Abstract #2629

A multiplex immunofluorescence assay to assess immune checkpoint inhibitor-targeted CD8 activation and tumor co-localization in FFPE tissues

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Abstract

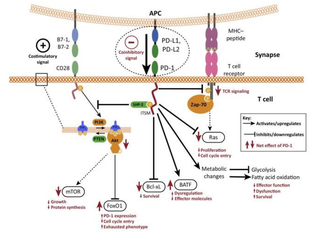
Background: Immune checkpoint inhibitors promote antitumor immune responses by enhancing T-cell activity. Measuring the pharmacodynamic effects of these drugs is challenging, as it requires assessing both immune cell and cancer cell populations. To evaluate T-cell activation in tumor tissue from patient biopsies, we developed a robust multiplexed immunofluorescence assay.

Methods: Our assay uses novel oligo-conjugated antibodies (Ultivue) for simultaneous quantitation of TCR activation (phospho-CD3zeta), immune checkpoint signaling via PD-1 (p-SHP1/p-SHP2), and the net stimulation/inhibition resulting from the integration of these two pathways in CD8 cells (p-ZAP70), while also providing the proximity of CD8 cells to tumor tissues, identified by β-catenin. The method was clinically validated using custom tissue microarrays (TMA) containing tumor biopsies of 3 different histologies (CRC, NSCLC, and breast).

Results: From a total of 192 tumor core biopsies, 20/64 NSCLC, 9/64 CRC, and 3/65 breast TMA cores were found to have a significant number of CD8+ tumor infiltrating lymphocytes (TILs) at baseline (≥50 cells in the examined section). In 18 of the 20 NSCLC cores, ≥50% of CD8 cells both inside and outside of the tumor were activated (CD3z-pY142+). In 6/9 CRC cores, ≥50% of CD8+ cells inside tumor tissues were activated, and in 4/9 CRC cores, ≥50% of CD8+ cells in stroma were activated. In 2/3 breast tumor cores, 90% of CD8+ cells inside tumor tissues were activated; in the remaining core, 90% of CD8+ cells in stroma were activated. Interestingly, all 192 cores had minimal to no expression of activated Zap70 (pY493) in CD8+ cells.

Conclusions: Depending on tumor histology, baseline biopsy samples may contain variable numbers of activated CD8+ TILs (CD3z-pY142+), which may reside inside or outside of tumor regions and express very low levels of Zap70-pY493. Anti-PD-1 therapy is predicted to enhance T-cell cytotoxic activity, as demonstrated by an increased number of TILs and elevated Zap70-pY493 expression. This assay is being used for pharmacodynamic evaluations in ongoing immunotherapy clinical trials. Funded by NCI Contract No HHSN261200800001E.

Background

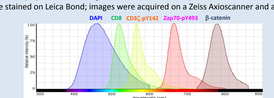


Chinai JM, et al; *Trends Pharmacol Sci*, 2015.

PD-1 modulation of TCR signaling. Binding of PD-L1 ligands to PD-1 leads to the binding of SHP-2 to phosphorylated ITSM and overall inhibition of T cell receptor (TCR) signaling through blockade of CD3ζ chain phosphorylation and Zap-70 association.

Materials and Methods

Development of oligo-conjugated 5-plex quantitative immunofluorescent assay (IFA): Validated antibodies to CD8, CD3z-pY142, Zap70-pY493, and β-catenin were conjugated to specific oligonucleotides and detected by complementary fluor-conjugated oligos (FITC, TRITC, Cy5, and Cy7, respectively; Ultivue). DAPI was included in the panel to assess cellularity by nuclear staining. Control tissue and cell pellet slides, and human tumor TMAs (Indivumed), were stained on Leica Bond; images were acquired on a Zeiss Axioscanner and analyzed by Definiens Software.



Tissue and cell pellet controls for quantitative multiplex IFA (qmIFA) development: β-catenin staining of tumor tissue for tumor segmentation was validated using an MTU951 Multi-tumor Tissue Microarray (US Biomax). CD3z-pY142 and CD8 tissue staining were validated on human tonsil. Zap70-pY493 staining was validated on anti-CD3/CD28 bead-activated T-cell pellets. The 5-plex IFA staining (including DAPI) was clinically validated on human tumor tissue microarrays from 3 different histologies: CRC, NSCLC, and breast (Indivumed).

