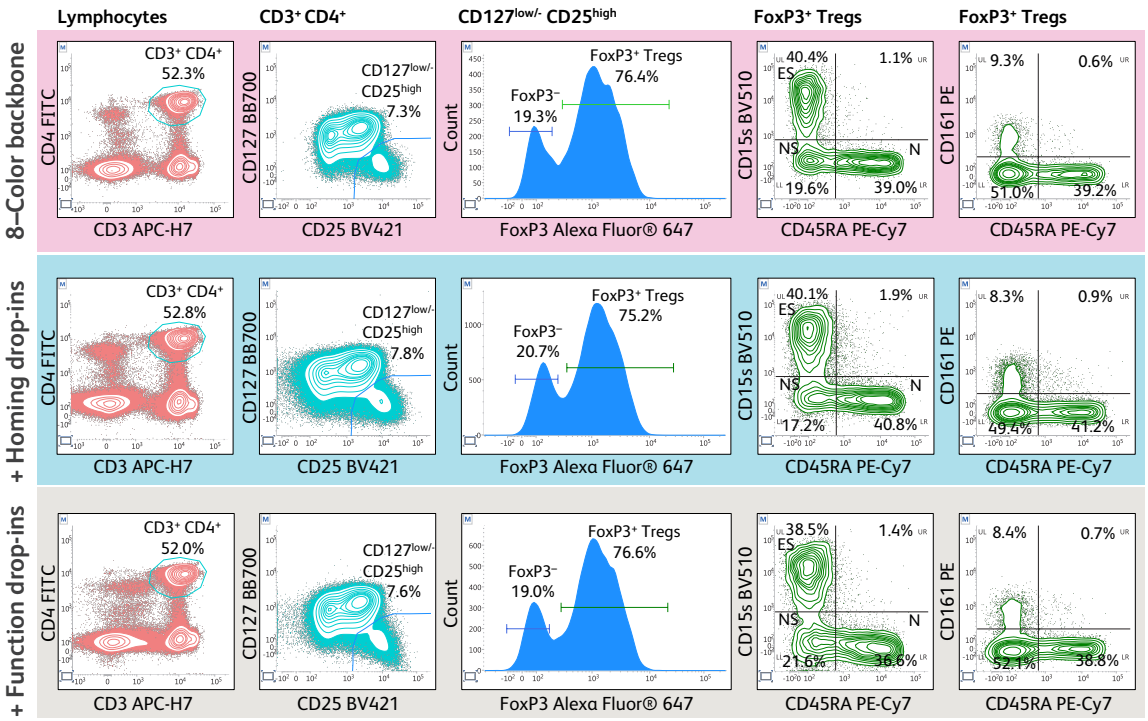


Figure 1. Eight-color Treg backbone panel resolution

The 8-color backbone panel enables identification of Tregs *($CD3^+CD4^+CD127^{low/-}CD25^{high}FoxP3^+$)* and the different subsets of Tregs based on CD45RA, CD15s and CD161 expression. $CD45RA^-CD15s^+$ suppressive Tregs, $CD45RA^-CD15s^-$ nonsuppressive cells and $CD45RA^+CD15s^-$ naïve Tregs are labeled as ES, NS and N, respectively. Population statistics are shown as percent of parent population for the respective subsets. Resolution of the 8-color Treg backbone panel as well as relative quantitation of the different Treg subsets *(top row)* were not significantly impacted by the addition of the 4-color Treg homing *(middle row)* or function *(bottom row)* drop-in panels.

