Quantification of Rare PD-L1 and Other Immunology Biomarker Expressing Leukocytes and CTCs in Peripheral Blood of Cancer Patients

Adam Jendrisak, Nadia Ebrahim, Angel Rodriguez, Rachel Krupa, David Lu, Mahipal Suraneni, Jiyun Byun, Ryon Graf, Yipeng Wang, Mark Landers, Ryan Dittamore

Epic Sciences, Inc., San Diego, California

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Background

- Expression of PD-L1 on tumor tissue and immune cell markers (CD3, CD4, CD8, etc.) on tumor infiltrating lymphocytes (TILs) are often associated with improved response to PD-1 and PD-L1 checkpoint inhibitors. However, PD-L1 diagnostic tests suffer from high co-morbidities and from significant false positives and false negatives.
- Expression of PD-1 and Tim-3 on TILs is associated with immune exhaustion.
- Utilizing a non-invasive liquid biopsy, we sought to examine the expression of checkpoint markers (PD-L1, Tim-3) and immune cell markers (CD3, CD8) on circulating tumor cells (CTCs) and leukocyte cell populations for the purpose of developing an improved predictive and pharmacodynamic biomarker for approved PD-1/PD-L1 checkpoint inhibitors and novel immunotherapy drugs in development.
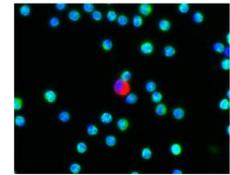
Methods

- Blood samples were drawn from 3 healthy donors, 18 non-cancerous lung disease, 33 lung cancer, and 1 bladder cancer patients and sent to Epic Sciences for processing with Epic Sciences' immunoassays.
- Panels of immunoassays include staining of checkpoint markers (PD-L1, Tim-3) and immune cell markers (CD3, CD8).

1) SLIDE PREPARATION
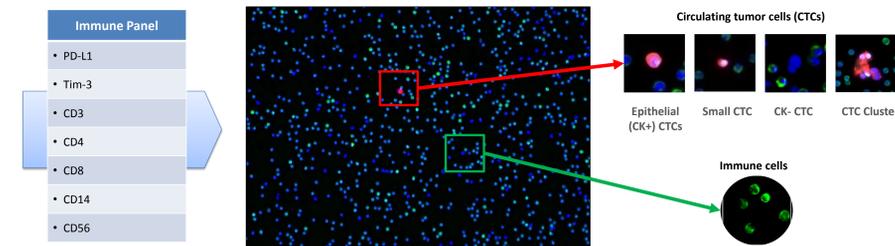2) CELL STAINING
CK, CD45, DAPI, PD-L1, etc.
3) SCANNING
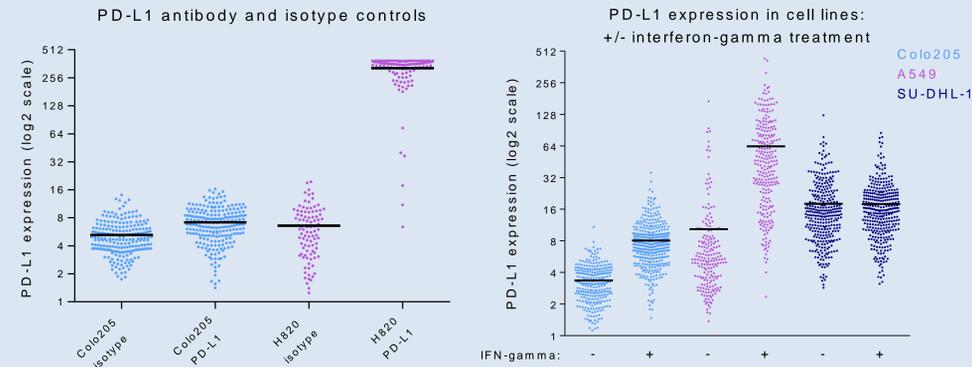
4) SINGLE CELL DIGITAL PATHOLOGY