Quantitative analysis of 5-plex IFA: Image analysis was performed in Definiens Architect 2.4.2 Tissue Studio IF. Tumor tissue was identified by β-catenin staining, and stroma was identified by absence of β-catenin staining. The tissue of each core was classified as tumor or stroma using the Composer Training in ROI detection. In Cellular Analysis, nuclei were detected and assigned to either tumor or stroma. Cell Simulation was based on cytoplasmic staining. In addition, TILs were identified by CD8 or CD3z-pY142 staining; cells that were CD8+ CD3z-pY142+, CD8+ CD3z-pY142-, or CD8- CD3z-pY142+ were identified in Cell Classification using the coexpression feature. These cells were assigned to either the tumor or stroma based on β-catenin staining.

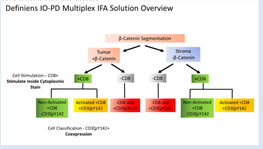


Figure 1. Comparison of β-catenin staining by IHC versus oligo-conjugated β-catenin antibodies detected at the Cy7 channel on various tumor cores

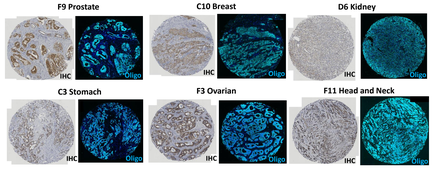
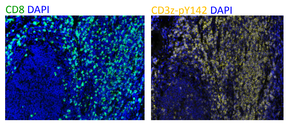


Figure 1. US Biomax MTU951 Multitumor Microarray stained with anti-β-catenin antibody detected by immunohistochemistry (left) or with oligo-conjugated antibody detected by AF750-conjugated complementary oligonucleotide (right; Ultivue).

Figure 2. Validation of phospho-biomarker staining of T-cell activation on human tonsil and antibody bead-activated T-cell pellets

Figure 2A. Validation of oligo-conjugated antibodies to CD8 (left panel) and CD3z-pY142 (right panel) on human tonsil tissue.



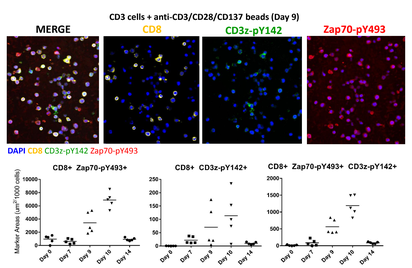


Figure 2B. Multiplex staining validation of oligo-conjugated antibodies to CD8, CD3z-pY142, and Zap70-pY493 on anti-CD3 and -CD28 bead-activated CD3 cells from normal donors. Lower panel shows marker quantitation on CD8 cells at various timepoints after antibody bead activation *ex vivo*.

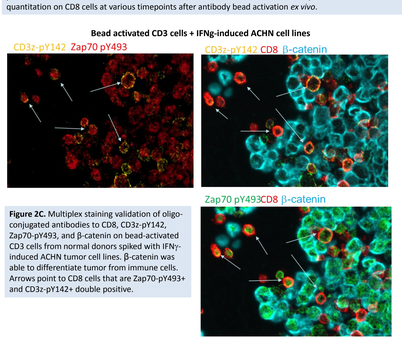
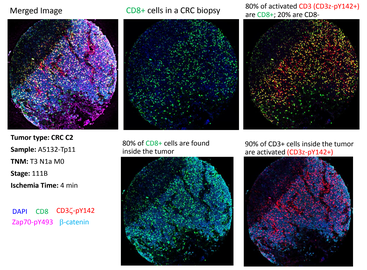


Figure 2C. Multiplex staining validation of oligo-conjugated antibodies to CD8, CD3z-pY142, Zap70-pY493, and β-catenin on bead-activated CD3 cells from normal donors spiked with IFNγ-induced ACHN tumor cell lines. β-catenin was able to differentiate tumor from immune cells. Arrows point to CD8 cells that are Zap70-pY493+ and CD3z-pY142+ double positive.

