Characterizing immunotherapy-induced lymphocyte infiltration at the single patient level using CANscript™, an *ex-vivo* human tumor model

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Background: The presence and activity of lymphocytes within the tumor is critical for clinical response to cancer immunotherapy, such as immune checkpoint blockade. Tumors with poor T-cell inflamed phenotypes, often referred to as a 'cold' tumor, is associated with modest clinical response. High baseline infiltration of effector T-cell lymphocytes is considered 'hot', and patients are predicted to respond more favorably to treatment. However, patient-to-patient response and durability remains highly variable. There is an urgent gap in available methods to study lymphocyte infiltration, trafficking and spatial heterogeneity induced by different cancer immunotherapies in individual patients. Moreover, there is a poor correlation between therapy-induced lymphocyte infiltration with clinical response, which could be shaped using personalized approaches to therapy.

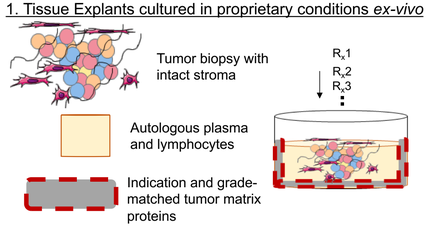
Methods: Here, we used CANscript™, an *ex-vivo* human tumor model that recapitulates and preserves the native, patient-autologous tumor microenvironment, including autologous patient-derived peripheral blood mononucleated cells (PBMC). Utilizing tissue from breast cancer patients classified as either 'cold' (N=5) or 'hot' (N=5), we studied lymphocyte infiltration under pressure of α-PD-1 immune checkpoint blockade (pembrolizumab) over a 72h time course compared to standard of care (SOC) drugs such as docetaxel. Using fluorescent labelling and flow cytometric analysis we characterized infiltrating lymphocytes, studying the role of T-cell repertoires under different environmental and immunotherapy pressures. We coupled these analyses with multiplex immunohistochemistry (CD3⁺, CD4⁺, CD8⁺) to map spatial heterogeneity of tumor cells and lymphocytes before and after treatment, *ex-vivo*.

Results: We determined that immune checkpoint blockade induced unique patterns of migration and infiltration of effector T-cells (T_{eff}) and T-regulatory (T_{reg}) cells in 'hot' vs 'cold' tumors. Furthermore, we determined that, in some instances, 'cold' tumors can be driven towards a 'hot' phenotype characterized by trafficking of active immune lymphocytes following treatment, which corresponded to differential ratio of T_{eff} to T_{reg} compared to baseline.

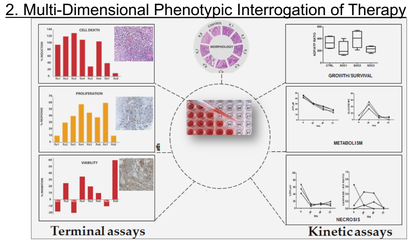
Concluding remarks: Taken together, these data demonstrate the utility of CANscript™ as a platform to characterize response to immunotherapy in a spatial context, providing insight into the migratory patterns of immune cell subsets at the individual patient level. Such an advance in our preclinical methods to study immuno-modulators may help guide treatment decisions for clinicians while simultaneously functioning as a platform to study and discover mechanisms of clinical efficacy for emerging drug combinations.

CANscript™

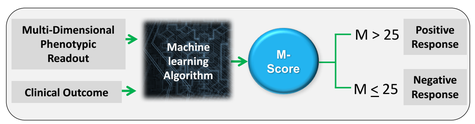
1. Tissue Explants cultured in proprietary conditions *ex-vivo*



2. Multi-Dimensional Phenotypic Interrogation of Therapy



3. Trained and Validated with 2000 Matched Patients



4. Published Assay Performance

Actual Clinical Response

	+	−
+	90	14
−	2	58

Positive Predictive Value = 87%
If CANscript result is positive, how often is it right?

Negative Predictive Value = 97%
If CANscript result is negative, how often is it right?

Sensitivity = 98%
How many TRUE POSITIVES did we catch?

Specificity = 81%
How many TRUE NEGATIVES did we catch?

