Simultaneous Characterization and Quantification of Immune Cell Subpopulations and PD-L1 Expressing CTCs in Peripheral Blood of Cancer Patients

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Background

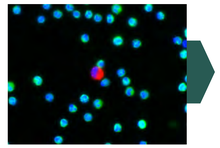
- Expression of PD-L1 on tumor, and immune markers in tumor tissue, are associated with improved response to PD-1 and PD-L1 checkpoint inhibitors. However, each alone has limited predictive utility.
- Multimodal characterization of both the tumor and host immune system is an unmet medical need for the improved prediction of response to immunotherapy.
- Metastatic lesions are likely to be under-sampled and require a liquid biopsy, given tumor heterogeneity and evolution and temporal changes in the host immune system.
- We sought to examine expression of PD-L1 on CTCs as well as characterize rare immune cell populations with a non-invasive liquid biopsy. Examining dynamic biomarker changes in longitudinal samples could enable the development of novel diagnostic tools for response prediction and pharmacodynamics studies related to immunotherapy.

Methods

Blood samples were drawn from 3 healthy donors, 5 non-cancerous lung disease patients, and 4 metastatic cancer patients (prior to and on-therapy with checkpoint inhibitor), and sent to Epic Sciences for processing with Epic Sciences' CTC and immune cell assays.

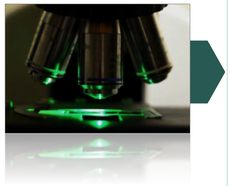
1) SLIDE PREPARATION

2) CELL STAINING

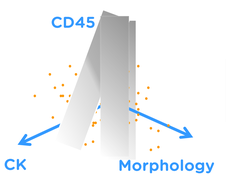


CK, CD45, DAPI, PD-L1, etc.

3) SCANNING



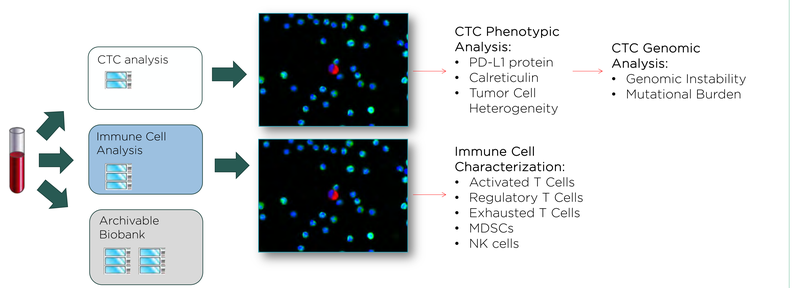
4) SINGLE CELL DIGITAL PATHOLOGY



Nuclear Features	Cytoplasmic Features	Cell Features
Nuclear Area	Cytoplasmic Area	AR Expression
Nuc. Convex Area	Cyto Convex Area	CK Expression
Nuc Major Axis	Cyto Major Axis	N/C Ratio
Nuclear Minor Axis	Cyto Minor Axis	
Nuclear Circularity	Cyto Circularity	
Nuclear Solidity	Cyto Solidity	
Nuclear Entropy	Cyto Entropy	
Nuclear Speckling	Cyto Speckling	
Nucleoli Presence	Cyto Presence	

Schematic of Epic's CTC platform: CTC enumeration, leukocyte classification, morphology and protein analyses

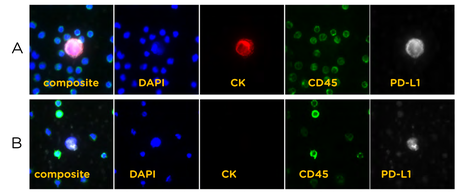
1) Nucleated cells from a 10 mL blood draw are plated onto 10-12 slides (approximately 3 million cells/slide)
2) Slides are stained with 4',6-diamidino-2-phenylindole (DAPI) and a combination of one or more additional markers, including cytokeratin (CK), CD45, PD-L1, CD3, CD4, CD8, CD14, Ki-67, PD-1, Lag-3, and Tim-3
3) Slides are scanned using a rapid fluorescent scanning method, which images each nucleated cell
4) All cells are analyzed with a multi-parametric digital pathology algorithm for morphological features and protein expression

