Background The use of probiotic supplementation by cancer patients is increasing, including amongst those undergoing immune checkpoint inhibitor (ICI) therapy. While probiotic supplementation has been identified as an important factor influencing cancer patient responses to ICI therapy in melanoma, the underlying mechanisms of how gut probiotics shape systemic tumor immunity and thereby modulate ICI therapy efficacy remain poorly understood.

Methods We used a preclinical melanoma model to identify various probiotic bacteria capable of suppressing tumor growth, and identified the mechanism by which the host-microbial crosstalk enables the most potent tumor-suppressing strain, *Lactobacillus reuteri*, to bolster a strong spontaneous antitumor immunity and increase anti-PD-L1 therapy efficacy. We interrogated the clinical relevance of our findings in a cohort of advanced melanoma patients that either responded or failed to respond to ICI therapy.

Results Probiotic bacterium, *L. reuteri*, induces antitumor immunity and promotes ICI therapy in B16 preclinical melanoma via inducing interferon-gamma production by CD8 T cells. *L. reuteri* translocates to, colonizes and persists within melanoma tumors, and this intra-tumoral localization of *L. reuteri* is both necessary and sufficient to mediate antitumor effects in melanoma. *L. reuteri*-mediated tumor suppression occurs in a tumor- and *L. reuteri*-antigen independent fashion. The mechanism by which *L. reuteri* induces this antitumor response is via catabolization of a dietary tryptophan catabolite, indole-3-aldehyde (I3A). I3A is required and sufficient to promote antitumor immunity and facilitate ICI therapy efficacy, and it mediates antitumor immunity via activation of aryl hydrocarbon receptor (AhR) within CD8 T cells. The translational relevancy of I3A’s impact on clinical melanoma is supported by our evidence for a role of I3A in promoting anti-PD-1 immunotherapy efficacy and survival in advanced melanoma patients.

Conclusions We show that probiotic bacterium *L. reuteri* can translocate to gut-distal melanoma tumors and reveal that its presence within the tumor is required to promote antitumor Tc1 cell immunity and facilitate ICI therapy in preclinical melanoma. Collectively, our findings elucidate a critical microbial-host crosstalk between the microbial released AhR agonist I3A and CD8 T cells within the tumor microenvironment that potently enhances spontaneous antitumor immunity and facilitates ICI therapy efficacy in preclinical melanoma.

Ethics Approval Animal care and experimentation were conducted in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Approval to treat patients was obtained from the University of Pittsburgh’s Hillman Cancer Center (HCC) Institutional Review Board (No. PRO14030075), and authors attest that signed informed consent was obtained from all patients involved in the study.

Consent N/A- no sensitive or identifiable information is included in this study.
Abstracts

1311 THE MELANOMA TUMOR MICROBIOME AS A PREDICTOR OF OUTCOMES IN PATIENTS WITH METASTATIC DISEASE TREATED WITH IMMUNE CHECKPOINT INHIBITORS

1Caroline Wheeler*, 2Rebecca Hoyd, 3Aik Choon Tan, 4Niti Ulrich, 5Gabriel Tirosco, 6Dwight Owen, 7Jennifer Osa, 8Martin McCarter, 9Vineeth Sukthankar, 10Alexandra Iseguchi, 11Carlos Chan, 12Yousef Zakharia, 13Rebecca Dodd, 14Sheetal Hardikar, 15George Weiner, 16Youngchul Kim, 17Ning Jin, 18Yunzhou Liu, 19Nicholas Denko, 20Marium Husain, 21John Carpten, 22Eric Singer, 23Lary Robinson, 24William (Bill) Dalton, 25Michelle Churchman, 26Daniel Spakowicz, 27Ahmad Tarhini. 1The Ohio State University, Columbus, OH, USA; 2Moffitt Cancer Center, Tampa, FL, USA; 3University of Utah, Salt Lake City, UT, USA; 4University of Colorado, Aurora, CO, USA; 5The University of Oklahoma, Oklahoma City, OK, USA; 6University of Iowa, Iowa City, IA, USA; 7University of Iowa, Holden Cancer Center, Iowa City, IA, USA; 8University of Southern California, Los Angeles, CA, USA; 9Rutgers Cancer Institute of New Jersey, Belle Mead, NJ, USA; 10M2Gen, Tampa, FL, USA

Background Emerging evidence supports an important role for the tumor microbiome in relation to oncogenesis, cancer immune phenotype, cancer progression and treatment outcomes in a number of malignancies. In this study, we investigated the metastatic melanoma tumor microbiome and potential roles in association with clinical outcomes, such as survival, in patients with metastatic disease treated with immune checkpoint inhibitors (ICIs).

