Title: *In vivo* label-free imaging reveals distinct CD8 T cell metabolic changes during effective radio-immunotherapy in cold murine melanoma versus hot colon carcinoma

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Scientific Abstract:

In vivo multiphoton autofluorescence microscopy provides label-free, single-cell imaging of metabolic changes. These metabolic changes are quantified via the metabolic coenzymes NAD(P)H and FAD which are autofluorescent molecules endogenous to all cells. Metabolic reprogramming is a hallmark of cancer. Immune cells also change their metabolism during cancer progression and this metabolic switch correlates to immune cell phenotype and function. Here, we study metabolic changes during administration of a potent radio-immunotherapy regimen in murine melanoma and colon carcinoma.

This therapy includes external beam radiation, hu14.18-IL2 immunocytokine (anti-GD2 mAb fused to IL2) or free IL2, and anti-CTLA-4, leading to *in situ* vaccination and cure of established murine tumors. We created a transgenic mCherry-labeled T cell mouse to study the critical T cell response. We implanted syngeneic B78 (GD2+) melanoma or MC38 (GD2-) colon carcinoma cells into the flanks of mCherry-labeled CD8 T cell reporter mice to induce tumors. Under anesthesia, skin flap surgery was performed, and tumors were imaged at multiple timepoints during therapy. Multiphoton imaging was performed to collect NAD(P)H, FAD, and mCherry signal. Fluorescence lifetime images were collected using time correlated single photon counting electronics. Tissues were also harvested and analyzed via flow cytometry, single-cell RNA sequencing, and multiplex immunofluorescence to corroborate imaging findings and characterize the immune infiltrate.

Our *in vivo* label-free imaging revealed that CD8 T cells from treated versus control tumors exhibit different metabolic phenotypes including changes in NAD(P)H and FAD protein binding and redox balance across these two solid tumors. In cold B78 melanoma tumors, *in vivo* metabolic imaging revealed that CD8 T cells are activated early but maintain an oxidative metabolic state consistent with exhaustion or a memory phenotype. In hot MC38 colon carcinoma, *in vivo* metabolic imaging revealed that CD8 T cells are activated early and then quickly transition to a glycolytic state that fuels their cytotoxic function. scRNAseq confirmed the metabolic changes we observed within our B78 model and provided additional insight into CD8 T and tumor cell changes during therapy. Flow cytometry illustrated significant changes in the immune infiltrates of both tumor types over time and treatment. These results show that label-free *in vivo* imaging enables single-cell quantification of metabolic changes during therapy - across multiple solid tumors. Combined with other traditional assays, we can elucidate key immune cell populations and the crucial timepoints during

therapy where changes are occurring. This imaging platform may be leveraged to help develop new immunotherapy combinations and study other cancer types where cures have not been realized.

Written Lay Abstract:

Researchers can study changes to molecules linked to our metabolism (how our body makes energy) by using a technique called "*in vivo* multiphoton autofluorescence microscopy". This uses a laser to "excite" the metabolism molecules inside our cells and make them light up under a microscope. When cells become cancerous, their metabolism is changed, and researchers can use this technique to study cancer cells.

A type of cancer treatment called radio-immunotherapy involves radiation plus immunotherapy. The radiation therapy helps kill cancer cells and activates the immune system. The immunotherapy further activates and helps the immune systems to find and kill the cancer cells but leave healthy cells alone.

For this study, the researchers wanted to know how radio-immunotherapy affects cancer cells by looking at how it changes their metabolism. The researchers used the *in vivo* multiphoton autofluorescence microscopy technique to see how cancer cell metabolism changed (and lit up under the microscope) when given radio-immunotherapy.

The researchers used mice with skin and colon cancers for this study. Using the microscopy technique, they were able to follow which molecules related to metabolism were affected by radio-immunotherapy and likely lead to destruction of the cancer cells.

These findings help researchers better understand how radio-immunotherapy works in cancer cells and how cell metabolism can be targeted and used in new cancer treatments. Future studies may test this microscopy technique on other types of cancers and treatments.

Visual Lay Abstract:

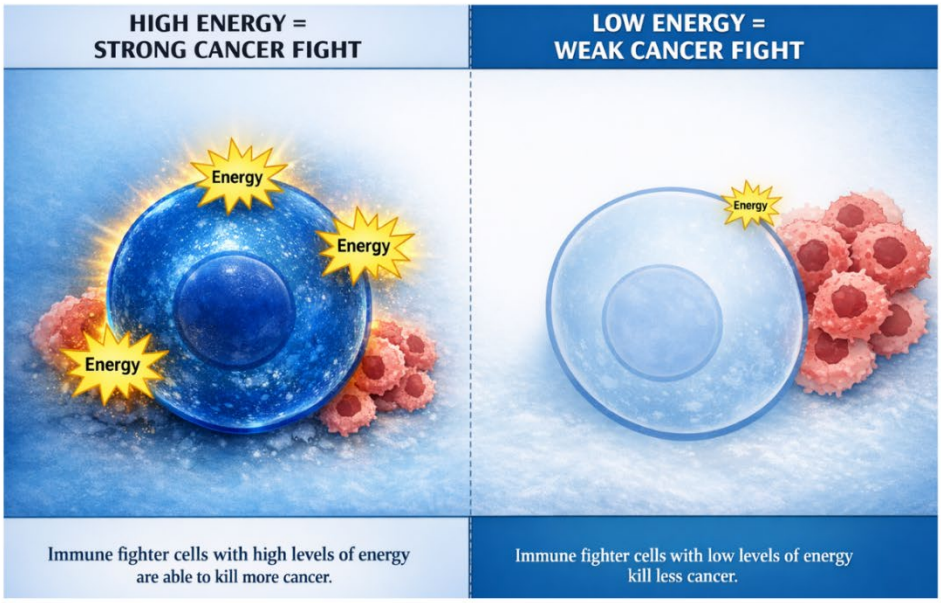
Why Energy Matters in Cancer Treatment

Immune cells need energy to fight cancer

Researchers studied CD8 T cells, a part of our immune defense that kills cancer cells. They found that CD8 T cells need energy to fight cancer better.

Seeing cell energy levels to choose the best treatment

The researchers created a microscope technique that shows how much energy the CD8 T cells have. This can help doctors choose the best treatments for a patient and lead to better medicines.



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