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Assessing Cytotoxic T cell Responses to Immune Checkpoint Inhibitors in Murine and Human Tumor Samples Using Metabolic Imaging

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Introduction

- Existing FDA approved biomarkers do not accurately predict which patients will respond to immune checkpoint inhibitors (ICIs)
- Ex vivo profiling initiatives to improve the prediction of response to treatment with ICIs currently require large amounts of tissue not routinely obtained from patients with cancer
- The Elephas Platform aims to improve ICI response prediction by imaging a patient's core needle biopsy treated with ICI ex vivo
- Multiphoton microscopy (MPM) allows for rapid visualization of biological processes in 3D living tissues in a non-destructive fashion, enabling longitudinal assessment of small specimens, such as core needle biopsies (CNBs)
- We used advanced MPM techniques, including label free and fluorescence lifetime imaging (FLIM), to measure T-cell activation and T-cell-mediated cytotoxicity in response to ICI in live tumor fragments (LTFs) from mouse syngeneic tumors, patient derived xenografts (PDX) and human tumor CNBs

Methods

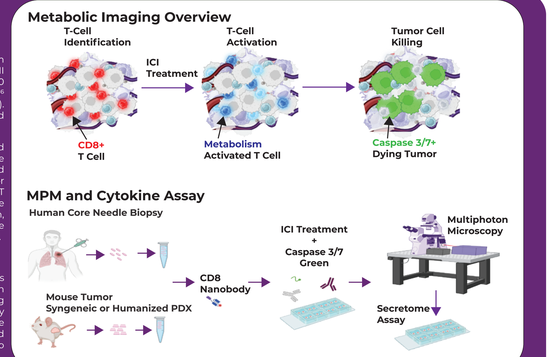
Specimens and treatment:

Humanized PDX tumors were derived by warm passaging PDX LTFs in the hind flank of NSG-MHC I/II DKO mice (Jackson Labs). When tumors reached ~50 mm³, mice were injected intravenously with 20 x 10⁶ peripheral blood mononuclear cells (PBMCs). Tumors were excised at ~500 mm³ and fragmented for imaging analysis.

Tumors from mouse (syngeneic lines or humanized PDX) or from human CNB were processed using the Elephas Platform to create LTFs which were sorted into well plates, cultured, and treated with αPD-1 or IgG control. For metabolic assessment of individual T cells following treatment, the cultured LTFs were imaged at 4 (Day 0) and 24 hours (Day 1). In addition, secretory factors in the culture supernatant were measured using a Luminex® human cytokine panel.

Imaging and analyses:

The intrinsically fluorescent metabolic co-factors nicotinamide dinucleotide (NAD(P)H) and flavin adenine dinucleotide (FAD) were imaged using multiphoton fluorescence lifetime microscopy (MP-FLIM). Data were analyzed via fluorescence lifetime analysis using two reference lifetimes (τ1 and τ2 for short and long lifetimes, respectively) to calculate NAD(P)H α1 and FAD α1.

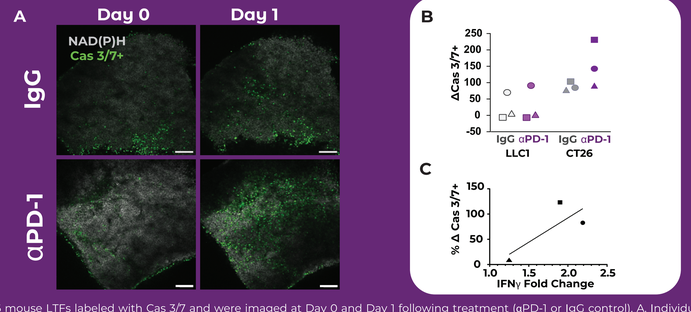


Cytotoxic T-cell-mediated killing of tumor cells was assessed with an exogenous marker for apoptosis (Caspase 3/7 Green) and imaged using MPM.

A CD8 nanobody conjugated to AF594 was used to label native cytotoxic T cells in a manner spectrally independent from NAD(P)H emission. Specificity of CD8 labeling was confirmed with humanized and non-humanized PDX tumors.

