Design and Validation of a Flow Cytometry Panel to Monitor Circulating Treg Frequencies as a PD Biomarker in Oncology or Autoimmune Disease

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1. INTRODUCTION

Key considerations when selecting an appropriate Pharmacodynamic (PD) Biomarker are accessibility of material and sensitivity of detection and quantification. The answers to these questions will dictate the design and validation of an appropriate detection method. This can be exemplified in two settings of colorectal cancer (CRC) and multiple sclerosis (MS); where regulatory T Cells (Tregs) are believed to play important roles in disease progression. While FoxP3 staining in tissue biopsies is a useful tool to determine Treg distribution and quantification, these invasive procedures are usually restricted for CRC and not an option for MS. However, circulating Treg levels have been reported to increase (CRC) or decrease (MS) according to disease progression. An accurate and robust flow cytometry panel can document altered cell frequencies in peripheral blood. This allows monitoring of disease progression in patients and their response to treatment. We describe a validated and robust flow cytometry panel to precisely monitor changes in Treg frequencies as a PD biomarker to complement immunohistochemistry methods.

2. METHODS

Immunofluorescence Microscopy:

Formalin fixed paraffin embedded (FFPE) tissue sections from healthy colon (n=2) and CRC colon (n=2) specimens were stained for using CD4 and FoxP3 fluorescent monoclonal antibodies (Fig.1). Samples were imaged using Vectra Polaris slide scanner and snapshots captured using Phenochart software to provide qualitative comparison of the expression of both markers in healthy colon and colorectal tumour tissue.

Flow Cytometry Analysis:

Blood from healthy human volunteers (HV) (n=6), MS (n=2) and CRC (n=2) patients was collected in K2EDTA blood collection tubes. Aliquots of blood from each sample were fixed and stained for surface markers as outlined in Table 1. Cells were then permeabilisation and stained for intracellular markers prior to being analysed on the Attune NTx. Samples were gated using the strategy highlighted in Fig. 2. Cell frequencies for each population of interest were calculated for HV and each disease indication as presented in Table 2.

Assessment of Flow Cytometry Assay Precision and Robustness:

To determine assay precision the following validation parameters were assessed and precision calculated as %CV in the HV sample cohort, data is presented in Table 3:

- Intra-assay precision, calculated from 3 technical replicates from each biological replicate across 1 analytical run
- Inter-assay precision, calculated from 3 technical replicates from each biological replicate across 3 analytical runs
- Acceptance criteria %CV ≤20

Clinical Material:

All samples were obtained through commercial sources subject to appropriate ethical review in the country of origin.

