Multiplex IHC Detection of Immune Checkpoint Receptors in the Tumor Microenvironment

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Abstract #5360

INTRODUCTION

Immune checkpoint blockade has been shown to partially reverse the exhausted T cell phenotype and consequently lead to a decrease in tumor burden.[1-5] Therefore, there is a need within the clinical research community to develop a better understanding of this T cell type. Here, we construct a seven-color multiplex IHC panel in order to simultaneously visualize cytokeratin (tumor mask), DAPI (nuclear counterstain), CD8 (cytotoxic T cell marker), the exhausted T cell markers PD-1, TIM-3 and LAG3, and the ligand for PD-1, PD-L1. This technique, which relies on IHC validated antibodies, not only enabled the concurrent detection of these markers but also provided high-resolution visualization of interactions between PD-1-expressing CD8+ T cells and PD-L1-expressing cells in the tumor microenvironment.

This seven-plex panel was used to analyze breast, lung and ovarian tumor tissue for the distribution, co-localization, frequency and proximity of these targets in relation to one another. While we often visualized co-expression of LAG3 and PD-1 on T cells, TIM-3 was frequently observed on other cells in the tumor microenvironment across all patient tumors.[3] These data provide valuable insight into the co-expression profiles of these markers in multiple tumor types and have potential implications for the use of combination therapies that aim to target both the innate and adaptive immune systems.

METHODS

A seven-plex panel including DAPI, TIM-3, PD-1, CD8, LAG3, PD-L1 and cytokeratin was optimized and applied to formalin-fixed paraffin embedded (FFPE) tumor sections of various tumor types (10 breast cancer sections, 10 lung cancer sections, and 10 ovarian cancer sections). FFPE tissue was deparaffinized and rehydrated. After antigen retrieval, tissue is stained with a primary antibody followed by the HRP conjugated secondary antibody. The tyramide serial stain protocol allows for the use of multiple rabbit monoclonal antibodies in a single panel. The HRP enzyme catalyzes the reaction of tyramide to a reactive form, which binds tyrosine residues on and near the target. The tyramide is conjugated to a fluorophore, which can then be detected and imaged. A Mantra™ quantitative pathology workstation (PerkinElmer, Waltham MA) outfitted with InForm™ Image Analysis software (PerkinElmer, Waltham MA) was used to spectrally unmix the fluorescent signal and provide quantitative data. One field per section was imaged and analyzed. This approach allowed for the phenotyping of cells and co-expression analysis. During this quantitative analysis, tumors were stratified according to CD8 expression, with >100 CD8+ cells/field termed “hot” and <100 CD8+ cells/field termed “cold”. The following antibody clones from Cell Signaling Technology, Inc. were used for staining in this panel:

- TIM-3 (D5D5R™) XP® Rabbit mAb #45208
- PD-1 (D4W2J) XP® Rabbit mAb #86163
- CD8 (C8/144B) Mouse mAb #70306
- LAG3 (D2G4O™) XP® Rabbit mAb #15372
- PD-L1 (E1L3N®) XP® Rabbit mAb #13684
- CK (C11) Mouse mAb #4545

CONCLUSIONS

A mIHC panel focusing on exhausted T cell markers was constructed and used to stain various tumor types. Highly detailed images of the tumor microenvironment were obtained and quantified in order analyze patterns of co-expression of co-inhibitory immune checkpoint receptors and to phenotype the immune infiltrate. A myriad of distinct patterns of co-expression of immune checkpoint receptors were observed, underscoring the need for highly multiplexed assays.