Evaluation of the modular 12-color Treg homing panel

As depicted in **Figure 2a**, different transcription factors regulate the differentiation of CD4⁺ T cells into specific T-helper subsets *(Th1, Th2, Th17, Th22)* that express different chemokine receptors and produce different cytokines. Previous studies have shown that the Treg compartment is comprised of subsets referred to as Th-like Tregs that express the same chemokine receptor(s) as their CD4⁺ T-helper cell counterpart(s), thereby enabling migration of the Treg subsets to peripheral tissues and sites of inflammation.[10-13] These Th-like Tregs can be identified within the CD45RA⁻ eTreg population, based on expression of the same homing receptor(s) as their T-helper cell counterpart(s). Similar to the conventional T-helper cell subsets, Th-like Tregs are classified as CD183⁺ Th1-like, CD194⁺CD196⁻ Th2-like and CD194⁺CD196⁺ Th17/Th22-like cells. CD183 (CXCR3) and CD196 (CCR6) can drive the homing of Th-like Tregs to sites of inflammation within peripheral tissues via interactions with their ligand(s) CXCL9, CXCL10, CXCL11[27] and CCL20[28] respectively. Similarly, CD194 (CCR4) mediates the migration of Th2-like Tregs toward its ligands CCL17 and CCL22.[29] The distribution of the Th-like Treg subsets can be altered in certain diseased states, as shown for melanoma and colorectal cancer,[11] thereby highlighting the potential diagnostic and prognostic value of monitoring these subsets.

In our study, Th-like Tregs were identified using a gating strategy described previously.[10] The 12-color homing panel successfully delineated the different Th-like Treg subsets *(Th1-like, Th2-like and Th17/Th22-like)* (***Figure 2b**, upper panel)*, based on their surface expression of the different chemokine receptors. Using the same gating strategy, the 12-color homing panel also enabled identification of the Th1, Th2 and Th17/22 subsets derived from the conventional CD4⁺ T-helper cells (Tconv cells) *(**Figure 2b**, lower panel)*.

As shown in **Figure 2a**, Tregs may be thymic-derived (RTE Tregs) or peripherally induced (pTregs).[1] CD31 on naïve T cells enables the distinction of RTE cells from peripherally expanded naïve T cells.[31] Similarly, RTE Tregs can also be identified based on the surface expression of CD31, as shown in **Figure 2b**, upper panel. Overall, the 12-color homing panel was able to resolve all the populations of interest, including the Th1-, Th2-, Th17/Th22-like Tregs as well as RTE Tregs.