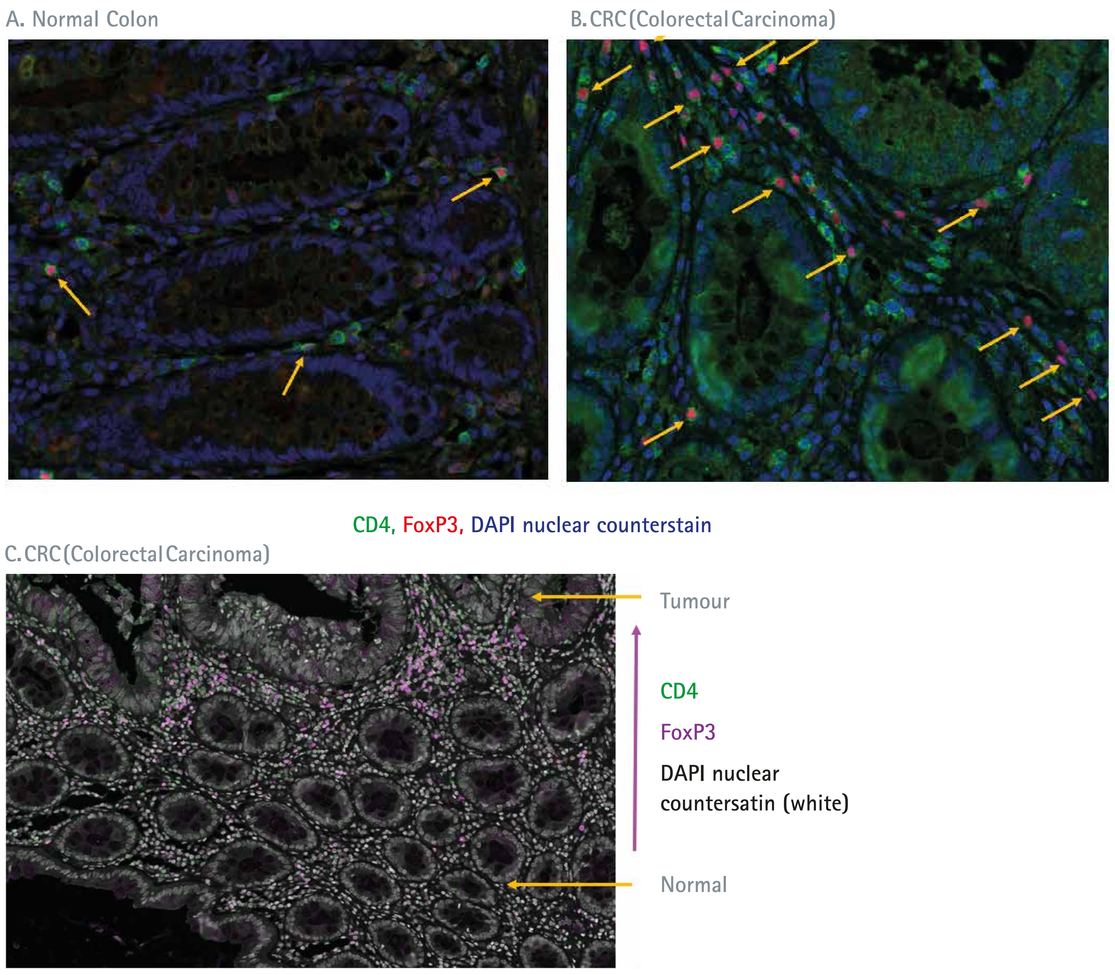

Figure 1. Immunofluorescent FoxP3 and CD4 staining of FFPE colon in normal and CRC tissue.
Immunofluorescent staining of FoxP3 and CD4 in sections of normal healthy colon **(A)** verses CRC patient colon **(B and C)**. FoxP3 staining appears more pronounced in the CRC patient over normal tissue. Image C represents the CRC patient captured at a lesser magnitude to illustrate Treg distribution at the tumour site.

Marker of Interest	FoxP3	CD45RA	CD45	CD25	CD127	CD4	CD3
Fluorophore	AF488	PE	PercP-Cy5.5	APC	BV421	BV510	BV605
Clone	259D	HI100	HI30	BC96	A019D5	SK3	SK7
Supplier	BioLegend	BioLegend	BioLegend	BioLegend	BioLegend	BioLegend	BioLegend

Table 1. Flow Cytometry Antibody Panel.
Samples were prepared for analysis using the antibody panel described below. Red blood cells were lysed using FACS Lyse solution (BD Biosciences) and leukocytes were fixed and permeabilised using FoxP3 / Transcription Factor Staining Buffer Set (ThermoFisher Scientific).