- As expected, higher amounts of PD-1, TIM-3 and LAG3 were observed in “hot” vs. “cold” tumors.[1-2,5]
- LAG3 expression was lower in all examined tumor types compared to expression of PD-1 or TIM-3.[4]
- TIM-3 expression was often observed on CD8-/CK- cells of the tumor microenvironment in addition to that on CD8+ T cells. These CD8-/CK- cells could be macrophages, CD4+ T cells, or other types of stromal cells.[3]
- A greater percentage of CD8-/CK- cells in “hot” tumors were PD-L1+ than compared to “cold” tumors.[5]

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2. Molecular and cellular insights into T cell exhaustion. (2015). Wherry J, Kurachi M. *Nat Rev Immunol.* 15, 486–499.
3. Ocaña-Guzman, Torre-Bouscoulet, Sada-Ovalle. (2016) TIM-3 Regulates Distinct Functions in Macrophages. *Front Immunol.* 7:229.
4. LAG3 (CD223) as a cancer immunotherapy target. (2017). Andrews LP1, Marciscano AE2, Drake CG3, Vignali DA. *Immunol Rev.* 276(1):80-96.
5. Innate and adaptive immune cells in the tumor microenvironment. (2013) Thomas F Gajewski, Hans Schreiber, and Yang-Xin Fu. *Nat Immunol.* 14(10): 1014–1022.

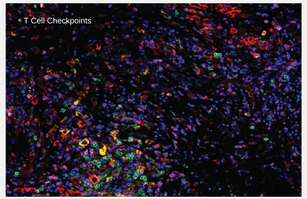

Figure 1A. T cell checkpoints in infiltrating ductal carcinoma of the breast. DAPI – Blue , CK – Cyan, PD-1 – Green, TIM-3 – Red, CD8 – Magenta, PD-L1 – Yellow

Figure 1B.

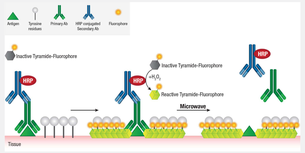

Figure 1C.

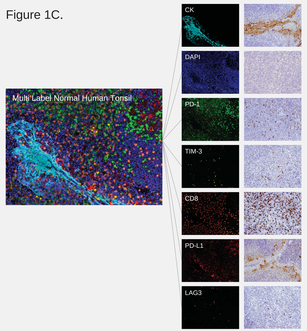

Figure 1B. Basic principles of tyramide-based fluorescent mIHC .

Figure 1C. Normal human tonsil stained with an exhausted T cell phenotype mIHC panel demonstrating that seven fluorescent signals can be clearly unmixed via spectral imaging. The fluorescent multiplex staining mirrors the corresponding chromogenic staining performed on serial sections from the same tissue block.

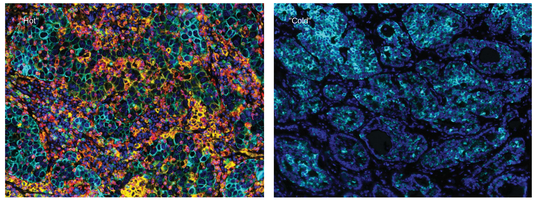

Figure 2A. Representative images of “hot” (L) and “cold” (R) non-small cell lung carcinoma (NSCLC) tumors. DAPI – Blue, CK – Cyan, PD-1 – Green, TIM-3 – Yellow, CD8 – Orange, PD-L1 – Red, LAG3 – Magenta

Increased Expression of Immune Checkpoint Receptors in Inflamed “Hot” vs. Non-inflamed “Cold” Tumors

Figure 2B.

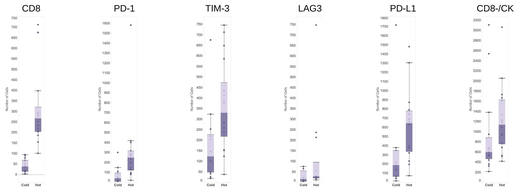

Figure 2C.

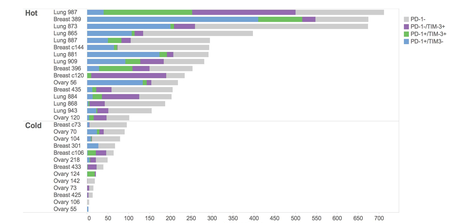

Figure 2B. “Hot” and “cold” tumors were analyzed for the numbers of CD8, PD-1, TIM-3, LAG3 and PD-L1 positive cells, as well as for the numbers of CD8-/CK- cells present.