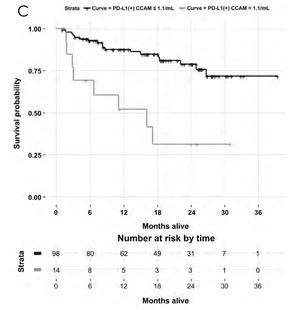


Schematic of Epic Single Cell Tools for Immune Monitoring

1) A single blood draw is used to make slides for CTC and immune cell analysis. Extra slides are archived for staining at a later date
2) Slides can be used for CTC and immune cell characterization as well as downstream genomic analysis

PD-L1(+) Peripheral Circulating Cells in Lung Cancer

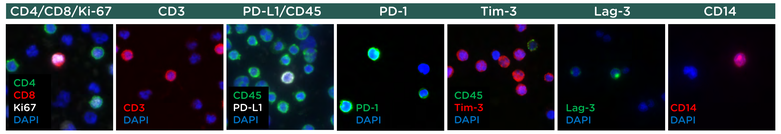




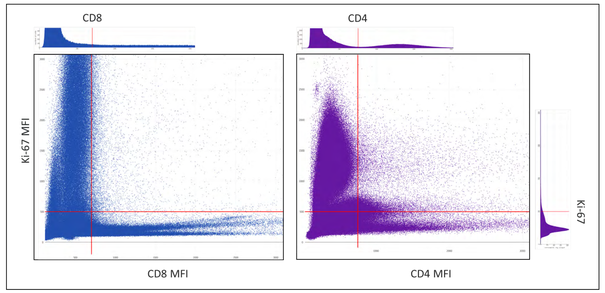
PD-L1 expression is detectable in two distinct cell populations in the peripheral blood of lung cancer patients and is associated with worse survival[1]. PD-L1 expression was detected within peripheral circulating cells associated with malignancy (CCAM) in 26/112(23%) non-small cell lung cancer patients. Two distinct populations of nucleated, non-hematolymphoid, PD-L1 expressing cells were identified; **A)** cytokeratin positive (CK+, PD-L1+, CD45-) and **B)** cytokeratin negative (CK-, PD-L1+, CD45-). PD-L1+ CCAMs are prognostic for overall survival in lung cancer patients. **C)** Kaplan-Meier estimates of overall survival of patients stratified by those with >1.1 PD-L1(+) CCAM (gray line) or ≤1.1 PD-L1(+) CCAM (black line).

[1]Boffa D. J. Cellular Expression of PD-L1 in the Peripheral Blood of Lung Cancer Patients is Associated with Worse Survival. *Cancer Epidemiology, Biomarkers & Prevention*. 2017; 26(7):1139-1145.

Tools to Characterize Rare Immune Cell Populations

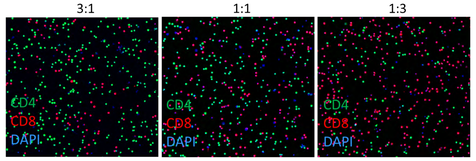


Representative images of immune cell staining in control cell lines and healthy donor or patient leukocytes. Cells were plated and stained with DAPI and one or more immune cell markers including CD4, CD8, Ki-67, CD3, PD-L1, CD45, PD-1, Tim-3, Lag-3, and CD14.



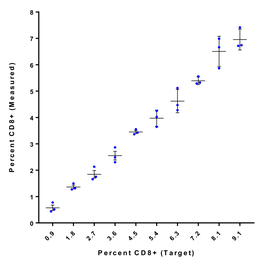
Representative scatter plots demonstrating CD4, CD8, and Ki-67 threshold determination in a healthy donor sample. Healthy donor leukocytes were plated and stained with CD4, CD8, Ki-67, and DAPI. Mean fluorescent intensity (MFI) was plotted for approximately 3 million cells to generate histograms and determine positivity thresholds for each marker, and to quantify $CD4^+$ and $CD8^+$ cell counts. Thresholds were applied to scatter plots for quantification of double positive values for $CD4^+Ki67^+$ and $CD8^+Ki-67^+$.

Cell Ratios (CD4:CD8)



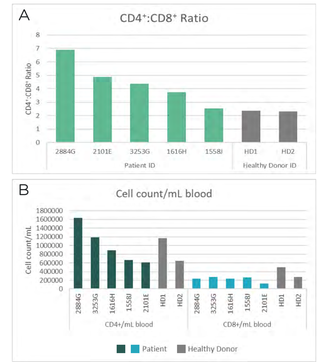
	3:1	1:1	1:3
% of CD4 Cells	70%	49%	23%
% of CD8 Cells	23%	44%	72%

Detecting large scale immune cell changes with the CD4, CD8 Assay. Contrived samples were prepared with immunomagnetically purified CD4 and CD8 cells plated in ratios of approximately 3:1, 1:1, and 1:3. Table shows measured cell counts as a percentage of total cells (approximately 1 million total cells per slide).