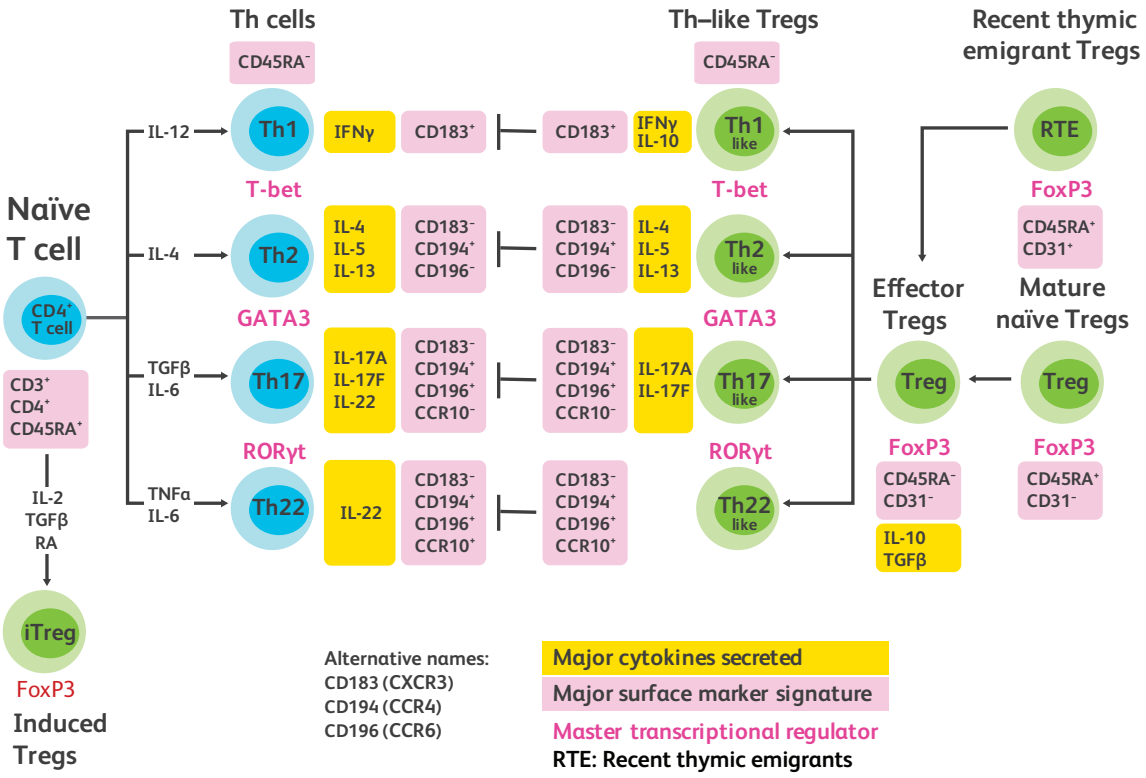


Figure 2a. Evaluation of the modular 12-color Treg homing panel

Parallel T-cell and Treg differentiation paths, and signature markers as identified by recent research and detailed in legend *(bottom center)*. Tregs share similar differential expression of chemokine receptors as their conventional T-helper cell counterparts, which helps these cells to colocalize to peripheral tissues and sites of inflammation. They also share similarities in transcription factor expression and cytokine secretion profiles. Tregs can be developmentally originated from the thymus or peripherally as induced Tregs *(pTreg)*. RTE Tregs can be identified based on CD31 expression. Effector Tregs *(CD45RA⁻ Treg cells)* show high level of heterogeneity in terms of phenotypic diversity of surface marker expression.

Note: Figure adapted from references 10, 11, 12 and 30.

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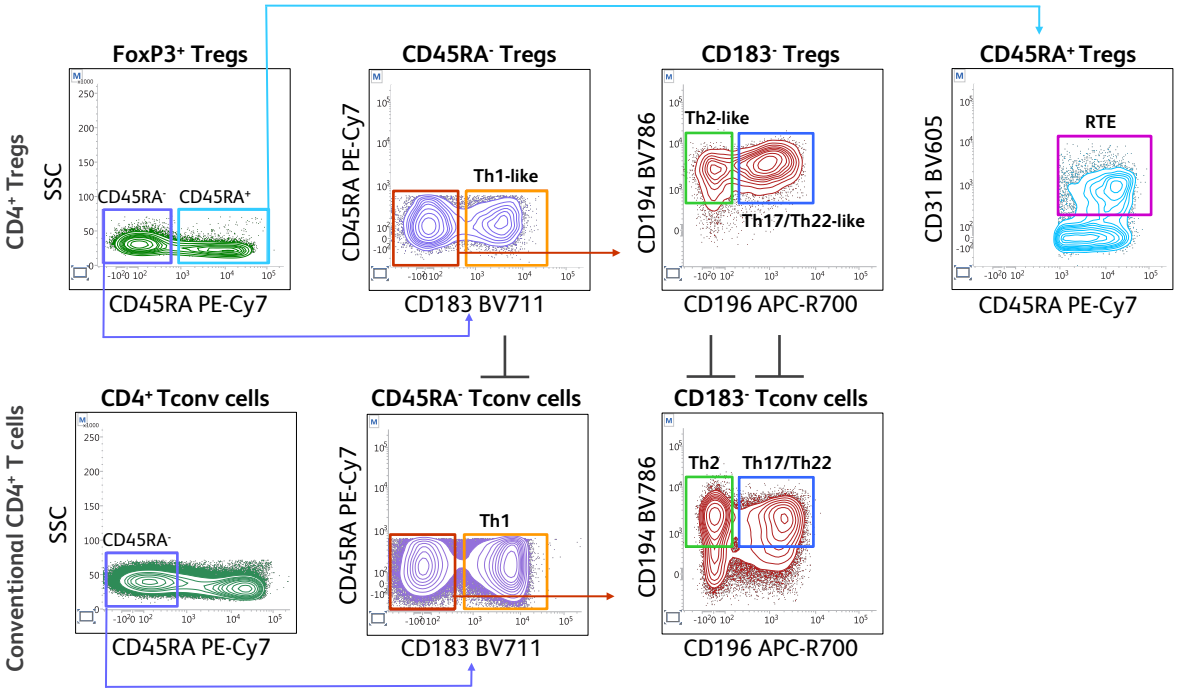

Figure 2b. The homing panel successfully identified three subpopulations of Th-like Tregs and RTEs within the Treg population *(upper panel)*. Conventional T-helper cell subsets Th1, Th2 and Th17/Th22 were also identified within the conventional CD4+ T cells *(Tconv cells, lower panel)*.