CD45 — CK — Morphology

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	

5) BIOMARKER ANALYSIS ON CTC AND LEUKOCYTES

Immune Panel
- PD-L1
- Tim-3
- CD3
- CD4
- CD8
- CD14
- CD56

Circulating tumor cells (CTCs)
Epithelial (CK+) CTCs — Small CTC — CK- CTC — CTC Cluster
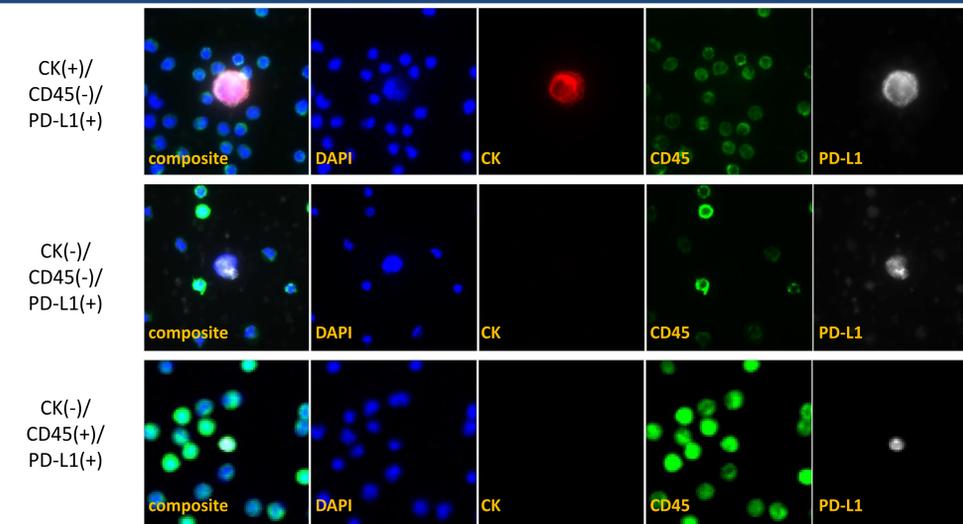
Immune cells

Schematic of Epic CTC platform CTC enumeration, morphology and protein analyses workflow:
1) Nucleated cells from one 10mL blood draw were plated onto 10-12 slides with each slide having ~3 million cells
2) Slides stained with DAPI and a combination of two or three other markers, including cytokeratin (CK), CD3, CD8, CD45, PD-L1 and Tim-3
3) Slides scanned
4) All cells measured by a multi-parametric digital pathology algorithm for morphological features and protein expression
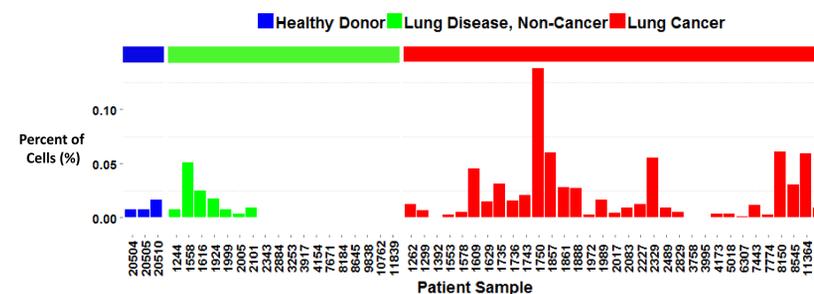5) Biomarker analyses were performed on both CTCs and leukocytes

PD-L1 Protein Assay Development

PD-L1 antibody and isotype controls

PD-L1 expression in cell lines: +/- interferon-gamma treatment
Colo205
A549
SU-DHL-1

Figure 2: Demonstration of PD-L1 assay specificity: (A) PD-L1-specific antibody and species-matched isotype control were tested in negative (Colo205) and high (H820) PD-L1-expressing cell lines. No specific staining was seen in negative control cell lines or with isotype control antibody. **(B)** Interferon (IFN)-gamma treatment increases PD-L1 expression in Colo205 and A549 cell line cells. PD-L1 expression in IFN-gamma-treated SU-DHL-1 cells remain unchanged, likely due to the up-regulation of cytokine signaling suppressor genes in this particular cell line.

PD-L1 Expression in CTC and Leukocytes of Lung Ca

CK(+)/CD45(-)/PD-L1(+)
composite — DAPI — CK — CD45 — PD-L1

CK(-)/CD45(-)/PD-L1(+)
composite — DAPI — CK — CD45 — PD-L1

CK(-)/CD45(+)/PD-L1(+)
composite — DAPI — CK — CD45 — PD-L1

Percentage of PD-L1(+) Leukocytes in 54 Sample Cohort

Healthy Donor — Lung Disease, Non-Cancer — Lung Cancer

Percent of Cells (%)

Patient Sample

	Minimum	Maximum	Median	Mean
Healthy Donor	0.0076%	0.0166%	0.0077%	0.0106%
Lung Disease (non-Cancerous)	0.0000%	0.0510%	0.0000%	0.0067%
Lung Cancer	0.0000%	0.1378%	0.0118%	0.0213%

