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A GPC2 ANTIBODY-DRUG CONJUGATE REPROGRAMS THE NEUROBLASTOMA IMMUNE MILIEU TO ENHANCE MACROPHAGE-DRIVEN THERAPIES

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Background Antibody-drug conjugates (ADCs) have emerged as an effective and safe anticancer therapy. ADCs may induce immunogenic cell death (ICD) that promotes additional endogenous antitumor immune responses. We have identified GPC2 as a target in neuroblastoma and developed an anti-GPC2 pyrrolobenzodiazepine (PBD) dimer-bearing ADC (D3-GPC2-PBD). Here, we explored whether the D3-GPC2-PBD ADC induces ICD, reprograms the neuroblastoma tumor immune microenvironment (TIME) and synergizes with other immunotherapies.

Methods Mouse neuroblastoma cell lines NXS2 and 9464D were engineered to express GPC2 to study D3-GPC2-PBD immunoregulatory properties. Induction of ICD was evaluated *in vitro* by quantifying calreticulin and HSP70/90 membrane translocation and HMGB1 and ATP release, and *in vivo* performing vaccination/re-challenge experiments. ADC modulation of the neuroblastoma TIME was studied using RNA-seq, cytokine profiling, CyTOF, and flow cytometry. ADC efficacy was tested alone and in combination with anti-CD40 agonists and anti-CD47 antagonists. We also studied ADC-mediated ICD in human neuroblastoma cells *in vitro* and elucidated synergy with macrophages and CD47 blockade *in vitro* and *in vivo* in a neuroblastoma patient-derived xenograft (PDX).

Results In GPC2-expressing murine neuroblastoma cells, the GPC2 ADC induced markers of ICD (mean ICD marker fold-change of 5.3 vs vehicle-treated controls). *In vivo*, subcutaneous vaccination with ADC-treated NXS2-GPC2 cells prevented tumor growth in 72% and 70% of mice re-challenged with naïve GPC2 isogenic and empty-vector NXS2 cells, respectively. Transcriptomic and immunophenotypic analyses of ADC-treated NXS2-GPC2 allografts revealed reprogramming of the TIME to a pro-inflammatory state. By RNA-seq, ADC-treated tumors showed upregulation of genes related to immune cell recruitment and activation (e.g., *Ccl2*, *Cd40*, *Cd80*, or *Cd69*) and cytokine arrays showed upregulation of CXCL16, CD40 and CCL12. By CyTOF and flow cytometry, ADC increased intratumor infiltration of activated CD40⁺, MHCII⁺ macrophages and CD69⁺, CD8⁺ T-cells. In this model, inhibition of antitumor macrophages and T-cells impaired ADC efficacy, whereas combination of GPC2 ADC plus CD40 agonist antibodies induced 100% of complete responses. In human neuroblastoma cells, ADC treatment induced phagocytosis of NB-EbC1 ($P=0.0004$) but not SMS-SAN cell lines, which correlated with higher calreticulin translocation in NB-EbC1 cells. The addition of CD47 blockade increased ADC-mediated phagocytosis of SMS-SAN cells ($P=0.0001$). Finally, we observed that low doses of the D3-GPC2-PBD ADC combined with CD47 antagonist antibodies significantly increased survival of mice bearing neuroblastoma PDXs compared to ADC treatment alone ($P=0.012$).

Conclusions We elucidated the immunoregulatory properties of GPC2-targeted ADCs and showed robust efficacy of this ADC in combination with rationally selected antibodies that modify host macrophage anti-tumor immunity.

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Ethics Approval Animal experiments were conducted using protocols approved by the CHOP Institutional Animal Care and Use Committee (IACUC; Approved IACUC Protocol #643) with adherence to the NIH guide for the Care and Use of Laboratory Animals accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Consent For human samples, donors provided informed consent through the University of Pennsylvania Immunology Core.

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