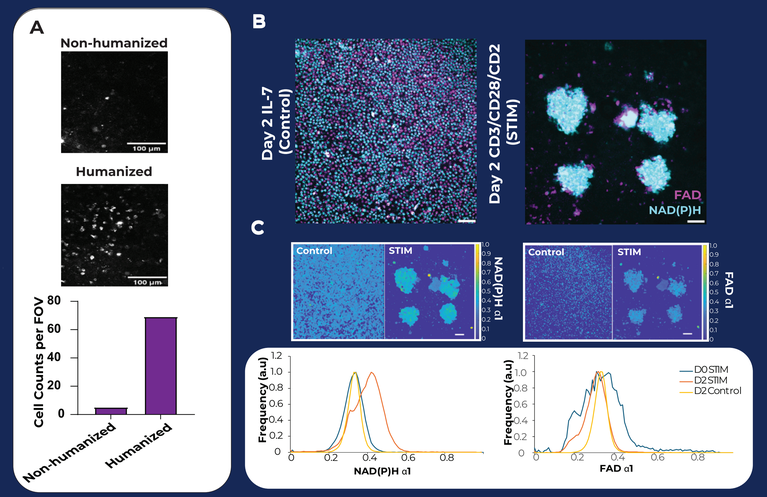
Cas 3/7+ events were enumerated following segmentation of Cas 3/7+ nuclei and were normalized to NAD(P)H+ tissue area.

1 Increased cytotoxicity as assessed by Cas 3/7 correlates with IFNγ response in αPD-1-treated mouse LTFs



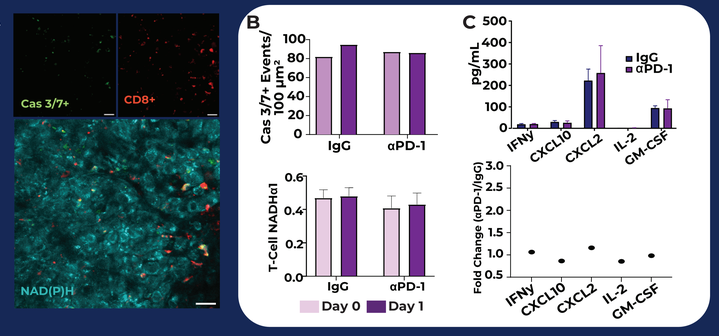
CT26 mouse LTFs labeled with Cas 3/7 and were imaged at Day 0 and Day 1 following treatment (αPD-1 or IgG control). A. Individual optical planes from IgG control (top panels) or αPD-1 treated (bottom panels) fragments are shown. Cas 3/7+ events were enumerated following segmentation of Cas 3/7+ nuclei. Scale bar = 100 µm B. Cas 3/7 positivity in IgG and αPD-1-treated LTFs are reported as the difference in number of Cas 3/7+ cells/mm² between days 0 and 1, for 3 independent CT26 tumors (responsive to αPD-1). LTFs from LLC-1 tumors (non-responsive to αPD-1) were run as a negative control. Cas 3/7+ events were normalized to tissue area. Each shape represents data from a pool of LTFs collected from independent CT26 or LLC1 tumors. C. Graph shows a correlation plot of fold-change in IFNγ (αPD-1 over IgG) in treated CT26 LTFs against percent change of Cas 3/7+ events for αPD-1 relative to IgG. Each shape represents data from a pool of LTFs collected from independent CT26 tumors.

