Background Intravital multiphoton autofluorescence microscopy provides in vivo, 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 of tumor and immune cells is closely associated with cancer progression and cell phenotype.1–3 We aim to study metabolic changes during administration of an effective, triple-combination immunotherapy within murine melanoma tumors.4 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 therapy4–5, so we created an mCherry-labeled T cell mouse model to study this response. Here, intravital multiphoton metabolic imaging was used to image concurrent tumor and CD8+ T cell metabolic trends during administration of immunotherapy.

Methods We implanted syngeneic B78 (GD2+) melanoma cells into the flanks of mCherry-labeled CD8+ T cell reporter mice (C57Bl/6 background) to induce tumors. Under anesthesia, skin flap surgery was performed and tumors were imaged at several time points during therapy. Multiphoton imaging was performed to collect NAD(P)H, FAD, mCherry, and collagen signal through a 40X objective (figure 1A). Fluorescence lifetime data was collected using time correlated single photon counting electronics. Tissues were harvested and analyzed via flow cytometry and multiplex immunofluorescence to corroborate intravital imaging findings and characterize the immune infiltrate.

Results Here we demonstrate that our intravital imaging is sensitive to metabolic changes in both B78 melanoma and CD8 T cells from immunotherapy treated tumors versus control tumors. These metabolic differences include changes in protein binding and redox balance (figure 1B) within treated tumors. We also show remodeling of collagen (figure 1C), a major component of the extracellular matrix, during immunotherapy that may be explained by an observed decrease in macrophages. Flow cytometry and multiplex immunofluorescence illustrate changes in the immune infiltrate composition, activation, and cytotoxicity during therapy (figure 1D).

Conclusions These results show that intravital metabolic imaging enables single cell quantification of metabolic changes in tumor and immune cells during therapy. Combined with other traditional assays, we can elucidate key immune cell populations and the crucial timepoints during therapy where changes are occurring. With continued efforts, this imaging platform may be leveraged to develop new combinations of immunotherapies.

Acknowledgements This work is supported by the Morgridge Institute for Research (Interdisciplinary Fellowship awarded to A.R.H.) and the NIH (R01 CA205101 and R35 CA197078). The authors thank the University of Wisconsin Carbone Cancer Center (UWCCC) Support Grant P30 CA014520, the UWCCC Translational Research Initiatives in Pathology laboratory – supported by the UW Department of Pathology and Laboratory Medicine and the Office of The Director NIH (S10OD023526), the UWCCC Flow Cytometry Laboratory, and the Genome Editing and Animal Models Laboratory for core services.

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

Abstract 72 Figure 1 In vivo multiphoton imaging and flow cytometry of tumor and CD8 T cell populations during immunotherapy. A) Experimental workflow showing B78 flank tumor implantation and growth, administration of triple therapy, and representative in vivo
fluorescence intensity image of B78 melanoma tumor growing in a mCherry reporter mouse. Image shows mCherry-expressing 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 μm. B) *In vivo* single cell B78 tumor fluorescence lifetime data shows increased NAD(P)H protein binding in treated CD8 T cells with decreased protein binding in treated tumor cells (left panel). Corresponding *in vivo* optical redox ratio fluorescence intensity data shows redox balance within the tumor microenvironment (right panel). Both CD8 T cells and tumor cells from treated tumors exhibited a decreased optical redox ratio, compared to control tumors, indicating they are more oxidized (n = 2 mice, B78 tumor cells = 1039, CD8 T cells = 132, mean ± SD, Mann-Whitney U Test). C) *In vivo* second harmonic generation images of B78 tumors show collagen morphology and alignment without labels. Collagen fibers from control tumors show little change from Day 0 pretreatment to Day 12 of PBS treatment while collagen fibers from treated tumors show remodeling of collagen towards a healthier tissue phenotype by Day 12 of immunotherapy treatment (healthy tissue collagen is typically very tortuous while tumor tissue collagen is typically very aligned). D) Flow cytometry data of B78 tumors shows differences in immune infiltrate populations in immunotherapy treated mice at Day 12 versus PBS control mice at Day 12 (n = 4 mice per condition, average%CD45+ cells). Immunotherapy treated mice show an increase in CD8 T cells and NKTs while macrophage numbers decrease.