Paper # 2005

DEFINING THERAPEUTIC VULNERABILITIES OF PERSISTER CELLS IN HIGH-RISK NEUROBLASTOMA

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Background: High-risk neuroblastoma is a pediatric cancer arising from the developing sympathetic nervous system with a 50% relapse rate that is typically fatal. At least two subpopulations of neuroblastoma cells were previously described that can transdifferentiate, adrenergic and mesenchymal, the latter being more resistant to chemotherapy. Nevertheless, mechanisms of therapy resistance remain largely unknown.

Objectives: Identify and characterize the malignant cellls that are responsible for relapsed high-risk neuroblastoma.

Design/Method: We used single nucleus RNA and ATAC sequencing to identify and characterize the cells that survive chemotherapy, termed here “persister cells”, from a cohort of 20 matched diagnostic and post-induction chemotherapy high-risk neuroblastoma patients and two patient derived xenograft (PDX) models from diagnostic tumors. Confirmatory functional studies using flow cytometry, small molecule inhibition and Western Blot were performed in eight representative cell lines derived from neuroblastomas at diagnosis and treated with standard-of-care chemotherapy. An RNA-seq dataset of 153 high-risk neuroblastoma patients was used to validate key pathways.

Results: Residual malignant cells in the post-chemotherapy tumor samples clustered into three main groups separated by response to therapy. The most prevalent group of persister cells in responders (N=16) displayed low MYC(N) activity even in the presence of *MYCN* amplification. This group also demonstrated decreased expression of the adrenergic core regulatory circuit genes including *PHOX2B, ISL1, HAND2*, along with marked activation of TNF-alpha via NF-kB signaling. We validated decreased expression of *MYCN* (2-fold decrease, $p<0.0001$) and *PHOX2B* (3.1-fold decrease, $p<0.0001$) in PDXs following chemotherapy. MYCN protein levels were decreased and nuclear p65 levels were elevated in cell lines that survived chemotherapy. Downstream targets of NF-kB, including BCL-XL, were upregulated in persister cells in 12 of 16 responding tumors from high-risk patients. BCL-XL protein levels were elevated in cell lines following chemotherapy. Furthermore, combining chemotherapy with pharmacologic inhibition of I-kappa-kinase 2 (ML120B) resulted in increased killing of persister cells.

In addition, we classified 153 diagnostic high-risk neuroblastomas as predominantly adrenergic or mesenchymal using RNA-seq, showing that mesenchymal tumors were enriched with NF-kB pathway activation signatures. We then validated high nuclear p65 levels in 3 untreated mesenchymal cell lines. Finally, key genes of the mesenchymal signature (CD44, VIM) were upregulated in patient tumors following chemotherapy.

Conclusion: NF-kB signaling is increased in mesenchymal neuroblastoma subpopulations, induced by chemotherapy, and mediates *de novo* and acquired chemotherapy resistance in high-risk neuroblastoma. We postulate that concomitant silencing of this pathway and/or key downstream targets such as BCL-XL could eliminate persister cells and prevent disease relapse.

Paper # 2006

ARMED ONCOLYTIC ADENOVIRUS AS EXPERIMENTAL THERAPY FOR PEDIATRIC MALIGNANT GLIOMAS

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Background: Despite radiation and chemotherapy, Diffuse Intrinsic Pontine Glioma (DIPG) are the most lethal brain tumors in children. New treatment modalities are urgently needed. Our group has just finished a phase-I trial for naïve DIPG using Delta-24-RGD adenovirus (NCT03178032). The trial showed that the administration of oncolytic viruses is safe and resulted in prolonged OS, but failed to induce complete remission in a percentage of the children. GITR and OX-40 are potent T-cell activators. For that reason, we generated Delta-24-GREAT and Delta-24-RGDOX—encompassing GITRL and OX-40L, respectively— in the backbone of Delta-24-RGD. We hypothesized that the combination of T-cell activators and oncolytic viruses will result in a robust anti-tumor immune response.

