ImmunoINTEL™, a Flow Cytometry Based Platform that Identifies and Quantifies the Most Critical Cell Subsets and Related Functional Potential in Dissociated Solid Tumors

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ABSTRACT

Standard immunohistochemistry (IHC) approaches to tumor-infiltrating leukocyte (TIL) phenotyping yield limited information as they utilize antibody (Ab) panels not broad enough to identify and functionally characterize the complexity of TIL subsets. A flow cytometry platform was developed that delivers quantitative, clinically relevant information to support the following: 1) Phenotypic and functional analysis of TILs to characterize the immune status within the tumor microenvironment (TME), and 2) Quantification of TIL and tumor cell (co-)expression profiles of targetable immunomodulatory receptors and ligands (IMR-L).

Standardized flow cytometry set-up, QC and sample processing procedure were established. Automated instrumentation and sample processing procedures were implemented in a barcode-based workflow utilizing Hamilton STARlet automated liquid handlers fully integrated with an SMS/LIMS and multi-dimensional flow cytometry data analysis software. Six Ab panels, ranging from 6-12 colors were designed, optimized, and validated to profile PBMC and TIL subsets.

Phenotypic composition of the following cell subsets in the TME was delineated and quantified using qualified antibody panels for: (1) T, B, and NK cells; (2) T regulatory cells; (3) myeloid cell subsets (classical/nonclassical monocytes, M1/M2-macrophages); (4) myeloid cells with suppressive phenotypes (M- and G-MDSC); (5) myeloid/plasmacytoid DCs; (6) epithelial and mesenchymal cells. The relative quantities of the TIL subsets were tabulated based on tumor type. The ImmunoINTEL™ panels were complemented with drop-in IMR and IMR-L markers (CD279/CD274 [PD1/PD-L1], TIGIT/CD112-CD155, CD366 [TIM-3]/Galectin-9, CD172a [SIRPa]/CD47, CD223 [LAG-3]/HLADR) to profile the functional status of TILs, and to study the cytotoxic potential of tumor TILs. Reliable separation of IMR/IMR-L positive TILs from negative TILs and IMR/IMR-L positive tumor cells from negative tumor cells was observed, with notable heterogeneity, across cell and tumor types. This included the elevated expression of several IMR-Ls on TILs and IMRs on epithelial and stromal cells, suggesting tumor- and TIL-intrinsic mechanisms modulating checkpoint interactions.

Stringent flow cytometry QC processes were developed and implemented to ensure quality and precision of phenotypic and functional analyses of dissociated TILs and tumor cells, capturing the most critical metrics of intra-tumor immune responses and providing quantitative characterization of IMR and/or IMR-L interactions for more refined selection of potential responders to immune checkpoint inhibitors.

INTRODUCTION AND OBJECTIVES

Importance of immune cell landscape within the tumor microenvironment (TME) for development of treatment directing biomarkers

- **Characterization of tumor-infiltrating leukocytes (TILs) in the context of TME is key for targeted immunotherapies. Phenotypic subset analysis of TILs can be correlated with clinical outcomes and used as a treatment directing assay for immunotherapeutics:**
  - Increased CD8+ and CD56+ TIL density is predictive of positive response to I-O treatment.
- **The presence of immune cells alone is insufficient information; the functional status of TILs is critical to predict clinical response:**
  - Are the CD8+ T cells activated or exhausted?
  - Are the CD8+ T cells being suppressed within the TME by Tregs, M2 Mϕ, and MDSCs?
  - What are the immuno-modulatory receptor/ligand (IMR/L), cytokine signatures and profiles of TILs?

Pierian Biosciences's ImmunoINTEL™ platform provides critical information on immune cell functional status within the TME:

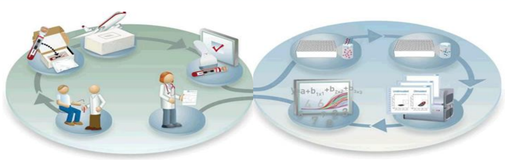
(1) Assessment of tumor-infiltrating NK, T cells (T regs, CD8+ cytotoxic T cells, exhausted T cells), MDSC, and various subsets of DCs and Mϕ in tumor parenchyma;
(2) Evaluation of the proportion of IMR/L-positive immune and tumor cells;
(3) Analysis of activation/ exhaustion markers, cytokine, and granzyme expression.

METHODS AND WORKFLOW

The Pierian Platform: ImmunoINTEL™, a flow cytometry based platform that delivers clinically relevant information on immune cell functional status within the TME

- **Automation:**
  - State-of-the-art HTP Attune NxT 16 parameter Flow Cytometers with Autosampler for rapid automated processing of samples;
  - Fully automated liquid handling workstations from Hamilton Robotics;
  - Web-based proprietary and customizable software for batch sample/plate processing and analysis.
- **Scalable laboratory infrastructure** built to support GLP governed work, clinical trials, and clinical diagnostics that is fully integrated into an SMS/LIMS system and workflow.
- **Highly standardized procedures and tools** (QC'd and validated/qualified GXP reagents: antibody panels, tumor dissociation/enrichment reagents; modulators and cell controls).
- In-house **quality management system** which meets international standards and is supported by ISO-EC 17025-2017 Laboratory Accreditation (underway).
- **Computational Sciences platform** for developing predictive algorithms and visualization of high complexity, multi-dimensional data sets.

