Title: Heat Stress drives Genetic Changes of Cancer Cells and impacts Tumoroid Growth and anti-tumor Phagocytosis

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Introduction:

Elevated temperature is common in inflammation and at least some solid tumors. Heat can alter cancer cell structure and genetics, perturbing the cytoskeleton and even mitosis (Pawlik et al., 2013; Gupta et al., 2008). Heat might drive tumor heterogeneity which is relevant, for example, to combining hyperthermia therapies with chemotherapy (Repasky et al., 2013) because surviving cancer cells will potentially evolve resistance that complicates effective treatment. Heat stress also occurs naturally in tumors; breast and lung tumors measure 2°C warmer than surrounding healthy tissue (Stefanadis et al., 2003). Given the effects of heat and its physiological relevance, it's important to clarify whether hyperthermic stress induces heritable genetic changes and contributes to cancer progression.

Macrophage immunotherapy is emerging as a possible therapy for solid tumors, because of phagocytosis. As hyperthermic treatments have been demonstrated to induce apoptosis of cancer cells and "shrink tumors" (DeNardo et al., 2008), we hypothesize that heat could impede 3D tumoroid growth and improve the ability of macrophages to phagocytose cancer cells.

We tested the impact of heat on B16 mouse melanoma cells in 2D and 3D culture to quantify the impact on genetic change at single cell resolution as well as tumor growth. To better understand how heat influences immune interactions, we co-cultured macrophages with the tumoroid during and post-stress to examine whether heat treatment altered the tumoroid environment in ways that impact macrophage infiltration and function. Engineered macrophages with knockout of the macrophage checkpoint receptor SIRP-α (receptor protein for CD47) were used to maximize phagocytosis.

Materials and Methods:

To evaluate the effects of heat stress on genomic instability in B16 mouse melanoma cells cultured in 2D, cells were seeded in six-well plates and exposed to 42 °C (severe hyperthermia) for 3 or 8 hours, then returned to 37 °C (body temperature) for a 48–72 hour recovery. Cells were then fixed, DNA-stained, and imaged via fluorescence microscopy. A recently developed chromosome reporter (ChReporter) system was used to identify genetic changes through imaging and flow cytometry. This system involves genetically engineering cell lines to express GFP fused to the Lamin-B1 gene found on mouse chromosome 18. Using ImageJ, the stressed cells were analyzed for gain/loss colonies of the GFP signal, micronuclei/enlarged nuclei formation, and cell division rate. Flow cytometry was also used to quantify the proportion of GFP-negative cells.

To assess the impact of heat stress in 3D culture, mCherry-expressing B16 cells were seeded into anti-adhesive, U-bottom 96-well plates to generate tumoroids and incubated overnight at 37 °C. After 18 hours, tumoroid formation was confirmed, and samples were exposed to 42 °C for 3 or 8 hours before returning to 37 °C. In some conditions, SIRPα-knockout conditionally immortalized macrophages (SKO) and/or TA99 monoclonal antibodies (used to opsonize melanoma cells) were added for co-culture. To assess the effect of heat on immune-mediated clearance, an additional group of

previously unheated tumoroids was heated to 42 °C for 2 hours in the presence of macrophages. Tumoroids were imaged every 24 hours via fluorescence microscopy and analyzed using ImageJ to quantify growth or reduction due to phagocytosis.

Results and Conclusions:

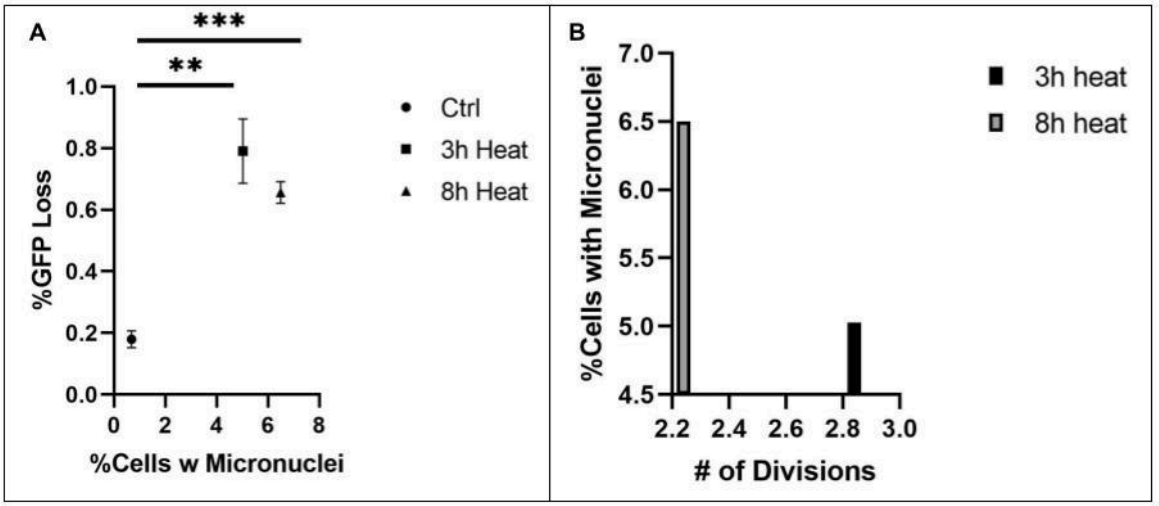

Figure 1. Impact of heat on B16 Melanoma Cells in 2D. **A)** Percent cells with micronuclei determined through image analysis graphed against percent GFP loss determined through flow. **B)** Number of divisions in each heat treated group graphed against percent cells with micronuclei as determined through image analysis.

Flow cytometry showed that cells exposed to 3 or 8 hours of heat had significantly higher rates of GFP-negative cells compared to unheated controls (Figure 1A). Imaging analysis revealed increased micronuclei in heat-exposed cells, with longer exposure correlating with higher micronuclei counts.

Slower overall growth was also observed in cells with longer exposure to heat based on the lower counts in the 8 hour heat exposure condition (Figure 1B). Yet this condition has more micronuclei at 8 hours compared to 3 hours. The increase in micronuclei rate, GFP-negative colonies, and slower overall growth in heated B16 cells supports the hypothesis that heat causes stress to cancer cells, increasing chromosomal instability and genetic heterogeneity.

In our 3D tumoroid model, 3 hours and 8 hours of heat also cause B16 tumoroids to be significantly smaller than control tumoroids after 5 days ($P<0.01$). Importantly, heat delays tumor growth with continued effects well beyond the early treatment period. This suggests that hyperthermic conditions negatively impact the ability for daughter and grand-daughter cells to proliferate and divide. Such treatments in conjunction with chemotherapy seems sensible.

Addition of macrophages and cancer-binding antibodies to tumoroids revealed that macrophages alone can suppress tumor growth after heating. When antibodies were also added, tumor growth suppression was similar regardless of heat. The results raise the possibility that heat triggers exposure or release of tumor-derived factors that polarize macrophages toward a more phagocytic

phenotype. This mechanism has been described recently for cancer cells with drug-induced micronuclei (Hayes et al., 2023). Alternatively, a persistent suppressive effect of heat on tumoroid growth can make a constant rate of phagocytosis more efficient. These possibilities require further investigation to elucidate the mechanisms by which heat influences macrophage anti-tumor activity.

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