Figure 3. qmIFA Clinical Sample Feasibility on CRC TMAs

3A. Indivumed CRC Core C2 stained with IPD qmIFA



3B. High-Resolution Images of CRC TMA C2

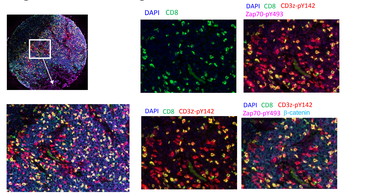
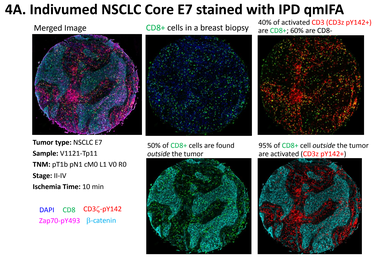


Figure 4. qmIFA Clinical Sample Feasibility on NSCLC TMAs

4A. Indivumed NSCLC Core E7 stained with IPD qmIFA



4B. High Resolution Images NSCLC TMA E7

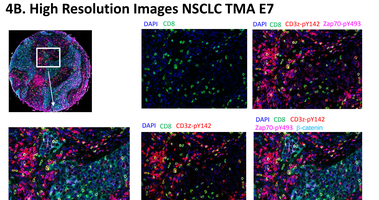


Figure 5. Definiens Image Analysis and Quantitation of CRC, NSCLC, and Breast TMA Cores

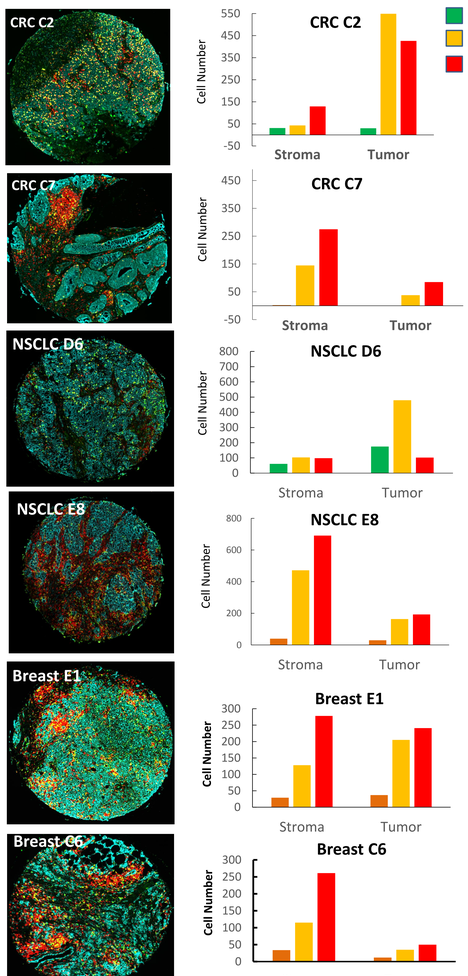


Table 1. Quantitation of total number of activated CD8 T cells in relation to tumor tissue or surrounding stroma in select CRC and breast TMA cores

