**Prussian blue nanoparticles-based nanoimmunotherapy and its application for treating neuroblastoma**

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**Introduction**

Neuroblastoma accounts for about 6% of childhood cancers and is a leading cause of cancer-related mortality in children. The overall survival of patients with disseminated or metastatic neuroblastoma over one year of age is less than 10% with conventional therapies. Thus there is an urgent need for novel therapies for this patient population. In response to this need, we have developed a nanoimmunotherapy, wherein we utilize Prussian blue nanoparticles for photothermal therapy (PBNP-PTT), which thermally ablates primary tumors releasing tumor antigens and endogenous adjuvants (including ATP and HMGB1). In addition the PBNPs are coated with immunological signals (e.g. CpG oligodeoxynucleotides; CpG) that boost antitumor immunity. These PBNP-based effects are administered in combination with checkpoint blockade immunotherapy (e.g. anti-CTLA-4) that reverses exhaustion of immune effector cells, particularly T cells. We hypothesize that these “nano” and “immune” components function in concert to eradicate primary tumors, prevent tumor recurrence, and potentiate a robust “abscopal” effect that can treat disseminated disease. We test our hypothesis in syngeneic, murine models of neuroblastoma (Neuro2a and 9464D).

**Methods**

Building on our previously published studies (Cano-Mejia *et al.* 2017, 2019; Sweeney *et al.* 2018),we assembled CpG on PBNPs using a layer-by-layer technique. *In vivo*responses were tested in syngeneic Neuro2a and 9464D models of neuroblastoma, where tumor-bearing mice were treated with CpG-coated PBNPs for PTT (CpG-PBNP-PTT) with or without checkpoint blockade (aCTLA-4). Tumor growth, survival, and immune responses were measured in both primary and synchronous (two tumor) models of neuroblastoma. Immune responses post-treatment were assessed in the tumors, spleens, and draining lymph nodes using techniques including flow cytometry, ELISPOTs, and cytotoxicity assays.

**Results and Discussion**

In the Neuro2a model of neuroblastoma, CpG-PBNP-PTT resulted in complete tumor regression in a significantly higher proportion (70% at 60 days) of treated animals relative to controls. Further, the long-term surviving, CpG-PBNP-PTT-treated animals rejected Neuro2a rechallenge suggesting that our nanoimmunotherapy generates immunological memory (Cano-Mejia *et al.* 2019). Similar results were observed in the 9464D model of neuroblastoma indicating generalizability of our approach. When used in a synchronous Neuro2a model of neuroblastoma, wherein the primary tumor was CpG-PBNP-PTT-treated, the secondary tumor received no PTT, and aCTLA-4 was administered systemically, 50% mice treated with CpG-PBNP-PTT + aCTLA-4 exhibited complete eradication of both primary and secondary tumors compared to control treatment groups, which exhibited significantly lower survival rates(less than 25%). Our findings point to the importance of simultaneous cytotoxicity, antigenicity, and adjuvanticity using CpG-PBNP-PTT in combination with aCTLA-4 immunotherapy in generating robust and persistent antitumor immune responses against disseminated neuroblastoma.

**Conclusion**

We have described a nanoimmunotherapy for neuroblastoma that combines the ablative properties of PBNPs, the immunostimulatory properties of CpG, and aCTLA-4 checkpoint blockade therapy. Our novel therapy co-localizes complementary antitumor immune effects that results in complete tumor regression and long-term survival in both primary and synchronous models of neuroblastoma.

**References**

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