Methods Baseline tumor samples were collected from 71 patients with metastatic melanoma prior to treatment with ICIs. Bulk RNA-seq was conducted on the FFPE tumor samples. Clinical outcome following ICI treatment was evaluated as overall survival (> 24 versus < 24 months). The RNA-seq reads were processed to carefully identify exogenous sequences using ExoTIC (Exogenous sequences in Tumor and Immune Cells). Reads that did not align to the human reference genome were filtered of (1) common laboratory contaminants, (2) taxa that inversely correlate with input RNA quantity, and (3) taxa commonly found in the negative controls of microbiome experiments. DESeq2 was used to perform a differential abundance analysis on the comparison groups at every taxonomic level.

Results The 71 patients with metastatic melanoma ranged in age from 24 to 83 years, 55% were male, and 55% survived > 24 months following the initiation of ICI treatment. Exogenous taxa were identified in the tumor RNAseq, including bacteria, fungi, and viruses (figure 1). Within the tumors responsive to immunotherapy (> 24 months survival), we found a significant enrichment of several microbes, including Fusobacterium nucleatum, Porphyromonas asaccharolytica, Nocardia m(angy)en(sis), and Mollivirus sibericum. Comparatively, the cohort of non-responsive tumors (< 24 months survival) was found to have a significant intra-tumoral enrichment of Fungi, as well as the bacteria Delftia lacustris, Enterobacter hormaechei, Pseudomonas fluorescens, and Moraxella osloensis (figure 2).

Conclusions Investigating the melanoma tumor microbiome utilizing baseline tumors (prior to initiating ICI) we found significant variations in the exogenous taxa associated with patient outcomes following ICI treatment. Our findings warrant further investigations and potentially support therapeutic strategies to modify the tumor microbiome in order to improve treatment outcomes with ICIs. Ongoing research is evaluating whether these correlations are causally associated with outcomes and evaluating their effect on the tumor immune microenvironment and immune cell infiltration.

REFERENCES

Abstract 1311 Figure 1 A stacked bar plot showing the relative abundances of exogenous taxa found in tumor RNAseq. Taxa are shown on the phylum level and are ordered by the relative abundance of <i>Uroviricota</i>/i>.

Abstract 1311 Figure 2 Differential abundance analysis of taxa found within tumor RNAseq data by the exotic pipeline. Colored points represent significantly (p-value < 0.05) enriched taxa with a high (>1.00) fold-difference in abundance between the groups.


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Background Symbiotic microbes that colonize gut promote host immunity. Cancer disrupts this homeostasis to alter microbial populations (“dysbiosis”) that suppresses anti-cancer immunity. Yet, the mechanisms of how dysbiosis suppresses anti-tumor immunity remain unclear. Group 2 innate lymphoid cells (ILC2s) are innate lymphocytes that reside in tissues including the gut, respond to the alarmin interleukin-33 (IL-33), and maintain microbial homeostasis in barrier surfaces. ILC2s also activate anti-tumor immunity in multiple cancers. Yet, links between dysbiosis, ILC2s, and anti-tumor immunity, remain unexplored.

Methods To investigate if cancer dysbiosis modulates ILC2s, we examined dysbiosis in pancreatic ductal adenocarcinoma (PDAC), where dysbiosis correlates with fewer intratumoral T cells and worse survival. PDAC is also infiltrated by anti-tumor ILC2s. Briefly, we analyzed dysbiosis by 16S-rRNA gene sequencing in fecal samples of PDAC mice deficient or not for ILC2s or IL-33. To study the effect of dysbiosis on ILC2s, we ablated dysbiosis using antibiotics or reconstituted it in germ-free mice, we explored ILC2s migration using parabiosis.

Results We found that PDAC induced Bacteroidetes overgrowth, thus phenocopying PDAC dysbiosis in patients. Interestingly, PDAC-dysbiosis suppressed intestinal ILC2s frequencies, as dysbiosis ablation with antibiotics increased, and fecal transplantation in germ-free mice conversely decreased intestinal ILC2 frequencies. Reciprocally, we found that ILC2 and IL-33-deficient mice evidenced Bacteroidetes overgrowth at steady state, thus phenocopying the PDAC-induced dysbiosis. Interestingly, ILC2 and IL-33-deficient mice also evidenced accelerated PDAC growth, and worse survival compared to wild-type mice (WT). Thus, PDAC-dysbiosis suppresses intestinal ILC2s that serve to maintain optimal gut homeostasis.