Pierian Biosciences ImmunoINTEL™ technology



Pre-Analytical Steps
- Sample collection
- Sample shipment
- Sample receipt and accessioning

Tumor Sample Processing
- Semi-automated dissociation of tissues into single-cell suspensions using benchtop gentleMACS™ dissociator

Analytical Steps
- Sample Processing
  - Automated tumor , whole blood, PBMC, or BMMC plating
  - Automated cytotoxic/modulator plating
  - Automated staining (ImmunoINTEL™)
- Sample Acquisition: Flow Cytometry (ImmunoINTEL™)

RESULTS

Fig. 1. Cloud-based software used in ImmunoINTEL™ for automated experiment design and multivariate data analysis enables HTP processing and minimizes errors

Templating and rapid plate cloning; export all meta-data to layout files (antibodies, modulators, sample, cytometer settings, and compensation).

Comprehensive and integrated cloud-based data analytics. Visualization and interactive proprietary algorithms.

Fig. 2. ImmunoINTEL™ metrics extraction, reporting & visualization for high complexity multi-dimensional data sets are performed in an automated system

Gating Trees for Metrics Extraction

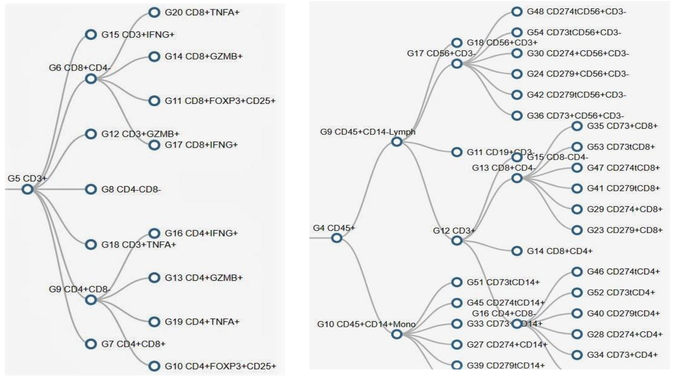
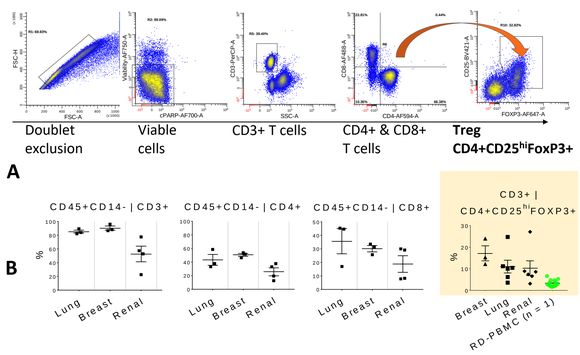
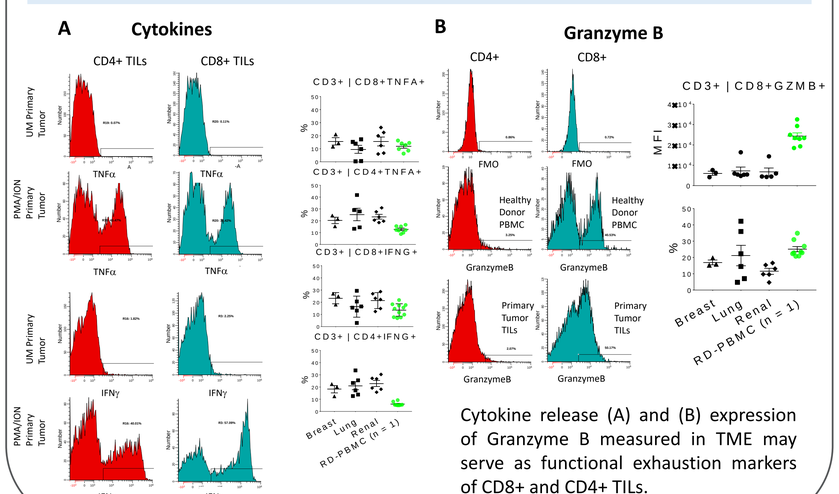


Fig. 3. Analysis of tumor-infiltrating immune-suppressive T regulatory cells (Treg) and tumor resisting CD8+ cytotoxic T cells in several tumor types



Elevated levels of CD4+CD25^{hi}FoxP3+ Treg suggest: (A) immunosuppressive signature with tumor spreading; (B) poor prognosis and objective response. (RD-PBMC = PBMC reference donor used across all assays).