Fig. 1 CANscript™ is a multi-dimensional live tissue platform to study personalized response to anticancer therapy at the individual patient level. 1) The explant live tissue assay reliably recreates the entire tumor ecosystem including microenvironment components, stroma, and immune contexture. 2) Drugs are interrogated by quantitatively assessing tumor compartment activity in a multi-dimensional approach. 3) The multi-dimensional quantitative interrogation was performed on ~2000 patients who received the same treatment in clinic that was tested in the explant assay. The results from the explant assay were correlated to patient clinical response using a machine learning algorithm. M-Score was derived, which predicts positive vs. negative response. 4) To date, more than 4000 patients have been evaluated by CANscript™

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Tumor indication	Subtype/tumor location	% patient samples	# of samples tested in CANscript	Drug Tested
HNSCC	Alveolus	17%	N = 52	Keytruda (Pembro)
	Maxilla	38%		
	Other	45%		
Breast	ER/PR+ HER2-	80%	N = 5	Keytruda (Pembro)
	ER/PR-/HER2-	20%		

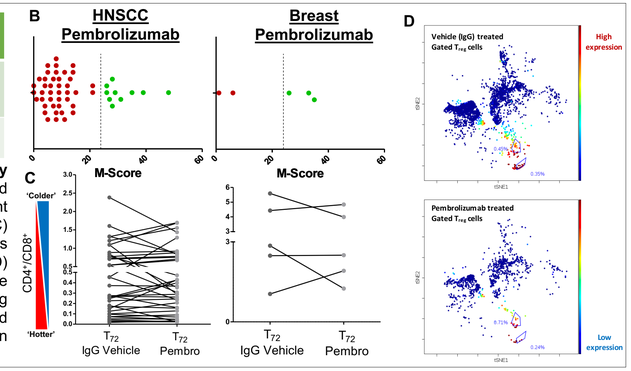
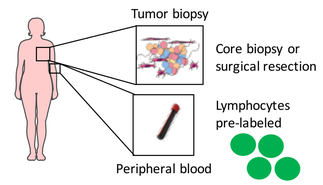


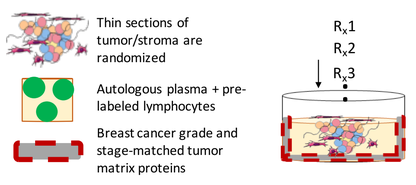
Fig. 2 Characterizing response to anti-PD-1 therapy in multiple tumor types A) Patient demographics and tumor (sub)types. B) M-Scores across multiple patient samples tested in CANscript™ using Pembrolizumab. C) Pair-wise expression of tumor infiltrated lymphocytes following vehicle (IgG) or Pembro treatment (72h). D) Single patient (breast ER/PR+, HER2-, all tissue dissociated cells) representative ViSNE plots, indicating differential T_{reg} population following vehicle (IgG) and Pembro treatment in CANscript™. Plot colored on CD25⁺ population.

Model Development - Lymphocyte Infiltration

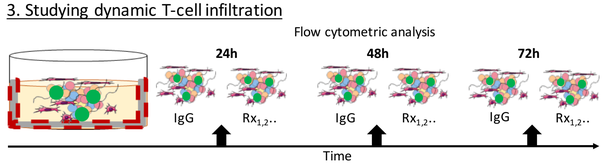
1. Live tissue from breast cancer patient



2. Therapy interrogation *ex-vivo* w/labeled lymphocytes in culture



3. Studying dynamic T-cell infiltration



4. Multiplex IHC for spatial heterogeneity

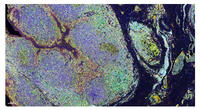


Fig. 3 Studying therapy-induced lymphocyte infiltration in a spatio-temporal context using CANscript™. 1) Fresh tumor containing stroma is harvested along with patient-autologous peripheral blood lymphocytes, which have been 'pre-labeled'. 2) CANscript™ is performed using the co-culture of tumor/stroma biopsy with pre-labeled lymphocytes 3) Tumor tissue is harvested at 24h intervals, dissociated and labeled for multi-colored flow cytometric analysis. Intrinsic TILs and stochastic TIL are captured. 4) Tumor tissue is at T_{72} is fixed and paraffin embedded. T-cell repertoire expression patterns are interrogated and qualitatively assessed using fluorometric multiplex IHC.

