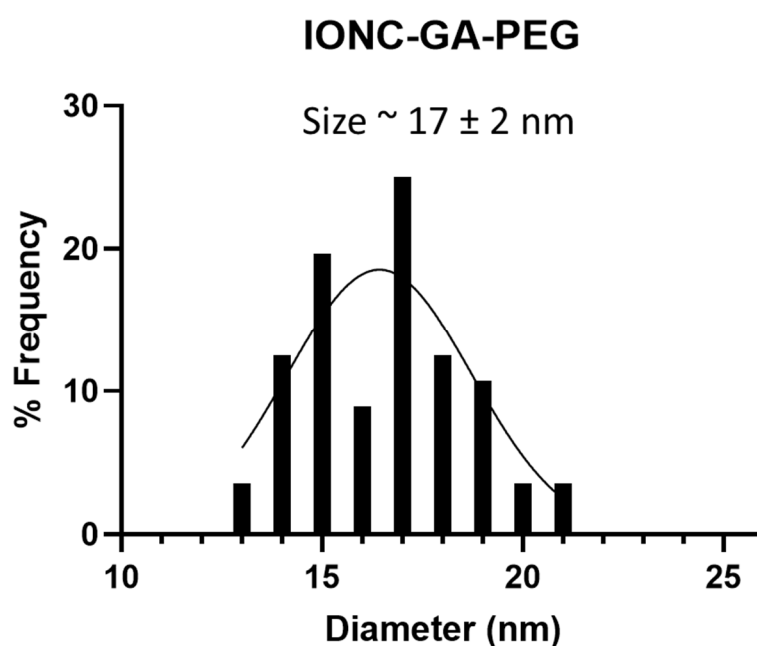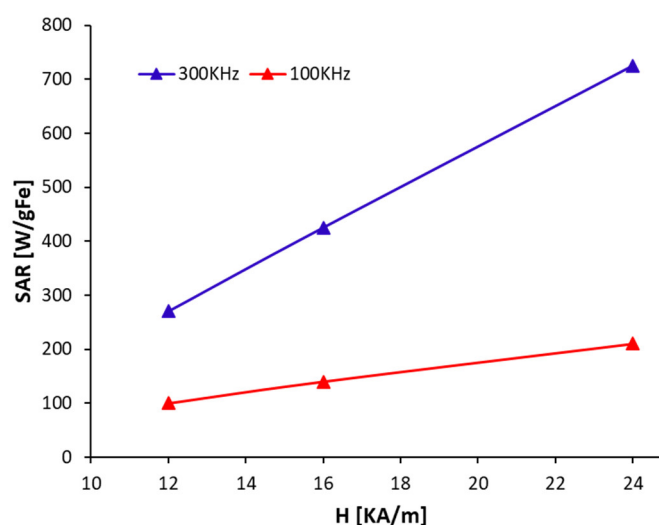# Supplementary Materials: Elucidating the Innate Immunological Effects of Mild Magnetic Hyperthermia on U87 Human Glioblastoma Cells: An In Vitro Study
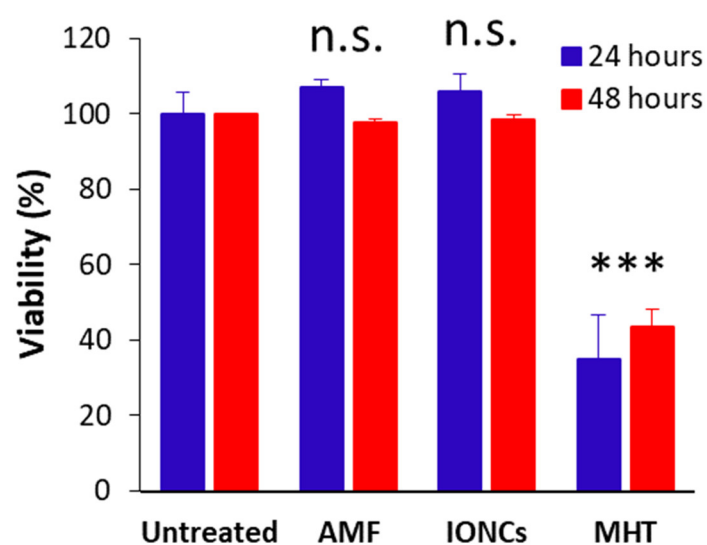
Stefano Persano, Francesco Vicini, Alessandro Poggi, Jordi Leonardo Castrillo Fernandez, Giusy Maria Rita Rizzo, Helena Gavilán, Niccolo Silvestri [1] and Teresa Pellegrino
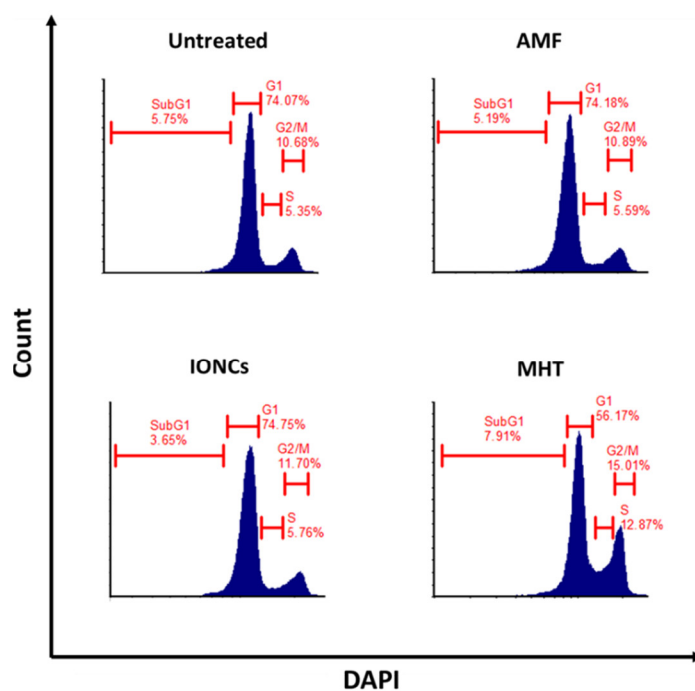