We next investigated how dysbiosis-induced ILC2s suppression modulates tumor growth. We previously reported that IL-33 responsive ILC2s infiltrate PDAC to activate antigen-specific CD8+ T cells. We identified these anti-tumor ILC2s as unique migratory ILC2s that traffic to tumors. We thus hypothesized dysbiosis may promote tumors by modulating ILC2s migration from the intestine reservoir. Consistently, in parabiotic mice, recombinant IL-33 (rIL-33) induced ILC2s to migrate hematogenously to PDACs in different tissues, and antibiotic ablation of dysbiosis lowered donor-derived ILC2s frequencies in recipient blood and intestine. Thus, dysbiosis modulates anti-tumor ILC2s frequencies in circulation and gut reservoirs. Interestingly, in WT PDAC mice, rIL-33 expanded intestinal ILC2s, restored microbiome composition, increased tumor-infiltrating ILC2s, and reduced PDAC growth.

Conclusions We find that cancer dysbiosis suppresses anti-tumor immunity by suppressing gut-derived ILC2s. Moreover, rIL-33 expands ILC2s in the gut and circulation to restore dysbiosis-suppressed ILC2s and controls PDAC. We thus introduce the therapeutic potential of IL33-based immunotherapies to reverse the dysbiotic state in cancer.

REFERENCES

CHARACTERIZATION OF THE ANTI-TUMOR IMMUNE ACTIVATION POTENTIAL OF AUR107, A NOVEL SMALL MOLECULE P300/CBP BROMODOMAIN INHIBITOR


Background Ubiquitously expressed histone acetyl transferases (HAT), E1A binding protein (p300) and its paralog CREB binding protein (CBP or CREBBP) are critical regulators of gene expression in both tumor and immune cells. Conditional deletion of either p300 or CBP in mouse Tregs or inhibition of their HAT activity resulted in impairment of Treg suppressive function, reduced peripheral Treg generation, and Treg apoptosis. These effects led to allograft rejection and decreased murine tumor growth. We have identified a novel small molecule p300/CBP bromodomain inhibitor, AUR107, as therapeutic agent for solid and hematological cancers. AUR107 has significant activity in a broad range of cancer cell lines with good selectivity. Here, we demonstrate the relevance of CBP/p300 bromodomain inhibition by AUR107 on function of Tregs cells and modulation of T helper cells in addition to its potent activity against various haematological and solid tumour models.

Methods AUR107 was profiled in human Treg differentiation assay, human Th17 assay and MDSC proliferation assay. AUR107 combination efficacy studies with anti-PD-1/anti-CTLA-4 antibodies are in progress in syngeneic models

Results Inhibition of CBP/p300 bromodomains by AUR107 resulted in decrease in differentiation of human Tregs in an ex vivo assay. AUR107 caused dose-dependent increase in the CD127+CD25-FoxP3- effector cells with corresponding decrease in the CD127+CD25+ cells in the differentiated CD4+ cells population. In the human PBMC assay, AUR107 caused increase in Th1 cell population with decrease in Th2 cell population. These observations indicate that inhibition of CBP/p300 bromodomains affects the function of regulatory T cells. Recruitment of regulatory T cells to tumors is known to be one of the major mechanisms of immune evasion by cancer cells, and hence AUR107 is expected to produce antitumour immunity. These results demonstrate that CBP/p300 bromodomain inhibition could be a novel approach for cancer immunotherapy in addition to their development as direct anticancer agents.

TRILACICLIB, AN INTRAVENOUS CYCLIN-DEPENDENT KINASE 4/6 INHIBITOR, ENHANCES ANTITUMOR RESPONSES BY MODULATING T CELLS

Sarah Ahn*, John Yi, Subing Cao. G1 Therapeutics, Inc., Research Triangle Park, NC, USA

Background Cyclin-dependent kinase (CDK)4/6 inhibitors, including trilaciclib, have been shown to augment antitumor immunity.1,2 In an open-label, phase 2 trial in patients with metastatic triple-negative breast cancer (mTNBC), administration of trilaciclib prior to gemcitabine plus carboplatin improved overall survival, potentially through protection and direct activation of immune function.3,4 Here, we report the effects of transient, trilaciclib-mediated CDK4/6 inhibition on immune function in vitro.

