

REFERENCE

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INHIBITION OF FOXP3 BY STAPLED ALPHA-HELICAL PEPTIDES ALTERS REGULATORY T CELL FUNCTION

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Background Regulatory T cells (Tregs) have been a therapeutic target of interest since early pre-clinical work revealed that their depletion led to enhanced tumor control. Despite continuing advances in the development of novel cellular-, antibody- and chemotherapeutic-based strategies to increase anti-tumor immunity, Treg presence and activity within the tumor microenvironment remains a complicating factor to their clinical efficacy. To overcome dosing limitations and off-target effects from antibody-based Treg deletional strategies, we investigated the ability to target FOXP3, the master regulator of Treg development, maintenance, and suppressive function using hydrocarbon stapled alpha-helical peptides (SAHs). We developed SAHs in the likeness of a portion of the native FOXP3 antiparallel coiled-coil homodimerization domain, in an effort to impede FOXP3 transcriptional function. SAHs overcome three major protein-protein interaction (PPI) therapeutic hurdles, namely: cellular penetrance, target specificity, and secondary structure stability. Our overall goal is to use these SAHs as investigatory drugs to demonstrate proof-of concept of the druggability of FOXP3. We aim to show their utility to further understand FOXP3 transcriptional dynamics and explore their potential use in altering the immune landscape in combination with other immune-focused therapies.

Methods Utilizing the FOXP3 crystal structure as a guide, we developed a number of single and double SAH peptides corresponding to the leucine zipper homodimerization domain (DD) of FOXP3 (SAH-FOXP3DDs). We tested the ability of SAH-FOXP3DDs to bind FOXP3, to access the intracellular compartment, to alter Treg transcriptional and phenotypic profiles, and to inhibit Treg-mediated immune suppression.

Results Select SAH-FOXP3DDs bound recombinant FOXP3 Δ N and the FOXP3 leucine zipper (LZCC) domain with high affinity and dose-dependently inhibited binding of FOXP3 to cognate DNA *in vitro*. Lead SAH-FOXP3DDs were cell permeable and showed no non-specific toxicity to T cells at high concentrations. Flow cytometric and qRT-PCR analysis of treated Tregs revealed dose-dependent changes in protein and gene expression of several FOXP3 targets suggestive of FOXP3-specific transcriptional alteration. Treatment of Tregs with lead SAHs effectively inhibited *in vitro* Treg-mediated T cell suppression and induced global gene expression changes corresponding to loss of function Foxp3 *in vivo*.

Conclusions This work supports the ability of SAHs to target transcription factors, particularly as a method of interrogating specific PPI functions, and provides strong proof-of-principle evidence that FOXP3 is druggable. SAH-FOXP3DDs will not only further our understanding of FOXP3 transcriptional

control but will serve as prototype therapeutics whereby we can explore their ability to amplify anti-tumor immunity in pre-clinical tumor models.

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DETERMINING THE EFFICACY OF ADCC BY THE 3D-EX EX VIVO PLATFORM UTILIZING TUMOROIDS OF FRESH PATIENT TUMOR SAMPLES WITH INTACT TUMOR MICROENVIRONMENT

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Background Antibody-dependent cell-mediated cytotoxicity (ADCC) is an effective tool where antibody-coated cells are targeted and killed by effector immune cells. The application of ADCC therapies has been expanded for both solid tumors as well as hematologic malignancies. However, the immunosuppressive mechanisms present in the immune tumor microenvironment (TME) pose a formidable challenge to immune cell efficacy in addition to hinderance of immune cell infiltration by tumor stromal elements. Hence, it is important to develop clinically relevant platforms to assess the efficacy of antibodies for ADCC. Here we utilized our 3D-EX platform using tumoroids of fresh patient tumor samples to assess ADCC-mediated tumor cell killing.

Methods All human tumor samples were obtained with proper patient consent and IRB approval. Fresh patient tumor tissue of various histologic types including non-small cell lung cancer (NSCLC) and colorectal cancer (CRC) was processed to generate uniform sized live 3D tumoroids measuring 150 μ m in size. Treatment groups included cetuximab alone or in combination with nivolumab and/or ipilimumab. Culture supernatants were collected for multiplex analysis of cytokine release in media. Multiplex flow cytometry was used to assess the activation profile of tumor resident immune cells in combination with high-content confocal imaging to determine extent of ADCC-mediated tumor cell death in the intact tumor extracellular matrix.

Results Using fresh patient-derived tumor organoids, we observed ADCC-dependent death of EGFR expressing tumor cells. Flow cytometric analysis of immune cell populations demonstrated treatment mediated activation of resident immune cells, which coincided with cytokine profiles determined by Luminex multiplex cytokine analysis. Additionally, tumor cell killing observed through high-content confocal imaging and quantitative image analysis showed tumor cell death with the 3D tumoroids.

Conclusions In this comprehensive study we demonstrate that the 3D-EX *ex vivo* model is a robust system to assess the efficacy of ADCC and to develop novel therapeutic combinations with other immuno-oncology therapies. Furthermore, implementation of this platform in clinical studies may also allow for determination of the most effective combinatorial immuno-oncology therapy strategies for specialized individual patient care.

Ethics Approval The study was approved by Chesapeake IRB Pro00014313.

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