INTRAVITAL MULTIPHOTON IMAGING OF INFILTRATING CD8 T CELL AND TUMOR CELL METABOLISM DURING IMMUNOTHERAPY IN A MURINE MELANOMA MODEL

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Background Intravital multiphoton microscopy (IMM) provides single cell imaging within intact living systems. IMM of the autofluorescent metabolic co-enzymes NAD(P)H and FAD, optical metabolic imaging (OMI), provides *in vivo* label-free imaging of metabolic changes. The metabolism of tumor and immune cells is closely associated with cancer progression and tissue site,^{1–4} so we aim to study metabolic trends during administration of an effective, triple-combination immunotherapy within murine melanoma tumors.⁵ This therapy includes external beam radiation, intratumoral hu14.18-IL2 immunocytokine (anti-GD2 mAb fused to IL2), and intraperitoneal anti-CTLA-4 leading to *in situ* vaccination and cure of GD2+ murine tumors.⁵ Previous work has shown that a T cell response is critical to the efficacy of this therapy,^{5–6} so we created an mCherry-labeled T cell mouse model to study this response. Here, IMM was used to image concurrent tumor and CD8+ T cell metabolic trends during administration of immunotherapy.

Abstract 43 Figure 1 *In vivo* multiphoton images of immune and tumor cell populations during immunotherapy. A) Representative *in vivo* fluorescence intensity images of B78 melanoma tumors growing in control and treated CD8 mCherry reporter mice show mCherry-labeled CD8+ T cells (red) infiltrating tumor tissue as well as autofluorescent metabolic coenzymes NAD(P)H (blue) and FAD (green) expressed by the tumor and T cells. Scale bar 25 μ m. B) Corresponding *in vivo* optical redox ratio intensity images show redox balance within the tumor microenvironment. Treated tumors exhibit an increased optical redox ratio which may indicate increased glycolytic activity during immunotherapy. C) Quantified single-cell B78 tumor (n = 353) and CD8 T cell (n = 18) autofluorescence data. Both CD8 T cells (p<0.0001) and tumor cells (p<0.0034) exhibit significantly increased optical redox ratio with treatment (n = 2 mice, median – center bold dashed line, 3rd quartile – upper dashed line, 1st quartile – lower dashed line, Mann-Whitney U Test).

Methods We created an mCherry-labeled CD8+ T cell mouse model through CRISPR/Cas9 knock-in. We then implanted syngeneic B78 (GD2+) melanoma cells into the flanks of these reporter mice to induce measurable tumors. Mice were anesthetized, skin flap surgery performed, and tumors imaged at several time points. IMM was performed using 750–1040 nm to excite NAD(P)H, FAD, and mCherry through a 40X (1.15 NA) objective. Fluorescence lifetime data was collected

using time correlated single photon counting electronics. Murine tissues were harvested and analyzed via flow cytometry and multiplex immunofluorescence to corroborate IMM findings and characterize the immune infiltrate.

Results Here we demonstrate the feasibility of our IMM platform to capture single cell metabolic changes during immunotherapy administration. Through our intravital imaging we show that CD8 T cell and tumor cell redox ratio (intensity of NAD(P)H/intensity of NAD(P)H + FAD) is significantly increased in treated compared to control mice (figure 1), possibly indicating increased glycolytic activity. We also show differences in protein binding within both CD8 T cells and tumor cells during treatment. Overall, this technology enables analysis of metabolic changes in CD8 T cells and tumor cells *in vivo* during administration of our immunotherapy regimen.

Conclusions These results provide additional support that the combination of intravital imaging with OMI allows for concurrent imaging of T cell infiltration and metabolic trends. Specifically, OMI enabled us to probe single cell metabolic changes occurring during our immunotherapy regimen. With continued work, this imaging platform may be leveraged to develop new combinations of immunotherapies.

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Ethics Approval All animal work was approved by the University of Wisconsin Institutional Animal Care and Use Committees.

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