Figure S1. Histogram of size distribution and Gaussian fitting curve of IONC-GA-PEG analyzed by TEM.
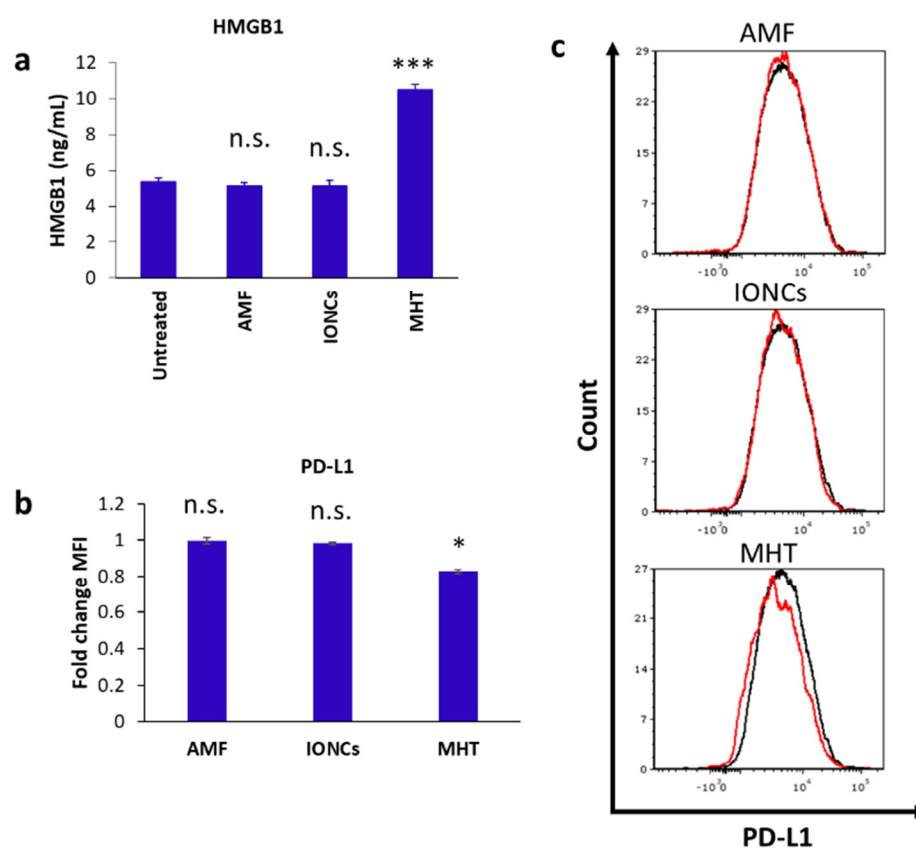