CRC C2	Biopsy	Stroma	% Stromal Cells	Tumor	% Tumor Cells
Total Cells	6,783	2,841	42.0%	3,942	58.0%
CD8+ CD3z-	61	31	1.10%	30	0.80%
CD8+ CD3z+	592	43	1.50%	549	13.90%
CD8- CD3z-	5575	2,638	34.9%	2,937	32.5%
CD8- CD3z+	555	129	4.50%	426	10.80%
CRC C7	**Biopsy**	**Stroma**	**% Stromal Cells**	**Tumor**	**% Tumor Cells**
Total Cells	5,132	2,762	53.8%	2,370	46.2%
CD8+ CD3z-	2	2	0.07%	0	0.00%
CD8+ CD3z+	183	145	5.25%	38	1.60%
CD8- CD3z-	4587	2,340	38.5%	2,247	41.0%
CD8- CD3z+	360	275	9.96%	85	3.59%
NSCLC D6	**Biopsy**	**Stroma**	**% Stromal Cells**	**Tumor**	**% Tumor Cells**
Total Cells	5,330	1,834	34.4%	3,496	65.6%
CD8+ CD3z-	236	61	3.30%	175	5.00%
CD8+ CD3z+	582	103	5.60%	479	13.70%
CD8- CD3z-	4312	1,572	20.2%	2,740	44.0%
CD8- CD3z+	200	98	5.30%	102	2.90%
NSCLC E8	**Biopsy**	**Stroma**	**% Stromal Cells**	**Tumor**	**% Tumor Cells**
Total Cells	5,868	3,432	58.5%	2,436	41.5%
CD8+ CD3z-	70	40	1.20%	30	1.20%
CD8+ CD3z+	636	472	13.80%	164	6.70%
CD8- CD3z-	4278	2,229	23.4%	2,049	25.7%
CD8- CD3z+	884	691	20.10%	193	7.90%
Breast C6	**Biopsy**	**Stroma**	**% Stromal Cells**	**Tumor**	**% Tumor Cells**
Total Cells	5,966	3,162	53%	2,804	47.0%
CD8+ CD3z-	46	34	1.08%	12	0.43%
CD8+ CD3z+	150	115	3.64%	35	1.25%
CD8- CD3z-	5459	2,752	40.0%	2,707	43.5%
CD8- CD3z+	311	261	8.3%	50	1.8%
Breast E1	**Biopsy**	**Stroma**	**% Stromal Cells**	**Tumor**	**% Tumor Cells**
Total Cells	8,058	2,237	28%	5,821	72.2%
CD8+ CD3z-	66	29	1.30%	37	0.64%
CD8+ CD3z+	333	128	5.72%	205	3.52%
CD8- CD3z-	7140	1,802	8.4%	5,338	63.9%
CD8- CD3z+	519	278	12.4%	241	4.1%

Table 2. Summary of T cells found in CRC, NSCLC, and breast TMAs

Histology	Number of cores	TMA cores with ≥50 total CD8+ Tcells	Stage	TMA cores with ≥50% of the total activated T cells in location	
				Tumor	Stroma
Breast	65	3	II-III	2	1
Colorectal	64	4	II	2	2
		5	III	4	1
NSCLC	64	20	II-IV	17	3

Summary and Clinical Implications

- We have developed a robust quantitative multiplex IO-PD immunofluorescence assay that quantitatively detects CD8+ cells and their activation status in relation to tumor tissues, as delineated by β-catenin. To analyze stained tissue, we have also developed algorithms by Definiens Architect to quantify activated CD8+ T cells (CD3z-pY142+) both inside tumor tissues as well as in surrounding stroma using CRC, NSCLC, and breast TMA samples from Indivumed.
- Based on the TMA tumor staining, 3/65 breast TMA cores had >50 total infiltrating lymphocytes in the biopsy, of which 2 cores had 90% of activated CD8+ T cells inside the tumor and 1 core had 90% of activated CD8+ T cells outside the tumor. There was minimal Zap70-pY493 expression in T cells.
- In the CRC TMA, 8/64 cores had >50 total infiltrating lymphocytes in the biopsy, of which 6/8 cores had >50% of activated CD8+ T cells inside the tumor and 2/8 cores had >80% of activated CD8+ T cells outside the tumor. Again, no Zap70-pY493 expression in T cells was found at baseline.
- In the NSCLC TMA, 20/64 cores had >50 total infiltrating lymphocytes in the biopsy, of which 17/20 cores had >50% of activated CD8+ T cells *inside* the tumor and 18/20 cores had >50% of activated CD8+ T cells *outside* the tumor. As in the other histologies, there was no Zap70-pY493 expression detected in T cells.
- Depending on tumor histology, baseline biopsy samples may contain variable numbers of activated CD8+ TILs (CD3z-pY142+), which may reside inside or outside of tumor regions and express very low levels of Zap70-pY493. This assay is being used for pharmacodynamic evaluations in ongoing immunotherapy clinical trials. The assay will be made available to the public via https://dctd.cancer.gov/ResearchResources-biomarkers.htm.

Acknowledgements

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