Real-time Imaging of Macrophage Immunotherapy Using a Novel Nitric Oxide Nanoreporter

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Statement of Purpose: Macrophage-centered therapeutic approaches that rely on immune modulation of tumor associated macrophages (TAMs) from a pro-tumorigenic phenotype (M2) to an anti-tumorigenic phenotype (M1) has facilitated a paradigm shift in macrophage-based cancer immunotherapy. However, limited clinical success was achieved due to low response rates observed in different types of cancers. The ability to measure immune response in real time is critical in order to differentiate responders from non-responders. However, there are currently no platforms to monitor real time macrophage immunotherapy response. Conventional imaging systems that measure anatomic readouts using CT and MRI are often not sensitive or selective to measure early response, while biomarker evaluation assays using serum samples are not reproducible. Hence there an immediate need to develop imaging techniques that can longitudinally monitor macrophage immunotherapy response. Nitric oxide (NO) produced as a result of activation of macrophages can be indicative of response. In this study, we report a NO Nano reporter design that incorporates a NO reporter element that emits at a visible wavelength used for *in vitro* characterization and a Near Infra-Red (NiR) NO reporter that is used for *in vivo* sensing.

Methods: Nitric oxide Nano reporter (NO-NR) were synthesized using a lipid film hydration technique facilitated by the self-assembly of PC, DSPE PEG and NO probe. RAW 264.7 MØ were used as *in vitro* model to monitor repolarization efficacy of different macrophage modulating drugs. Flow cytometry was used to quantify M1 and M2 markers. *In vivo* studies were performed in a 4T1 breast cancer model. The therapy consisted of administration of different macrophage modulating drugs (*anti-CSF1R, PLX, BLZ945 and iCSF1R*) along with the NO-NR. The mice were imaged at regular time points using the *In vivo* imaging system which measured the fluorescence emitted as a result of macrophage activation.

Results and Discussion: Nitric oxide Nano reporters were synthesized and was observed to be stable for extended periods of time in storage and physiological conditions. Additionally, the nanoparticle system was modified to incorporate a CSF1R inhibiting amphiphile (iCSF1R) in addition to the nitric oxide reporter in the same system to form the iCSF1R-NO-NR immunotheranostic system. Next, the screening potential of the NO-NRs was evaluated on M2 polarized macrophages treated with different drugs targeting the CSF1 axis (mentioned in the methods section). Co-administration of NO-NR along with different drug treatments revealed that NO-NRs were indeed capable of monitoring real time macrophage activation in real time by emitting fluorescence upon nitric oxide release. It was observed that while treatment with PLX showed improved repolarization at early time points, sustained repolarization was observed only on treatment with iCSF1R theranostic system. These results were further validated by flow cytometry to evaluate the M1/M2 expression profiles upon drug treatment. We next performed *In vivo* mouse studies in a breast cancer model. It was observed that there was a significance increase in fluorescent signal in mice treated with iCSF1R-NO-NR at a time point as early as 12h after the first dose. (**Figure 1**) A detectable signal in the tumor of mice treated with iCSF1R-NO-NR was nearly 3-fold greater than other treatment groups and was observed even at the end the of 5 days post treatment. These results were further validated by tumor progression data and *ex vivo* immune profiling by flow cytometry.

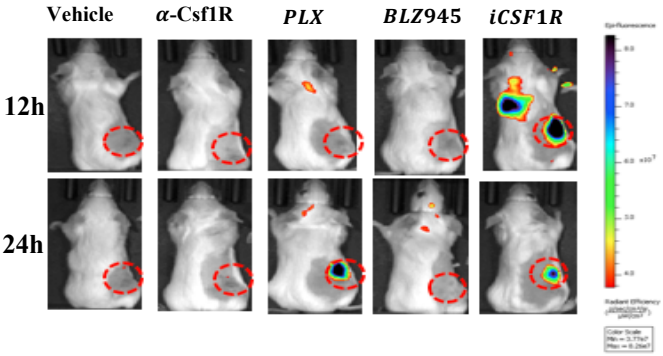

Figure 1: Representative images of mice from different treatment groups imaged at 12h and 24h post treatment. The tumors (dotted red circle) show fluorescent activation at time points when there is an enhanced anti-tumor efficacy as a result of macrophage activation

Conclusions: In summary, we have demonstrated the synthesis of a Nitric Oxide Nano Reporter (NO-NR) and an iCSF1R-NO-NR theranostic system facilitated by the self-assembly of co-lipids. The NO-NRs were as a tool to monitor the macrophage immune response in real time in *In vitro* conditions. Furthermore, macrophages treated with an iCSF1R amphiphile showed the best immunotherapeutic efficacy as an account of sustained inhibition of the CSF1R axis due to extended drug release mechanisms. These results were further validated in an aggressive murine 4T1 breast cancer model, where enhanced fluorescence signal was observed in the group treated with iCSF1R-NO-NR and signal was sustained until the end points where animals were sacrificed. *Ex vivo* studies that were performed evaluated the immune profile of tumor infiltrating macrophages which further validated the tumor progression data.