Objectives: Characterize the anti-tumor effect of Delta-24-GREAT and Delta-24-RGDOX and determine the mechanisms of action.

Design/Method: We used murine (NP53, XFM, PKC) and human (TP54, TP84, DIPG IV) DIPG cell lines. Infectivity assay was determined using Ad-GFP-RGD adenoviral vector. We examined the in vitro expression of viral ligands, and the replication properties and cytotoxic activity of the armed viruses. DIPG cells were orthotopically implanted in the pons of mice. The armed oncolytic virus was injected intratumorally. Mice were followed for survival. Flow cytometry was used to examine the tumor microenvironment.

Results: Adenovirus infected more than 80% of the murine and human DIPG cells. Dose escalation experiments showed decreased viability with increasing doses of oncolytic viruses. The infection transduced efficiently GITRL and OX-40L in more than 80% of the treated cells.

NP53-bearing mice showed increased survival and 30% of long-term survivors (P=0.003, median OS PBS 25.5 days vs 35.5 days for treated mice). In agreement with these data, the survival of XFM tumor-bearing mice was prolonged upon the infection with Delta-24-RGDOX (P=0.018, median OS PBS 9 days vs 12.5 treated mice). Finally, flow cytometric analyses of the treated tumors showed increased frequency and activation of T-cell populations. Challeging experiments showed the development of an anti-tumor immune memory.

Conclusion: Delta-24-GREAT and Delta-24-RGDOX infect, replicate in, and kill glioma cells. Infection is followed by the transduction of the T-cell activators GITRL and OX-40L. Furthermore, Delta-24-RGDOX treatment displayed a superior anti-DIPG activity than parental virus and it was mediated by enhanced antitumor immune response. Our data should propel the development of clinical study to test the safety and efficacy of oncolytic viruses armed with GITRL and OX40L in patients with DIPG.

Paper # 2007

NOVEL THERAPY FOR CYTARABINE-RESISTANT AML UTILIZING THE COMBINATION OF AZD5991 AND ONC213

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Background: Resistance to cytarabine (AraC)-based chemotherapy is the main cause of treatment failure in acute myeloid leukemia (AML). Thus, new effective therapies against AraC-resistant AML are needed. The anti-apoptotic Bcl-2 family protein Mcl-1 and oncoprotein c-Myc are overexpressed in AML, associated with AraC resistance, disease relapse, and poor prognosis. Therefore, targeting Mcl-1 and c-Myc in AraC-resistant AML is a rational therapeutic option. Moreover, recent studies have demonstrated that AraC-resistant AML cells rely on oxidative phosphorylation (OXPHOS). Thus, targeting OXPHOS may represent another rational approach to combat AraC-resistant AML cells. AZD5991 is a selective Mcl-1 inhibitor and has shown efficacy in AML. Our preliminary studies demonstrated that knockdown of Mcl-1 or inhibition of c-Myc significantly enhances the antileukemic activity of AZD5991 against AML. Thus, the combination of AZD5991 with a therapeutic agent that downregulates Mcl-1 and c-Myc should enhance the antileukemic activity of AZD5991. ONC213 is a novel imipridone which downregulates Mcl-1 and c-Myc.

Objectives: To determine the *in vitro* and *in vivo* antileukemic activity of combined ONC213 and AZD5991 in AML cells with either intrinsic or acquired resistance to AraC.

Design/Method: The proposed study focuses on *in vitro* antileukemic activity of this novel combination against AraC-resistant AML cells and the underlying molecular mechanisms of action. We use MTT assays to measure viable cells, Annexin V/Propidium iodide (PI) staining and flow cytometry analysis to measure cell apoptosis, western blotting to measure protein levels, and Seahorse influx analyzer to measure OXPHOS.

