Intratumoral Delivery of Immunomodulatory mRNA Polymeric Nanoparticles for Breast Cancer

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Statement of Purpose: Triple negative breast cancer (TNBC) is an aggressive tumor type that exhibits recurrence, metastasis, and poor survival[1]. An antigen-free method of immunotherapy can stimulate the immune system to recruit lymphocytes, produce immunostimulatory factors, and recognize danger signals[2]. Furthermore, local delivery of immunotherapy reduces the systemic toxicity usually seen with non-specific immunotherapies while increasing potent efficacy to both the local tumors and metastases. To improve the interactions between tumor cells and cytotoxic lymphocytes, our nanoparticle platform aims to reprogram the tumor immune microenvironment (TIME) and surrounding cells to express a co-stimulatory factor and release an immunostimulatory agent, inducing tumor-associated Antigen Presenting Cells (tAPCs)[3]. The reprogrammed TIME is designed to stimulate cytotoxic lymphocytes for the treatment of primary TNBC tumors and metastases.

Methods: Biodegradable, lipophilic polymeric poly (beta-amino ester) (PBAE) nanoparticles (NPs) were used to co-deliver mRNA constructs encoding a signal 2 co-stimulatory molecule (4-1BBL) and a signal 3 immunostimulatory cytokine (IL-12), as well as the TLR9-agonist immune adjuvant CpG oligodeoxynucleotide (CpG ODN). The NPs are also formulated with PEG to increase movement in tissue. For i*n vivo* delivery, the NPs are combined with a thermoresponsive block copolymer poly(lactide-co-glycolide)–block-poly(ethylene glycol)–block-poly(lactide-co-glycolide) (PLGA–PEG–PLGA) that gels when injected into the tumor, which allows local retention of the immunomodulatory NPs. *In vitro* studies were conducted using murine 4T1 and E0771 TNBC cell lines. We extensively investigated various mRNA ratios, adjuvant ratios, copolymer concentrations, and NP doses *in vitro* to assess transfection efficiency and mRNA expression (**Fig. 1A**). For *in vivo* studies, mice were injected with 4T1 or E0771 cells in the mammary fat pad and allowed to grow for one week, and then NP treatment commenced with three NP/copolymer injections intratumorally, on days 7, 8, and 11. For *ex vivo* immune cell characterization studies, mice were sacrificed 7 days after the start of treatment, and cells from tumors and spleens were processed, stained, and analyzed using flow cytometry.

Results: An initial screen to explore the effect of various immunostimulating adjuvants on transfection and viability of 4T1 cells was successfully completed with NPs dosed at 30 w/w and 150ng/well (Fig. 1a). Adjuvants at 1:0, 1:1, 3:1, and 10:1 mRNA:adjuvant were explored with several molecules including CpG, cyclic dinucleotide (CDN) and polyinosinic-polycytidylic acid (poly(I:C)). We identified

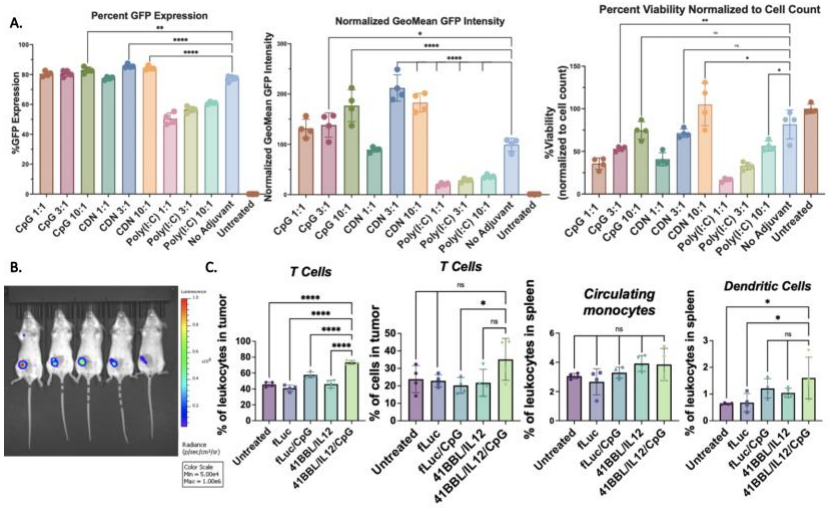

Figure 1: A) *In vitro* screen with NPs dosed at 150ng/well assessing transfection and viability at 24hrs **B)** IVIS analysis of lead NPs after intratumoral injection (i.t.). **C)** Flow cytometry analysis of immune populations in tumors and spleens at day 7 after i.t. injection with lead formulation. *=p<0.05, **=p<0.01, ****=p<0.0001)

adjuvant molecules and ratios that maintain high transfection and viability. When injected *in vivo* with the thermoresponsive polymer, the ideal formulation 3:1 mRNA:CpG adjuvant ratio NPs were sequestered locally at the tumor site, and high levels of transfection were seen at 24 hours, visualized using firefly luciferase (fLuc)) mRNA and In Vivo Imaging (IVIS) (**Fig. 1B**). We observe an increase in the general T cell population, including the $CD4^{-}CD8^{+}$ population, in the tumor and spleen, revealing improved recruitment to the primary tumor after 7 days (**Fig. 1C**). Furthermore, an increase in circulating macrophage and dendritic cell populations in the spleen suggests immune cell activation as a NP response.

Conclusions: The immunomodulatory mRNA NPs induce an anti-tumor effect after intratumoral administration, with the lead formulation successfully transfecting tumor cells *in vitro and in vivo*. Intratumorally delivering nanoparticles encapsulating mRNA encoding immunostimulatory agents and adjuvants via an injectable thermoresponsive gel has great potential for TNBC treatment.

References:[1]Seal, M. D., et al.. *Cancer J.* **2010**, *16* (1), 12–16.[2]Emens, L. A. **2021**, *27* (1), 59–66. [3]Tzeng, S. Y., et al. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117* (8), 4043–4052.