Evaluation of the modular 12-color Treg function panel

Given the well-documented heterogeneity of Treg phenotype and function, several studies have focused on the identification of markers that enable characterization of Treg functional dynamics. PI16 is one such marker, and immunosuppressive PI16+ Tregs show enhanced migration towards inflammatory chemokines.[17] CD147 is highly expressed on Tregs, and has been shown to be upregulated in CD45RA− *(effector)* cells as compared to CD45RA+ *(naïve)* Treg subsets and defines a highly suppressive population of cells.[18] Human lymphocyte antigen-antigen D related (HLA-DR) expression on Tregs is associated with contact-dependent immunosuppression, and higher levels of HLA-DR are directly correlated to immunosuppressive activity.[19,32] CD39 is an ectoenzyme expressed on Tregs, which in conjunction with CD73 mediates the hydrolysis of ATP into adenosine, a potent inhibitor of effector T-cell proliferation.[20] These four markers were therefore selected as drop-ins for the design of a 12-color Treg function panel based on their reported correlation with T-cell immunosuppression, and their expression was simultaneously investigated within the different Treg subsets and among different donor samples.

As shown in **Figure 3a**, the different subsets of FoxP3+ Tregs, including naïve *(nTreg; brown-colored gate)*, nonsuppressive *(nsTreg; blue-colored gate)* and effector suppressive *(esTreg; pink-colored gate)* subsets were analyzed for the expression of FoxP3 and CD161 from the 8-color backbone panel and the four Treg function markers from the drop-in panel *(CD147, HLA-DR, CD39 and PI16)*. As seen in the figure, histograms from a representative donor sample showed a direct correlation between the expression of CD15s in the esTregs and high levels of HLA-DR, PI-16, CD39, CD147 and FoxP3 expression. No correlation between CD15s and CD161 was observed, thus suggesting these markers identify two distinct subsets of functionally immunosuppressive Tregs. No expression of the drop-in function markers was observed in the nTreg cells, whereas intermediate expression was detected in nsTreg cells. The box bars indicate the average level of expression for each marker measured in three donors. This data recapitulates previous observations from mass cytometry studies[21-23] and more importantly, expands our knowledge of the interplay among different markers associated with Treg function.

The contour plots in **Figure 3b** highlight donor-specific differences in the expression of the Treg function markers *(CD15s, CD39 and HLA-DR)*. These donor-specific differences are quantitated in **Table 2**.