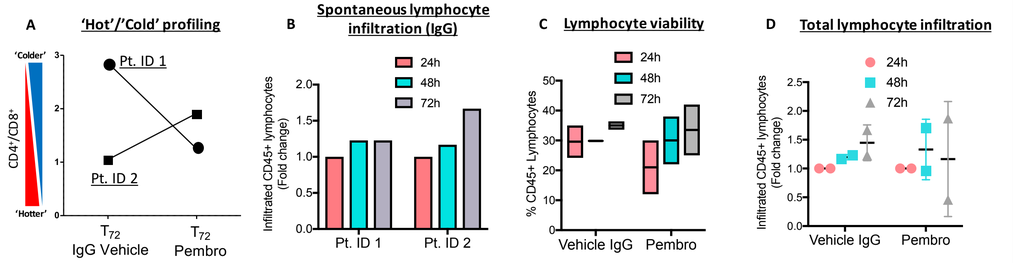


Fig. 4 Non-uniform, dynamic lymphocyte infiltration in patient-specific manner. A) Pairwise expression of two representative breast cancer patient tumor tissues profiled for their CD4⁺:CD8⁺ expression following 72h in CANscript treated with vehicle (IgG) or Pembro. Note the change from 'hot' and 'cold' phenotypes before and after treatment. B) Vehicle (IgG)-treated CANscript was assessed for spontaneous infiltration of CD45⁺ lymphocytes at 24h intervals at the patient-specific level C) Viability of all lymphocytes (infiltrated + intrinsic TIL) vehicle (IgG) or Pembro-treated CANscript. D) Total CD45⁺ lymphocyte infiltration in vehicle and Pembro-treated tumor tissues at 24, 48 and 72h.

Dynamic infiltration of lymphocytes in CANscript™

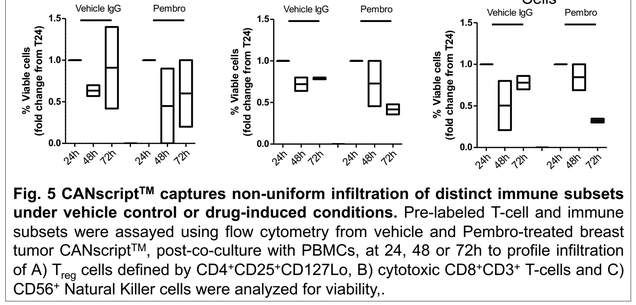


Fig. 5 CANscript™ captures non-uniform infiltration of distinct immune subsets under vehicle control or drug-induced conditions. Pre-labeled T-cell and immune subsets were assayed using flow cytometry from vehicle and Pembro-treated breast tumor CANscript™, post-co-culture with PBMCs, at 24, 48 or 72h to profile infiltration of A) T_{reg} cells defined by CD4⁺CD25⁺CD127Lo, B) cytotoxic CD8⁺CD3⁺ T-cells and C) CD56⁺ Natural Killer cells were analyzed for viability,.

Spatial tumor-immune heterogeneity visualized by multiplex IHC

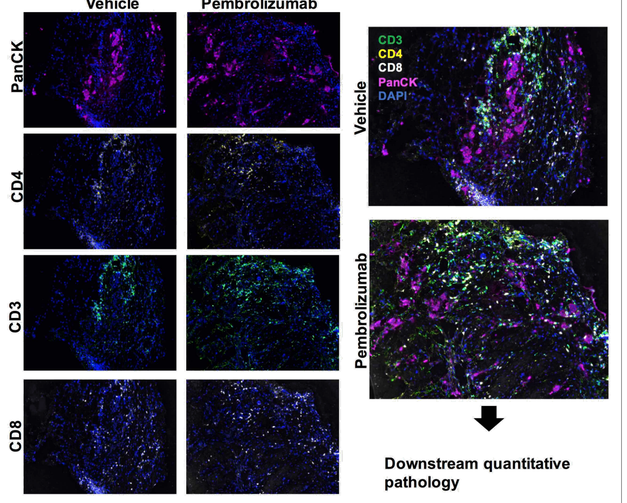


Fig. 6 Multiplexed IHC captures spatial heterogeneity of tumor infiltrating lymphocytes (TILs) in CANscript™ Formalin-fixed paraffin-embedded CANscript breast tumor tissues, vehicle (IgG) and Pembro-treated at T_{72}, stained for Pan-cytokeratin (magenta), CD3 (green), CD4 (yellow), CD8 (white), shown with DAPI (blue) and and overlay of all markers. Imaged using PerkinElmer Vectra Polaris, which enables downstream quantitative pathology.

Conclusions: Dynamic, spontaneous infiltration of lymphocytes into the tumor (aka TILs) are a key factor for immune-based tumor rejection. However, little is known about the role of immunotherapy or other anticancer agents as they modulate TILs. CANscript™ enables a platform to study the antitumor effect of different therapies along with stochastic lymphocyte infiltration at the individual patient-level. By integrating our algorithm-driven predictive clinical assessment (M-Score) we are able to provide unique understanding towards the role of dynamic TIL with clinical response to therapy. Our data demonstrate that high inter-patient variability in immune modulation, and response to immuno-modulators, suggests high importance of integrating a multi-dimensional approach to personalized cancer medicine.