Results: We found that Mcl-1 and c-Myc proteins were upregulated or unchanged in AraC-resistant AML cell lines. These resistant cell lines had decreased or no deoxycytidine kinase (dCK). ONC213 suppressed Mcl-1 and c-Myc in these AraC-resistant cell lines and could modestly overcome AraC resistance only in the presence of dCK. In contrast, ONC213 significantly and synergistically enhanced AZD5991-induced apoptosis in these cells. Knockdown of Mcl-1 significantly enhanced AZD5991-induced apoptosis which was further enhanced by c-Myc inhibition.

Conclusion: ONC213 synergistically enhances AZD5991-induced apoptosis in AraC-resistant AML cells mediated by suppression of both Mcl-1 and c-Myc. Studies are underway to determine the role of OXPHOS in the synergistic antileukemic activity of ONC213 and AZD5991 against AraC-resistant AML cells and *in vivo* efficacy of this promising combination therapy. The results of this study will form a solid foundation for the clinical evaluation of this promising combination therapy to combat AraC-resistant AML in children, which may also be applicable to AML in adults.

Paper # 2008

ENRICHMENT OF HIGH-RISK INNATE IMMUNE CELLS IN HISPANIC AND BLACK CHILDREN WITH B-ALL

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Background: Black and Hispanic children with B-acute lymphoblastic leukemia (B-ALL) experience worse outcomes compared to their non-Hispanic white (NHW) counterparts. Immune-based therapies have improved the outcomes of children with B-ALL, however, impact of racial/ethnic background on immune microenvironment is less studied.

Objectives: Characterize the immune microenvironment in the bone marrow (BM) of pediatric patients with B-ALL at diagnosis and evaluate differences in immune landscape based on race/ethnicity.

Design/Method: BM from 61 children with newly diagnosed B-ALL(Hispanic=21, Black=17, NHW=23) was obtained via the Aflac Biorepository. High-dimensional analysis was performed utilizing single cell mass cytometry with 61 markers to characterize T, NK and myeloid cells. Data was analyzed using Cytobank and high-dimensional visualization platforms such as ViSNE. Clinical data including self-reported race/ethnicity and NCI-risk classification were obtained for all samples.

Results: Multi-dimensional analysis was carried out for each cell population to dissect race/ethnicity-associated differences. ViSNE clustering of NK cells identified 3 different NK populations, including a distinct population of mature CD57+ NK cells with $Tbet^{hi}$, $HLADR^{hi}$,

granzymeB^{hi}, CD27- phenotype. The distribution of NK subsets was highly impacted by race/ethnicity. Hispanic (H) patients had higher proportions of CD57+ mature NK cells when compared with other cohorts, (40 ± 4% vs 33 ± 2%; p=0.03) with pronounced differences apparent within standard risk (SR) patients. H-SR had higher proportion of CD57+ NK cells compared to other SR patients (mean H-SR 43.4 ± 5.87% vs 26.3 ± 2.87% p= 0.0049). ViSNE clustering of myeloid cells identified 5 clusters based on patterns of cell surface markers, including a distinct CD11c+CD16+DR^{hi} inflammatory/non-classical myeloid population. Further analysis showed that NHW-SR patients have significantly lower proportions of CD16+DR+ myeloid cells compared to Hispanic, Black and NHW-HR patients (mean NHW-SR 3.67 ± 2.56% vs Others 10.8 ± 7.87% p= 0.0394). Notably, a phenotypically similar population has recently been implicated in leukemic progression in preclinical models (Witkowski et al, Cancer Cell 2020). In contrast to innate cells, T cell clusters were broadly comparable between different racial/ethnic cohorts.

Conclusion: These studies provide detailed single-cell proteomic analysis and highlight the impact of racial/ethnic background on immune microenvironment in pediatric B-ALL. Our data identify differences in innate immunity with enrichment of high-risk immune-populations in Hispanic and Black children and depletion of inflammatory myeloid populations in NHW-SR children with B-ALL. These variations may contribute to the observed differences in outcomes and may impact the application of immune therapies in racial/ethnic subsets.