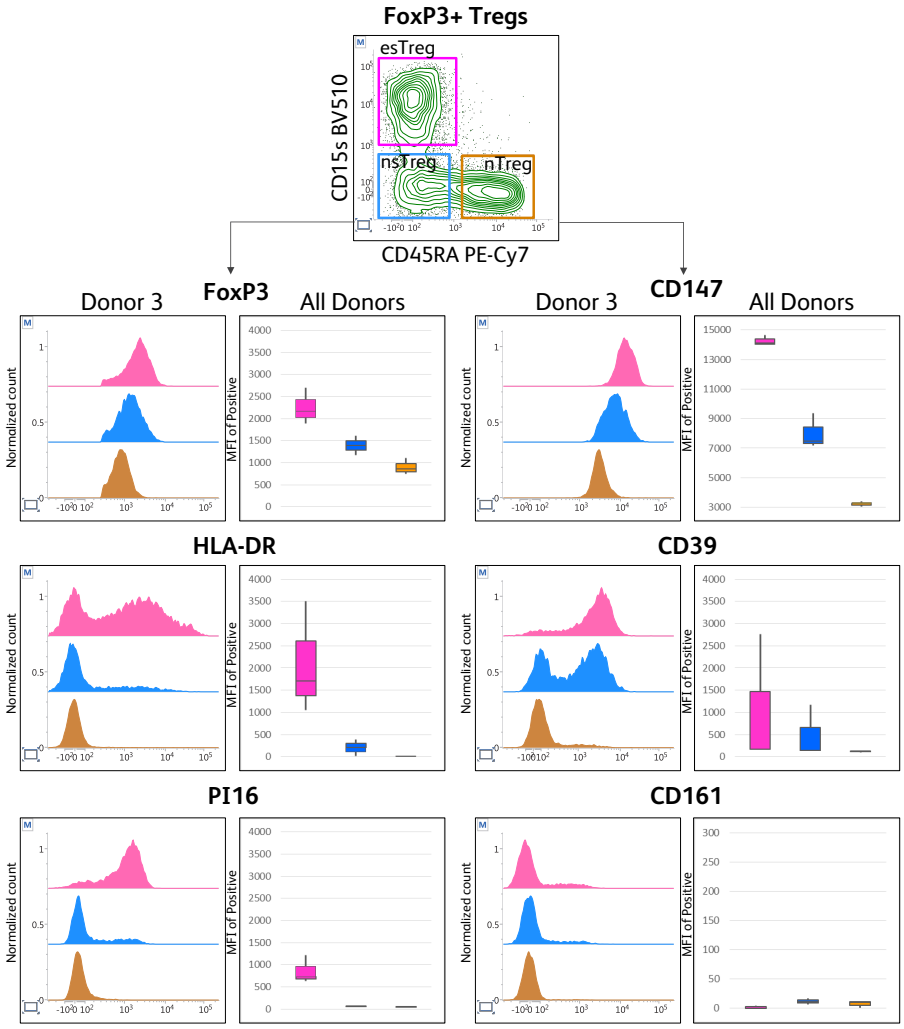

Figure 3a. Evaluation of modular 12-color Treg function panel
$CD3^+CD4^+$ $CD127^{low/-}CD25^{high}FoxP3^+$ Tregs were gated as naïve Treg *(nTreg, brown)*, nonsuppressive Treg *(nsTreg, blue)* and effector suppressive Treg *(esTreg, pink)* subsets based on expression of CD45RA and CD15s. The subsets were analyzed for expression of FoxP3, CD161, CD147, HLA-DR, CD39 and PI16 using three donor samples.

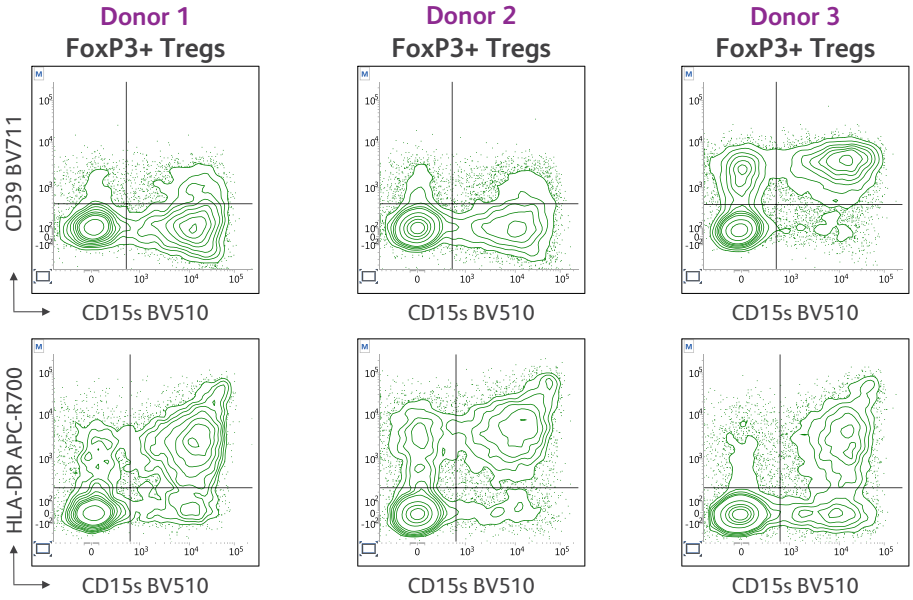

Figure 3b. Donor-specific differences in the expression of Treg function markers *(CD15s, CD39 and HLA-DR)*.

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Population		Percent parent		
		Donor 1	**Donor 2**	**Donor 3**
$CD39^+$	$CD15s^-$	5.1	5.5	19.6
	$CD15s^+$	12.7	8.5	35.2
$CD39^-$	$CD15s^-$	37.0	55.9	39.6
	$CD15s^+$	45.1	30.1	5.7
$HLA\text{-}DR^+$	$CD15s^-$	12.1	15.5	6.2
	$CD15s^+$	47.0	32.1	26.6
$HLA\text{-}DR^-$	$CD15s^-$	31.0	46.6	53.6
	$CD15s^+$	9.9	5.8	13.7

Table 2. Quantitation of donor-specific differences in the expression of Treg function markers.