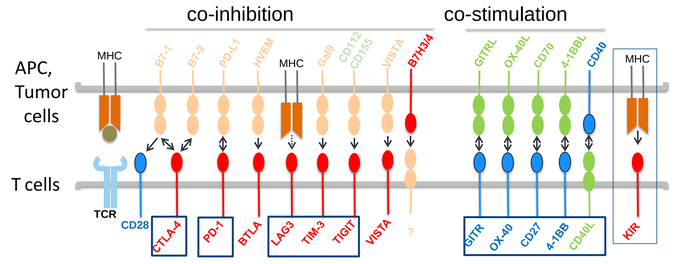
Fig. 4. ImmunoINTEL™ probes functional status and cytotoxic potential of TILs



Cytokine release (A) and (B) expression of Granzyme B measured in TME may serve as functional exhaustion markers of CD8+ and CD4+ TILs.

RESULTS

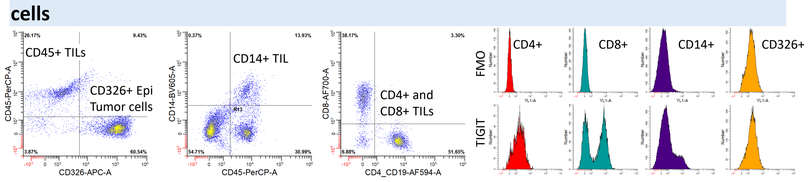
Fig. 5. Beyond PD-L1: ImmunoINTEL™ probes expression of multiple relevant immuno-modulatory receptor/ligand (IMR/L) pairs



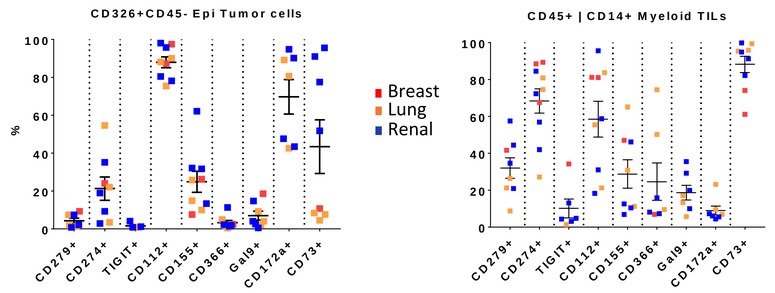
D. Pardoll, Nature Reviews Cancer 2013: 12: 252–264

Ability to profile immune cell subsets in the context of PD-1 and/or other IMRs expression → To provide information on Combination therapy

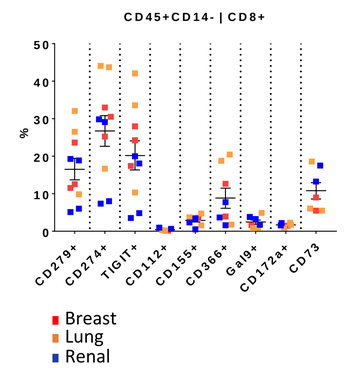
Fig. 6. ImmunoINTEL™ measures the expression levels of immune checkpoint/exhaustion markers and their cognate ligands on TILs and tumor epithelial cells



Gating examples for dissociated tumor — Example: TIGIT expression



Immune Checkpoint/Exhaustion Marker and Their Cognate Ligands



The ImmunoINTEL™ panels were complemented with drop-in IMR/exhaustion markers and their ligands (CD279/CD274 [PD1/PD-L1], TIGIT/CD112-CD155, CD366 [TIM-3]/Galectin-9, CD172a [SIRPa]/CD47, CD73) to profile the functional status of TILs. Reliable separation of IMR/IMR-L positive TILs from negative TILs and IMR/IMR-L positive tumor cells from negative tumor cells was observed, with notable heterogeneity, across cell and tumor types (breast, lung, and renal). This included the elevated expression of several IMR-Ls on TILs and IMRs on epithelial and stromal cells, suggesting tumor- and TIL-intrinsic mechanisms modulating checkpoint interactions.

CONCLUSIONS

- Pierian Biosciences' Technology and ImmunoINTEL™ platform allows for:
  (a) Single-cell phenotypic and functional analysis of immune & tumor cells within the TME;
  (b) Quantitative characterization of immunosuppressive phenotypes as well as the cellular expression of targetable immuno-modulatory (checkpoint) receptors and their ligands for more refined selection of target patient populations.
- The ImmunoINTEL™ platform can serve as a drug discovery/evaluation tool (a) in early stage immunotherapeutic exploratory studies; (b) for patient stratification in clinical trials; (c) for providing mechanism-based proof of principal in pre-clinical studies.
- The ImmunoINTEL™ platform adds an additional dimension to TIL analysis and generates accurate and highly reproducible data sets under validated conditions. The downstream software-based HTP analysis accelerates data analysis/extraction and increases the quality of TIL data obtained from patient-derived tumor specimens.
- Our data demonstrates the breadth of measurements collected with the ImmunoINTEL™ platform, which is beyond limited capacity of standard IHC.

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