M1 macrophage-loaded hydrogels reduce the viability and proliferation of hepatocellular carcinoma *in vitro* and *in vivo*

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Statement of Purpose: Hepatocellular carcinoma (HCC) is the second most common cause of death from cancer worldwide. Although M2 phenotype macrophages can promote tumor growth and invasiveness in HCC, M1 phenotype macrophages have demonstrated the inhibition of proliferation of HCC. The clinical use of M1 macrophages for the treatment of HCC would require a delivery vehicle to localize M1 macrophages to the tumor environment. This could be accomplished by encapsulating M1 macrophages in poly(ethylene glycol) diacrylate (PEGdA) and thiolated gelatin poly(ethylene glycol) (Gel-PEG-Cys) crosslinked hydrogels that can be polymerized with macrophage encapsulation directly at the therapeutic site. In this study, we determine if M1 macrophage-loaded hydrogels can inhibit HCC growth at different doses and co-culture times. We then determine the molecular mechanism of HCC proliferation inhibition by M1 macrophage-loaded hydrogels and the *in vivo* efficacy of the hydrogels against HCC tumors in two mouse models. The development of this cell-based biomaterial device could provide a potential alternative localized therapeutic for the treatment of HCC.

Methods Hydrogels were made at 0.5% (w/v) Iragcure 2959 photoinitiator (BASF), 10% (w/v) PEGdA, and 10% (w/v) Gel-PEG-Cys. THP-1 cells (ATCC) were loaded into hydrogels and differentiated into M1 macrophages via PMA, LPS, and IFNγ stimulation. HCC cell types Hep3B and 97L and hepatic MIHA cells (ATCC) were co-cultured with hydrogels using 10 mm well transwell plates (Dot Scientific, Inc.). Analysis of co-cultures was done using LIVE/DEAD® (Invitrogen) staining, the MTT assay (Acros Organics), Griess Reagent for nitric oxide (Sigma-Aldrich), and ELISA kits for supernatant analysis (R&D Systems, Inc.). Cultures treated with TNFα, IL6 or nitric oxide were analyzed via LIVE/DEAD® staining and the MTT assay. Gene expression was determined through RNA extraction using an RNeasy Mini Kit (Quagen) followed by reverse transcription using an iScript™ cDNA synthesis kit (Bio-Rad) and rt-qPCR using a StepOnePlus Real-Time PCR System (ThermoFisher Scientific). A dorsal window chamber (DWC) nude mouse ectopic liver cancer model was used using MHCC97L cells (ATTC) labeled with GFP and imaged through a CZ LSM 710 confocal system. A subcutaneous tumor nude mouse model was used with MHCC97L cells imaged using a Xenogen IVIS *in vivo* imaging system. All animal study protocols were approved by the Animal Welfare Committee of the University of Hong Kong.

Results: THP-1 cells encapsulated in hydrogels were successfully differentiated into M1 macrophages determined by CD68 expression. Hydrogels loaded with as low as $5x10^5$ M1 macrophages/hydrogel significantly reduced Hep3B and 97L viability with a significant increase in nitric oxide, IL6, and TNFα in the supernatants after 4 days of co-culture. Increases in IL6 and TNFα were observed in the co-culture supernatants after as little as one day of co-culture. Hep3B and 97L cells demonstrated a significant decrease in viability when treated with concentrations of nitric oxide and TNFα observed in the co-culture supernatants for 24 hours, but not when treated with the same concentrations of IL6. MIHA cells co-cultured with M1 macrophage-loaded hydrogels did not demonstrate the same decrease in viability as Hep3B and 97L, and did not demonstrate a decrease in viability when treated with nitric oxide at levels observed in co-culture supernatants. Genetic analysis demonstrated that the increase in IL-6 and TNFα in co-culture supernatants was due both to the M1 macrophages and Hep3B or 97L cells. Co-cultures induced a significant increase in M1 macrophage *IDO1* expression and a significant increase in Hep3B and 97L *CASP3* expression that was not observed in MIHA cells. In the dorsal window chamber model, necrotic sites in the HCC tumor were observed after 2 days of treatment with M1 macrophage-loaded hydrogels with increased necrotic sites in metastatic areas after 8 days of treatment. In the subcutaneous luciferase model, there was a significant reduction in tumor volume after 4 weeks of treatment with M1 macrophage-loaded hydrogels. Tunnel staining indicated that remaining HCC cells in the tumor were apoptotic.

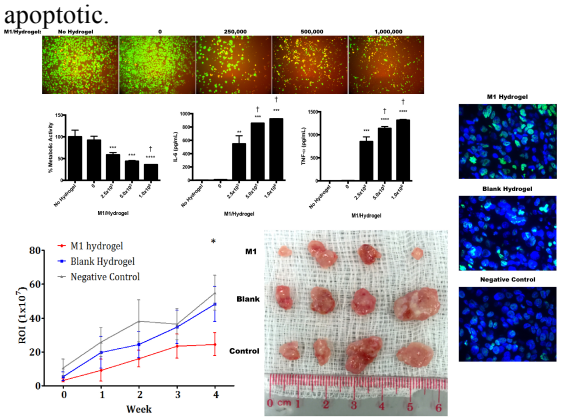

Figure 1. *In vitro* and *in vivo* efficacy of M1 macrophage-loaded hydrogels against hepatocellular carcinoma

Conclusions: M1-loaded hydrogels can induce apoptosis from the activation of caspase 3 in HCC cells through a combined production of TNFα and nitric oxide due to the upregulation of *IDO1*. Localized application of M1-loaded hydrogels reduced HCC tumor size, proliferation, and metastasis in mouse models, suggesting clinical viability of M1 macrophage-loaded hydrogels for the localized treatment of HCC.

References: Gish RG, J Clin Oncol. 2007;25:3069-3075. Yeung OWH. J Hepatol. 2015;62(3):607-616. Engstrom A. Int J Oncol. 44(2):385-392. Qi X et al. Theranostics. 2016;6(11):1934-1946. Sun et al. Carcinogenesis. 2008;29(11):2096-2105.