High-dimensional analysis of Treg function panel

Conventional analysis of multicolor flow cytometry data relies on manual gating and event representation as a series of bivariate plots. This process is subject to bias and may result in variability and lack of reproducibility. Additionally, for panels with multiple markers *(n)*, this can be a time-consuming process as it would require analysis of n*(n-1)/2 bivariate plots to account for all the possible marker combinations in a multicolor experiment. Newer computational methods based on sound mathematical principles *[such as t-distributed stochastic neighbor embedding (tSNE) and Barnes-Hut implementation of tSNE (bhSNE)]* offer unbiased ways to facilitate the simultaneous analysis of multiple markers.

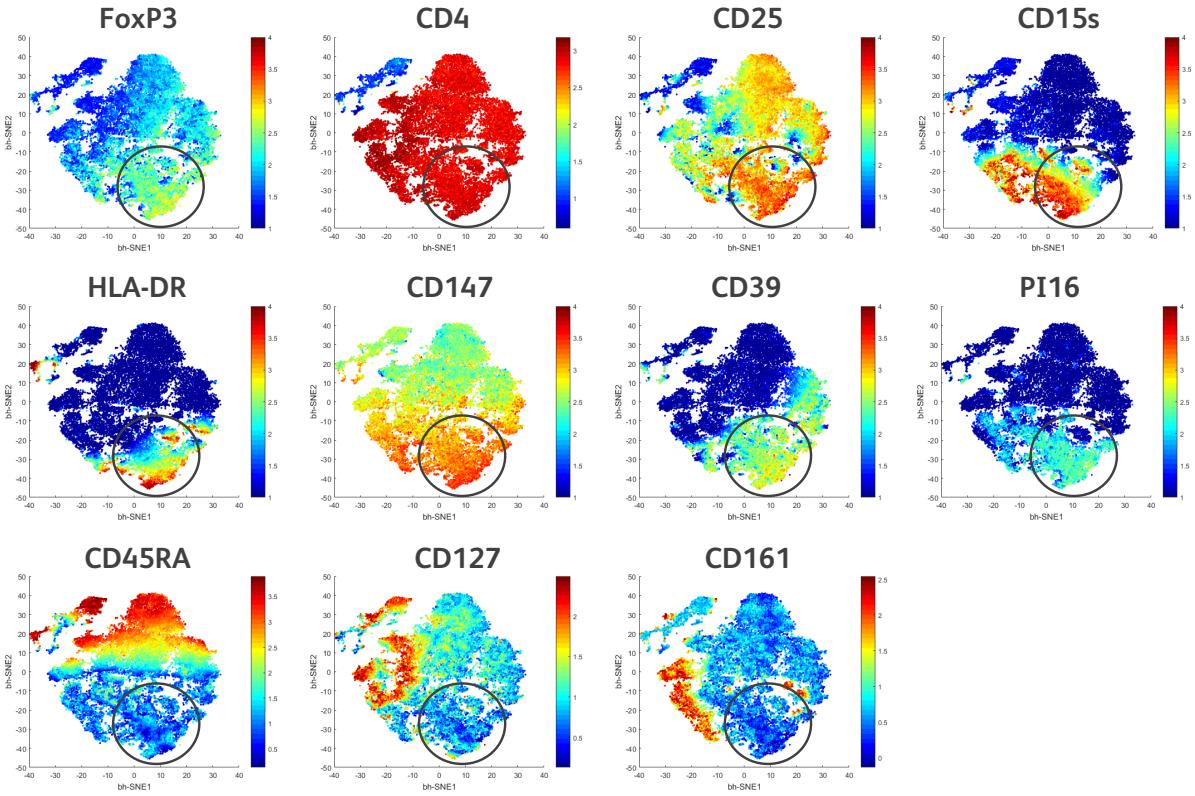

Figure 4. High-dimensional analysis of the Treg function panel markers for one donor.