Figure 2C. A bar graph of PD-1 and TIM-3 single and co-expression on CD8+ T cells in “hot” and “cold” tumors.

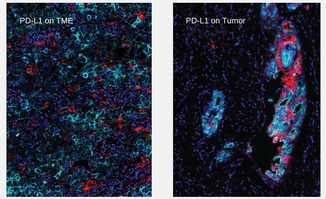

Figure 3A. PD-L1 expression in the tumor microenvironment (TME) in NSCLC (L) or the tumor in breast adenocarcinoma (R). DAPI – Blue, CK – Cyan, PD-1 – Green, PD-L1 – Red

Increased Expression of PD-L1 in the TME in “Hot” vs. “Cold” Tumors

Figure 3B.

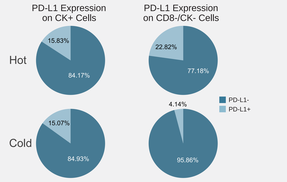

Figure 3C.

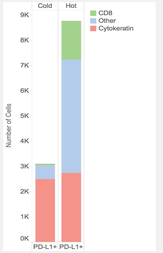

Figure 3B. The percentage of CK+ or CK-/CD8- cells per field in “hot” vs. “cold” tumors that were positive for PD-L1 expression.

Figure 3C. The number of PD-L1+ cells per field in “hot” vs. “cold” tumors that were also positive for CD8, CK or negative for both.

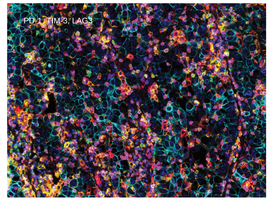

Figure 4A. Co-Expression of Immune Checkpoint Receptors in “Hot” Tumors in NSCLC. DAPI – Blue, CK – Cyan, PD-1 – Green, TIM-3 – Yellow, CD8 – Orange, PD-L1 – Red, LAG3 – Magenta

Expression of PD-1, TIM-3, & LAG3 on CD8+ and CD8-/CK- Cells

Figure 4B.

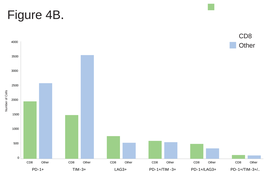

Figure 4C – F.

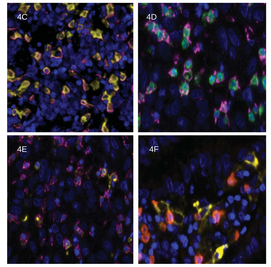

Figure 4B. The expression and co-expression profiles of immune checkpoint receptors in cells per field in “hot” tumors.

Figure 4C – F. Representative images showing (C) PD-1+/CD8+ T cells, (D) LAG3+/CD8+ T cells, (E) TIM-3+/CD8+ T cells and (F) TIM-3+/CD8- cells.

Unique Immune Cell Checkpoint Receptor Phenotypes Among Patient Tumors

Figure 5.

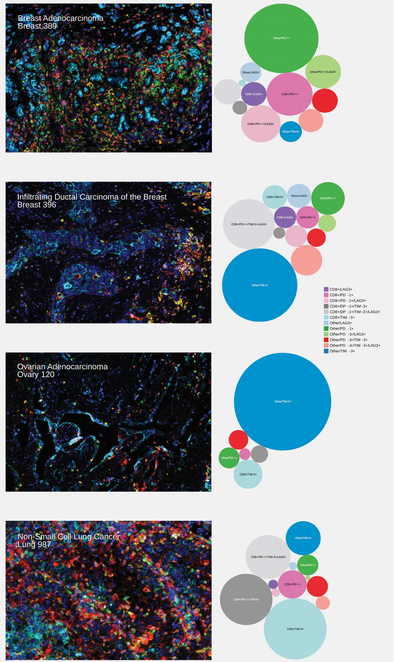

Figure 5. Bubble graphs of the cell phenotypes present in several tumor sections and the corresponding multispectral images.

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