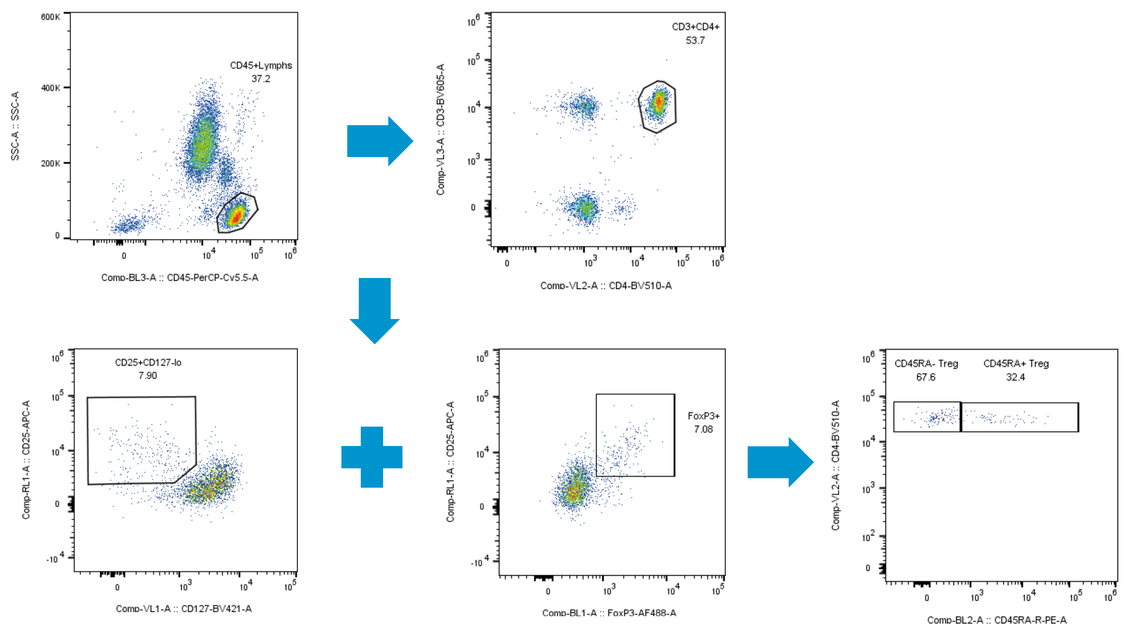

Figure 2. Flow Cytometry Gating Strategy.
The gating strategy described herein was applied to identify Tregs as $CD4^+CD25^+CD127^{-/lo}FoxP3^+$. Firstly, lymphocytes were identified and gated as $CD45^+SSClo$. From the Lymphocyte population, the $CD3^+CD4^+$ population was identified. From the $CD3^+CD4^+$ population, the $CD25^+CD127^{-/lo}$ and $CD25^+FoxP3^+$ populations were identified and gated, using Boolean gating, both gates were combined to define Tregs as $CD3^+CD4^+CD25^+CD127^-/loFoxP3^+$. The Treg population was stratified further as Effector ($CD45RA^-$) or Naïve ($CD45RA^+$) Tregs.

Analyte	Sample	Frequency	Mean NHV
$CD3^+CD4^+$ (%CD3)	MS 1	50.1	47.94
	MS 2	50.5	
	CRC 1	56.5	
	CRC 2	38.4	
$CD3^+CD4^+CD25^+CD127^{-/lo}FoxP3^+$ (%CD4)	MS 1	1.85	6.33
	MS 2	3.4	
	CRC 1	10.8	
	CRC 2	5.24	
$CD45RA^+$ (%Treg)	MS 1	12.2	37.73
	MS 2	44.1	
	CRC 1	29.7	
	CRC 2	23	
$CD45RA^-$ (%Treg)	MS 1	24.4	50.44
	MS 2	55.9	
	CRC 1	57.8	
	CRC 2	57.6	

Table 2. Comparison of cell frequencies between HV and diseased patients.
Frequencies of each population of interest were calculated in HV, MS and CRC patients, and variation between sample types compared.

Reportable Analyte	Intra-assay Precision		Inter-assay Precision	
	Overall %CV	Number of Replicates meets precision	Overall %CV	Number of Replicates meets precision
$CD3^+CD4^+$ (%CD3)	1.8	6	2.1	6
$CD3^+CD4^+CD25^+CD127^-/loFoxP3^+$ (%CD4)	7.8	6	10.1	6
$CD45RA^+$ (%Treg)	19.4	4	15.9	4
$CD45RA^+$ (%Treg)	14	5	16.4	4

Table 3. Precision of reported analytes.
Intra and Inter assay run precision were determined from 3 technical replicates for each HV donor (n=6). For each reportable analyte, assay precision was calculated as the %CV for each individual donor and overall precision, and values are displayed in the following table. Precision was considered acceptable where the %CV was ≤20% for the majority of biological replicates and overall precision.

3. RESULTS

FoxP3 and CD4 staining of colon tissue from CRC patients was compared to healthy colon tissue by immunofluorescent microscopy. An increase in FoxP3 staining in the CRC colon tissue was observed in comparison to healthy tissue (Fig.1), suggesting an increase in Treg frequency within the tumour microenvironment (TME). The increase in FoxP3 staining in diseased tissue compared to healthy tissue, suggests that detection of FoxP3 by immunofluorescent microscopy may be a useful biomarker for monitoring disease progression, or immunological changes within the TME, and could prove to be valuable when evaluating patient response to drug.

However, removal of patient tissue, or tumour biopsy, is an invasive procedure and sampling intervals are likely to be limited to screen and endpoint analysis. Additionally, in the case of MS, where Tregs are also thought to play a role in disease progression, tissue sampling is not an option. In such cases, measuring frequencies of circulating Tregs may be a suitable alternative to monitor disease progression, as suggested by Yu-Feng et al. (2019) and Betts et al. (2011).

Concept Life Sciences have successfully developed a flow cytometry panel which can reliably and precisely detect circulating Tregs defined as CD4+CD25+CD127-/loFoxP3+, as shown in Table 1 and Fig.2. Assay precision, calculated for each population of interest, met acceptance criteria for both intra and inter-assay precision as detailed in Table 3. Additionally, we have shown our ability to measure a broad range of Treg frequencies within various sample types such as MS and CRC, which are expected to return lower and higher values respectively compared to the healthy population. This suggests the assay is sensitive enough to detect fluctuations within in peripheral Treg populations. Quantifying differences within the circulating Treg population from the same patient overtime by flow cytometry may translate as a useful Pharmacodynamic biomarker allowing investigators to monitor changes in patient Treg populations in response to treatment.

REFERENCES

1. Yu-Feng, Sheng-Xiao, Xiao-Wen et al.; Multiple Sclerosis and Related Disorders 28 (2019) 75–80
2. Betts, Jones, Junaid et al.; Gut 61 (2012) 1163-1171