Tim-3 Expression in Healthy Donor Leukocytes

Healthy Donor Leukocytes
Composite — DAPI — CD45 — Tim-3

Healthy Donor Leukocytes
Composite — DAPI — CD45 — Tim-3
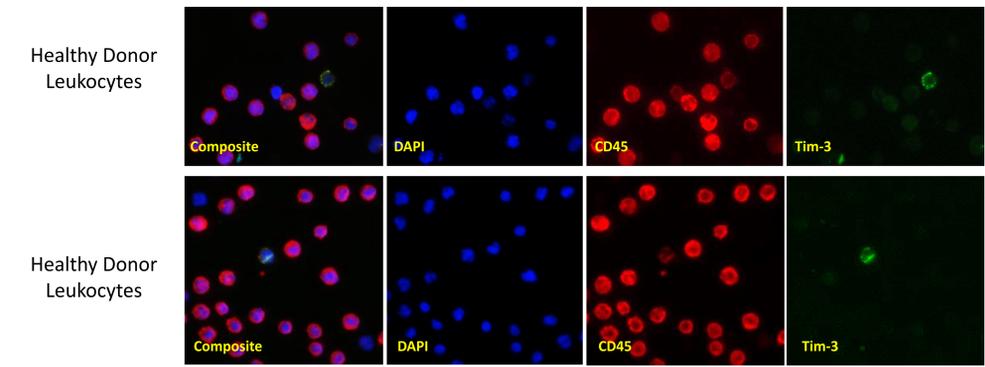
CD3 Expression in Jurkat Cells and Healthy Donor Leukocytes

Jurkat Cells (Positive Control)
Composite — DAPI — CD3

Healthy Donor Leukocytes
Composite — DAPI — CD3
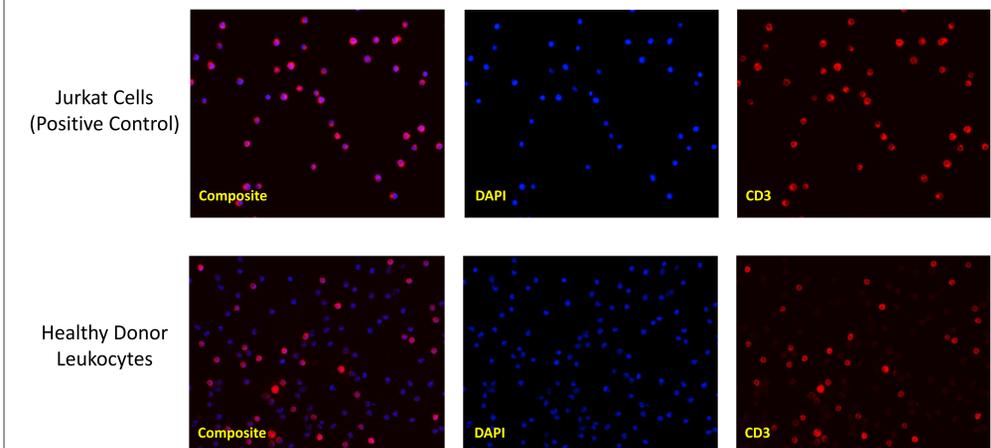
CD8, CD45 and PD-L1 Co-Expression in Bladder Ca Leukocytes

CD8(-)/CD45(-)/PD-L1(+)
Composite — DAPI — CD8 — CD45 — PD-L1

CD8(+)/CD45(+)/PD-L1(-)
Composite — DAPI — CD8 — CD45 — PD-L1
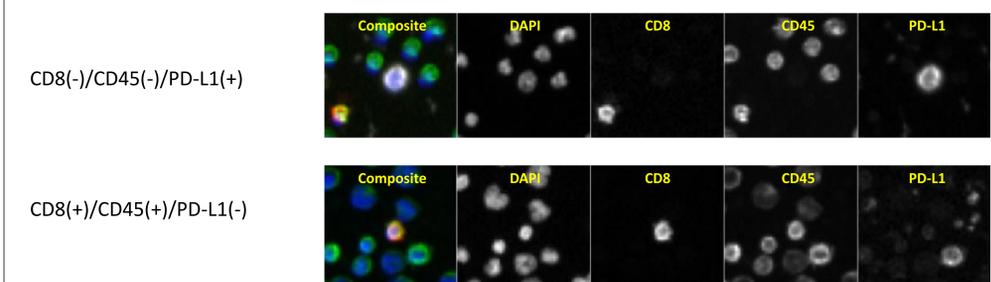
Conclusions

- Epic Sciences' platform has low limit of detection, ability to archive patient blood samples and ability to quantify biomarker expression on both CTCs and leukocytes simultaneously.
- Detection of leukocyte subtypes such as CD3, CD8, CD14 and CD56 cells, will allow us to further characterize PD-L1 and Tim-3 in T-lymphocytes and other immune cell types.
- Development of a liquid biopsy-based platform that is capable of simultaneously measuring immune biomarkers in CTCs as well as leukocytes will allow real time assessment of response to immune checkpoints inhibitors.