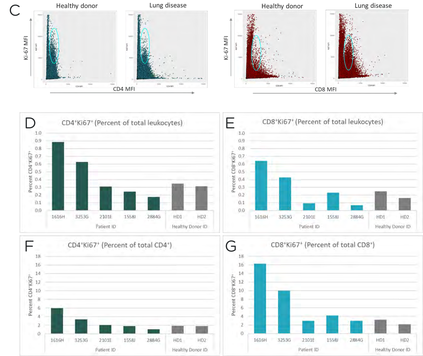


Detecting small scale immune cell changes: quantitation and linearity of the CD8 assay. $CD8^+$ cells were spiked into healthy donor leukocytes at target ratios of approximately 1 - 9%. After staining and quantification of $CD8^+$ cells, percentages of detected spiked $CD8^+$ cells for three technical replicates (Percent $CD8^+$ Measured) were plotted against target percentages (Percent CD8+ Target). Bars show mean and standard error of the mean.

CD4/CD8/Ki-67 in Lung Disease Patients

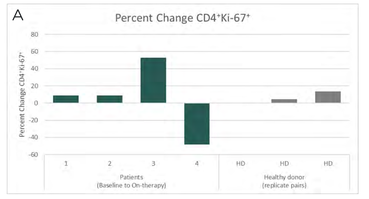


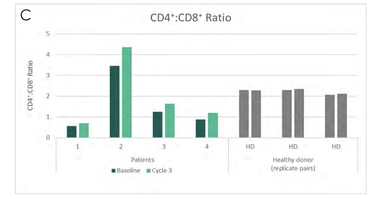
Quantification of CD4 and CD8 positive populations. Leukocytes from five lung disease patients and two healthy donors were plated and stained with CD4, CD8, Ki-67, and DAPI. $CD4^+$:$CD8^+$ ratios **(A)** and cell counts per mL of blood **(B)** were quantified.



Quantification of double positive populations. Scatter plots comparing $CD4^+Ki-67^+$ and $CD8^+Ki-67^+$ double positive populations in healthy donor and lung disease patient samples **(C)**. $CD4^+Ki-67^+$ **(D)** and $CD8^+Ki-67^+$ **(E)** leukocytes were quantified in healthy donor and patient samples and graphed as a percentage of total leukocytes. $CD4^+Ki-67^+$ and $CD8^+Ki-67^+$ double positive values were also graphed as a percentage of total $CD4^+$ **(F)** or total $CD8^+$ **(G)** leukocytes, respectively.

Changes in CD4/CD8/Ki-67 with PD-L1 inhibitor Tx





Changes in CD4, CD8, and Ki-67 positive populations were detected in metastatic cancer patient samples upon treatment with PD-L1 inhibitor. Baseline and cycle 3 samples were collected for four metastatic cancer patients undergoing PD-L1 inhibitor therapy. Percent change was calculated for $CD4^+Ki-67^+$ and $CD8^+Ki-67^+$ populations between baseline and cycle 3 samples **(A and B)**. $CD4^+Ki-67^+$ and $CD8^+Ki-67^+$ percentages were quantified per total $CD4^+$ and $CD8^+$ cells, respectively. Six replicates from a single healthy donor were stained along with patient samples to serve as controls. For percent change analyses, replicates were analyzed as pairs to mimic baseline and on-therapy samples. $CD4^+$:$CD8^+$ ratios were determined for patients and healthy donor samples **(C)**. Percent change between baseline and on-therapy was calculated for patient samples and healthy donor replicates were analyzed as pairs **(D)**.

Conclusions

- Multimodal characterization of both the tumor and host immune system is an unmet medical need for the improved prediction of response to immunotherapy.
- Detection of PD-L1 in circulating cells from NSCLC liquid biopsy samples was associated with worse overall survival, highlighting the potential utility of minimally invasive liquid biopsies for tumor profiling.
- Epic Sciences has developed tools to simultaneously detect, characterize, and quantify CTCs and rare immune cell subpopulations from a single blood draw.
- Examining dynamic biomarker changes with minimally invasive liquid biopsies in longitudinal samples could enable the development of novel diagnostic tools for response prediction and pharmacodynamics studies related to immunotherapy.