**EFFECT OF SUB-ABLATIVE HYPERTHERMIA ON PD-1/PD-L1 AXIS MODULATION IN THE TUMOR MICROENVIRONMENT**

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**Background** Lack of intratumoral immune checkpoint receptor (ICR) expression results in resistance to immune check point inhibitors (ICIs), limiting their clinical efficacy in many patients. Here we investigate the modulatory effect of percutaneous sub-ablative hyperthermia (SA-HT) on PD-1/PD-L1 axis as a potential strategy to modulate ICR expression towards ICI responsiveness.

**Methods** A three phase experiment was performed. In phase 1, an in-vitro experiment was performed using breast adenocarcinoma R3230 cells, exposed to variable thermal doses (41, 43 and 45 all ± 1 °C) for variable durations (5- 60 min, 5 times). Cells were propagated, and supernatant and cells were collected at 0-11d (6 times) post-treatment. Soluble PD-L1 (sPD-L1) in supernatant was analyzed using ELISA. In phase 2, 3 cell lines of different species and cell type (R3230/rat, Hepa 1-6/mouse and HeLa/human) were treated with effective thermal doses (41°C x 60 mins (HT41), 43°C x 30 mins (HT43) & 45°C x 15 mins (HT45)), informed by phase 1 results. Propagated cells were collected up to 14d post-treatment and evaluated for c-Met (a potent PD-L1 regulator) and PD-L1 expression by flow cytometry. Finally, a phase 3 in-vivo validation was performed by using 6 Fisher rats with 10-12 mm R3230 tumors, randomized to hyperthermia (HT; SA-HT @ 45°C x 15 mins) (n=3) or control (n=3). Animals were sacrificed at 7d and tumors harvested for FC.

**Results** In phase 1, specific SA-HT doses demonstrated effective PD-L1 modulation. Specifically, sPD-L1 demonstrated significant decrease in comparison to control at days 2, 4 and 7-9 at a treatment of HT45, HT43 and HT41, respectively, p <0.05 for all. In phase 2, response to SA-HT varied by tumor type. R3230 cells demonstrated downregulation of cMet/PD-L1 in HT45 at 7 days of post treatment (p<0.05). However, HeLa cells demonstrated upregulation of cMet/PD-L1 in HT43 and HT45 treated cells at 7 days post treatment (p<0.03) compared to control. Moreover, Hepa 1-6 cells demonstrated upregulation of cMet/PD-L1 in HT41 at day 16, but downregulation in HT45 at days 7 & 14, when compared to control (p<0.05 for all). Interestingly, all treatment groups demonstrated significant correlation of cMet and PD-L1 expression. In phase III, HT45 resulted in increased intratumoral CD8/PD-1+ cells, and upregulation of cMet+ and PanCK/PD-L1+ cells compared to sham, p <0.05.

**Conclusions** Sub-ablative hyperthermia effectively modulates PD-1/PD-L1 axis and may be a viable adjuvant against ICI resistance. Furthermore, SA-HT PD-L1 modulation may vary based on tumor type and strongly correlates with intratumoral cMET expression.