**Background**

Neuroblastoma is the most common extra-cranial solid tumor occurring in children and accounts for 15% of all pediatric cancer-related deaths. While the 5-year survival rate has greatly improved, the prognosis of children with high-risk neuroblastoma at diagnosis is less than 50%. Immune checkpoint therapy is a promising new application for pediatric oncology; however, neuroblastoma is often immunologically ‘cold’ and current immune checkpoint targets are not abundant in pediatric solid tumors. To improve response to checkpoint blockade and directly target tumor cells for elimination, this study evaluated the use of Natural Killer (NK) cells. NK cells prime the immune system for a successful response to cancer immunotherapy. NK cells activated with PM21-particle technology (PM21-NK cells) are highly cytotoxic, produce IFNγ and induce PD-L1 on cancer cells. Although highly cytotoxic, PM21-NK cells express inhibitory receptors that compete for ligands that trigger NK cell lysis, such as PVR, a ligand highly expressed on neuroblastoma cells. In this study we hypothesize knockout of inhibitory receptors that compete with the activating receptor DNAM-1 will enhance their antitumor effects against neuroblastoma.

**Methods**

CRISPR-based Knockout (KO) of TIGIT, PVRIG and/or CD96 in PM21-NK cells was performed and effector functions of these gene-edited NK cells were tested. Cytotoxicity against 3D neurosphere models using neuroblastoma cell lines derived from patients with a range of clinical phenotypes was assessed and compared to wild-type PM21-NK cells using a kinetic live-cell imaging assay. Other effector functions such as cytokine production and resistance to exhaustion will be examined.

**Results**

TIGIT, PVRIG, and CD96 receptors could be knocked out alone or in double or triple combinations with on average 89% efficiency. Each receptor as a single knockout improved NK cell cytotoxicity, with TIGIT KO followed by PVRIG KO having the most profound effect. Double knockout of TIGIT and PVRIG outperformed single knockouts of those receptors, with triple knockout of TIGIT, PVRIG, and CD96 incrementally improved cell killing. The knockouts of each of the receptors could affect cytokine production and resistance to exhaustion and these will be examined.

**Conclusions**

Overall, PM21-NK cells show promise for therapeutic use against neuroblastoma, and engineering to prevent the PVR-mediated inhibitory axis signaling further enhances their function.

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**Ethics Approval**

This study was conducted in accordance with the University of Central Florida Institutional Biosafety Committee and all biological materials used were approved under BARA 19–27 and Safety Protocol SPROTO2022200000044. The University of Central Florida Institutional Review Board determined this study is not research involving human subjects (STUDY00005502, STUDY00004553 and STUDY00004071).