Figure S2. SAR values of IONC-GA-PEG at frequencies of 100 (red triangles) and 300 (blue triangles) kHz, and field amplitudes of 12, 16 and 24 kA/m.
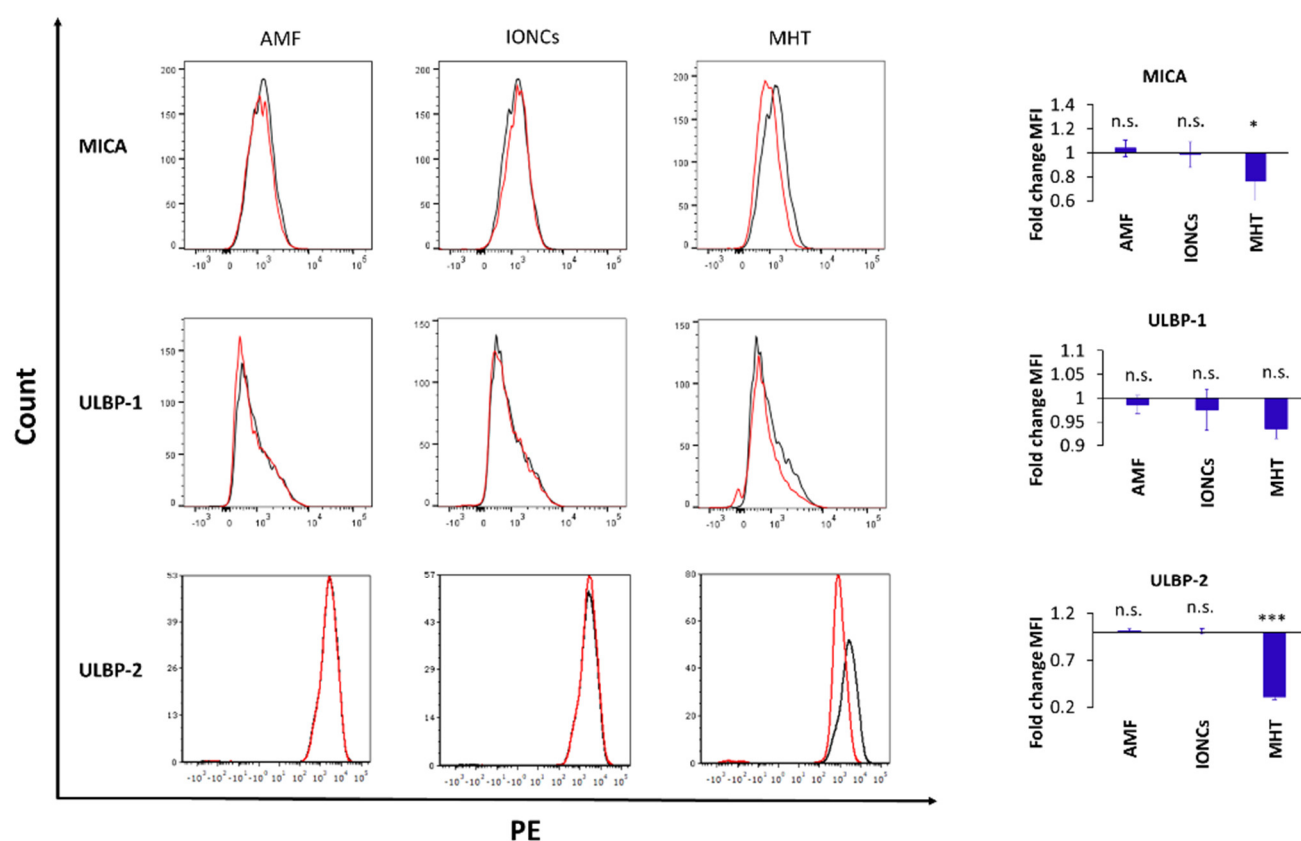
Figure S3. Evaluation of cell viability of U87 cells exposed to various treatment conditions, such as no treatment (untreated group), AMF, IONCs at a concentration of 2 g_{Fe}/L and MHT (2 g_{Fe}/L of IONCs + AMF). Cell viability was determined by CCK-8 assay at 24 and 48 hours post-treatment. Statistical analysis was conducted via two-tailed unpaired student's t test (*** $p < 0.001$; n.s.= not significant).
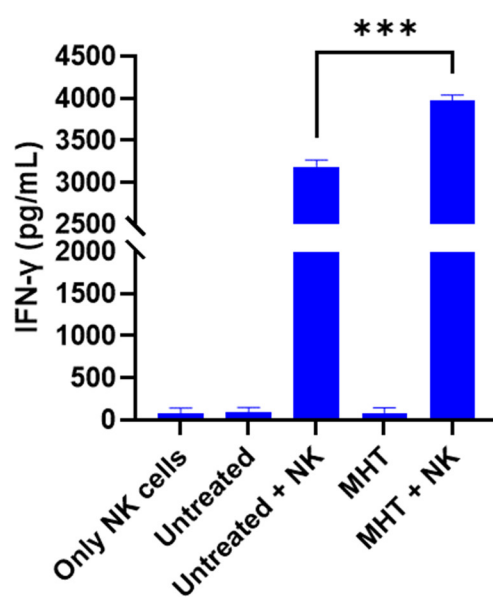