2 Specific labeling of T Cells and use of MP-FLIM to detect CD3/CD28/CD2 activation of T cells



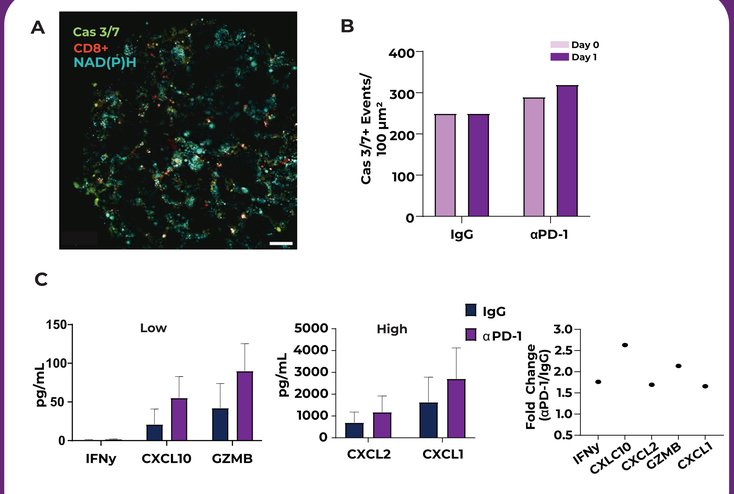
A. Representative images showing specificity of CD8 nanobody in stained, humanized and non-humanized NSCLC PDX tumors. CD8 counts were enumerated in the field of view (FOV). B. CD8+ T cells isolated from PBMCs treated with IL-7 (control) or CD3/CD28/CD2 + IL-7 (stimulated [STIM]) were imaged for NAD(P)H and FAD signals using FLIM at 0, 1, and 2 days post-treatment. Images show intensity projection of FLIM data in selected 2D culture fields for FAD and NAD(P)H . C. The α1 component corresponding to the relative population in the short lifetime state of FAD and NAD(P)H (fixed at 0.1 and 0.4 ns, respectively) was determined for each pixel using phasor fluorescence lifetime analysis (using pixel binning with kernel of six pixels). The images show the α1 components for Day 2 control and stimulation for FAD and NAD(P)H. The histograms show the pixel distribution of the α1 components for the same treatment conditions compared with Day 0 STIM. Scale bar = 50 µm.

3 Combined assessment of cytotoxicity and T-cell activation in a humanized PDX model non-responsive to ICI ex vivo



LTFs from an NSCLC humanized PDX were labeled with Cas 3/7 and CD8 nanobody, and then imaged at Day 0 and Day 1 following treatment (αPD-1 or IgG control). A. Single optical plane from a representative fragment is shown. Cas 3/7+ events and CD8 labeled T cells were enumerated following segmentation. Scale bar = 50 µm B. Upper bar graph shows Cas 3/7 positivity in IgG and αPD-1 treated LTFs on Day 0 and Day 1. Cas 3/7+ events were normalized to NAD(P)H area. Lower bar graph shows T-cell metabolism as measured by the NAD(P)H α1 component in IgG and αPD-1 treated LTFs on Day 0 and Day 1. CD8 nanobody intensity was used to identify and segment individual T cells for quantification of their NAD(P)H free and bound fraction via fluorescence lifetime analysis. The mean and standard deviation are shown for the NAD(P)H α1 component across all T cells quantified from 3 optical planes for all fragments. C. Secretory factors from the LTF cultures were analyzed using the Luminex human cytokine panel. Upper figure shows cytokine levels from 2 separate wells for IgG and αPD-1 for select cytokines. Error bars represent SEM. Lower figure shows fold change in cytokine levels between αPD-1 and IgG.

4 Changes in cytotoxicity and cytokine levels in response to ICI treatment in a human lung CNB



A. Representative image from human lung CNB showing CD8 labeled T cells, Cas 3/7 stained nuclei and NAD(P)H intensity. Cas 3/7 events and CD8 labeled T cells were enumerated following segmentation. Scale bar = 50 µm. B. Graph shows Cas 3/7 positivity in IgG and αPD-1-treated LTFs on Day 0 and Day 1. Cas 3/7+ events were normalized to NAD(P)H. C. Secretory factors from the LTF cultures were analyzed using the Luminex human cytokine panel. Graphs on left shows cytokine levels from 2 separate wells for IgG and αPD-1, split between low and high cytokines, and graph on right shows fold change in cytokine levels between αPD-1 and IgG. Error bars represent SEM.

Conclusions

- Using MPM in mouse LTFs, we show that IFNγ, a marker of T-cell activation, correlates with ICI-induced tumor cell death
- We demonstrate the potential for MPM to characterize the response to ICIs from the limited amount of tissue collected using clinically relevant human CNBs
- Responses to ICIs are shown to be consistent across modalities on our platform (imaging and cytokine secretion)
- The Elephas Platform uses MPM to assess ICI response in CNBs with the goal of supporting personalized cancer treatment

Acknowledgments

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