Gated FoxP3⁺ cells from the 12-color Treg function panel were subjected to high-dimensional analysis using the bhSNE method. Heat maps show relative expression levels of each marker analyzed. This approach allows a simple visual identification of clusters of cells defined by similar phenotype. For example, the cluster inside the black circle represents a subset of Tregs expressing high levels of CD4, CD25, CD15s, HLA-DR, CD147, CD39 and low levels of CD45RA, CD127 and CD161. This population is likely equivalent to the effector suppressive population defined in **Figure 2a**.

Figure 4 shows a bhSNE projection of FoxP3⁺ cells from a single donor *(donor 3)*. Analysis was performed on total FoxP3⁺ cells, to minimize possible bias during manual gating. Although FoxP3 is predominantly expressed by Tregs *(CD3⁺CD4⁺CD127$^{low/-}$CD25^{high})*, FoxP3 expression in rare populations of CD4⁺CD25⁻ cells and CD8⁺CD25⁺ cells has also been reported in both humans and mice.[33-35] bhSNE mapping of the FoxP3⁺ gated cells generated a two-dimensional plot in which each cell is depicted as an individual data point and each cell is colored based on the expression level of the marker being analyzed *(blue color indicates low level of expression; red color indicates high level of expression)*. Cells with similar marker-expression patterns cluster together in the bhSNE analysis. As shown in **Figure 4**, a cluster of cells expressing relatively high levels of FoxP3 can be identified within the total FoxP3⁺ cell population *(denoted by the black circles in **Figure 4**)*. The immunophenotype of this cell cluster can be determined by assessing the expression levels of all the other markers present in the panel. The data in **Figure 4** shows that cells in this cluster express high levels of CD4, CD25, CD15s, HLA-DR, CD147 and CD39 and relatively low levels of CD45RA, CD127 and CD161. This cell cluster most likely corresponds to the esTreg subset as defined in **Figure 2a**. Interestingly, a small subset of CD4⁻FoxP3⁺ cells was also identified, and more detailed phenotypic and functional studies would be required to determine the identity and biological role of these cells. Overall, our results demonstrate the utility of a dimensionality-reduction approach in facilitating the analysis of multiparametric data and in enabling the visual identification of cell clusters based on specific expression pattern(s) of marker(s).

Discussion

We developed a 12-color modular panel on the BD FACSLyric flow cytometer consisting of an 8-color backbone and two 4-color drop-in panels. The modular panel-design approach provided an efficient, flexible and standardized solution for complex analysis of cell populations of interest. The 8-color backbone panel enabled high-resolution identification of the Treg compartment and the underlying subpopulations. Supplementation with the 4-color drop-in panels for Treg homing and function markers allowed deeper characterization of Treg subsets, without any significant impact on the resolution of parent population(s). The ability to preserve resolution upon addition of drop-ins allowed us to expand the number of markers to be assessed in a single tube without having to redesign and reoptimize the backbone panel.

Addition of the 4-color homing drop-in panel enabled identification of RTE Tregs within the nTreg population, as well as Th-like subsets within the eTreg population as reported previously.[10,11,24] Addition of the 4-color Treg function drop-in panel enabled identification of a previously reported highly immunosuppressive subpopulation expressing high levels of CD15s, HLA-DR, PI-16, CD147 and CD39 within the CD45RA$^-$ eTreg population.[14-21] Moreover, optimal panel design combined with high instrument sensitivity enabled high resolution and identification of donor-specific variations in the different Treg subsets.

The modular panel-design approach provides flexibility to study any drop-in marker combinations while using the backbone panel as an anchor. Researchers can design their own 4-color drop-in panels to explore other facets of Treg biology, such as proliferation, maturation and/or stability. By combining multiple markers in a single tube, this 12-color modular assay on the BD FACSLyric system can help researchers discover biologically and clinically interesting relationships between existing and new Treg markers amid the underlying heterogeneity of the Treg population. Application of high-dimensional data analysis techniques facilitates a deeper and more comprehensive analysis of Treg biology, which may prove to be useful in designing improved and individualized cell-based therapies.

The BD FACSLyric flow cytometer offers high sensitivity and enables high-resolution identification of cell populations. With up to 12 different colors, it is an ideal solution for expanded immunophenotyping of clinically relevant cell populations.

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