Figure S4. Flow cytometry analysis of cell cycle in U87 cells after 24 hours post-treatment with AMF, IONCs or MHT compared to untreated cells. Representative figures of cell cycle distribution (SubG1. G0/G1, S, and G2/M) showing accumulation of MHT-treated cells in S and G2/M phases.

Figure S5. Release of HMGB1 in response to MHT treatment was evaluated by ELISA (**a**). The expression of PD-L1 on the surface of untreated cells or U87 cells exposed to different treatment (AMF, IONCs and MHT) was evaluated by flow cytometry (**b**,**c**). Data are shown as expressed as average fold change (mean ± SD) of the mean fluorescence intensity (MFI) of three independent experiments (*n* = 3) and as representative histograms. Statistical analysis was conducted via two-tailed unpaired student's t test (* $0.01 < p < 0.05$; *** $p < 0.001$; n.s. = not significant).
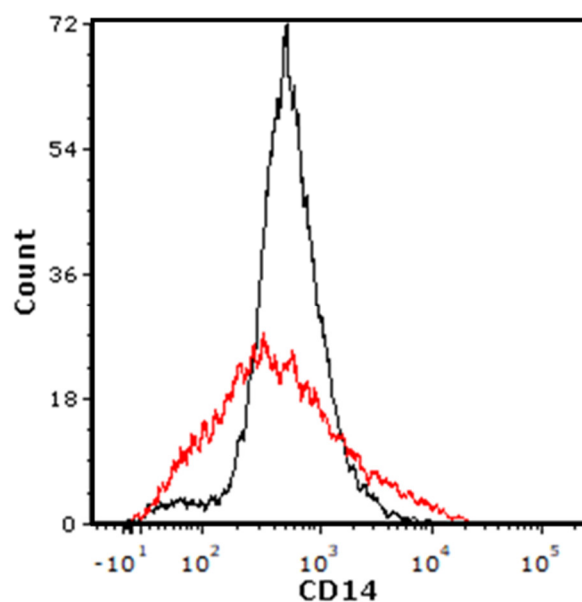
Figure S6. Flow cytometric evaluation of changes in surface expression of MICA, ULBP-1 and B7H6 in U87 cells exposed to different treatments (AMF, IONCs and MHT) compared to untreated cells at 24 hours post-treatment. Data are shown as representative histograms and expressed as average fold change (mean ± SD) of the mean fluorescence intensity (MFI) of three independent experiments (*n* = 3). Statistical analysis was conducted via two-tailed unpaired student's t test (* 0.01 < *p* < 0.05; *** *p* < 0.001; n.s.= not significant).

Figure S7. Analysis of IFN-γ secretion by IL-2-activated NK cells co-cultured with untreated or treated U87 GBM cells. Only NK cells and, untreated and MHT-treated U87 (MHT) cells alone were

included as control groups. Data are shown as mean ± SEM of three experiments. Statistical analysis was performed using a one-way ANOVA test (*** $p < 0.001$).

Figure S8. Evaluation of CD14 expression on THP-1 cells before and after macrophage differentiation by flow cytometry.