Methods Peripheral blood mononuclear cells (PBMCs) or naïve CD4+ and CD8+ T cells were purified from 6 healthy human donors and activated with CD2/3/28 beads with or without trilaciclib. To visualize phenotypic and functional changes, trilaciclib was added to naïve CD4+ and CD8+ T cells 0-, 1-, and 3-days post-activation. Activated T cells were collected and stained for flow cytometric analyses 3-, 7-, and 14-days post-activation. Supernatant from activated PBMCs was harvested after 72 hours and added to human breast cancer cells. Following 24 hours’ incubation, levels of programmed death-ligand 1 (PD-L1) and human leukocyte antigen (HLA) class I and II were quantified by flow cytometry, and CXCL9 and CXCL10 chemokines by enzyme-linked immunosorbent assay.

Results Irrespective of when trilaciclib was added to CD4+ and CD8+ T cells, significant increases in the frequency of CD45RO+ memory T cells were observed. Within CD45RO+ memory T cells, T cells incubated with trilaciclib had increased frequencies of CD62LloCD69hi effector CD4+ and CD8+ T cells, with increases in CD62LloCCR7lo effector memory T cells also observed. Furthermore, trilaciclib significantly increased CXCL9 and CXCL10 levels (P=0.0001). Surface expression of PD-L1 and HLA class I and II was increased in breast cancer cell lines cultured with supernatant from T cells activated in the presence of 50 and 100 nM of trilaciclib, resulting in a greater frequency of cells being double-positive for HLA class I and II or HLA class I and PD-L1.

Conclusions Trilaciclib may enhance antitumor immunity by modulating essential steps in the cancer-immunity cycle. Our data suggest trilaciclib may increase antigen presentation by promoting HLA class I and II expression and the recruitment of T cells to the tumor site via CXCL9 and CXCL10. Trilaciclib also augments the differentiation of T cells by promoting the formation of memory T cells. These data support a role for trilaciclib in improving antitumor efficacy, as observed in the phase 2 trial in mTNBC, and provide a rationale to combine trilaciclib with immunotherapy to enhance immunogenicity within the tumor microenvironment.

Acknowledgements We thank Dr. Jason Grayson for immune profiling services.

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Ethics Approval This study was approved by the Wake Forest University School of Medicine’s Ethics Board under IRB #00080511.

Background. Oncovita, a biotech company spin-off from Institut Pasteur, is developing a therapeutic vaccine in oncology based on Measovir®, a proprietary technology derived from the safe and highly immunogenic measles attenuated vaccine virus (MV). MV is a paramyxovirus with a negative strand RNA genome. The MV specific tropism for cancer cells is due to the overexpression of its entry receptor CD46 on the surface of most cancer cells.

Methods. We constructed MVdeltaC, a genetically modified MV by deletion of its virulence factor C. We evaluated its immuno-oncolytic activity in vitro on over 40 human tumoral cell lines, including mesothelioma, lung adenocarcinoma, bladder, ovarian, cervical cancer and hepatocarcinoma, and in vivo by i.p. or i.t. administration in different patient-derived xenograft (PDX) mesothelioma and bladder models, and finally in a syngeneic model of neuroblastoma in immunocompetent A/J mice. The mode of action (MOA) has been investigated ex vivo using human primary immune cells.

Results. in vitro, MVdeltaC exhibited a strong oncolytic capacity which was 2 to 3 times greater than standard MV against a series of human tumor cell lines. MVdeltaC was active in more than 70% of the tested cell lines. In vivo, a single low dose given intraperitoneally in NOD/SCID mice grafted with human malignant mesothelioma induced a strong reduction of tumor mass two weeks after treatment. This potential was confirmed in PDX models of mesothelioma and bladder tumors by weekly administration of MVdeltaC. Finally, in immunocompetent A/J mice, only three i.t. administrations resulted in tumor regression and even total disappearance in 66% of mice. The animals that completely rejected the tumors were re-challenged on the other flank 3 months later with the same amount of tumor cells. No tumor growth was observed, suggesting an immune protection. In ex-vivo MOA experiments with human autologous primary cells, we demonstrated that the infection of cancer cells by MVdeltaC triggers the release of danger signals and tumor associated antigens (TAA), activation of mDC and pDC, phagocytosis of dying cancer cells and cross-presentation of TAA to autologous T lymphocytes.

Conclusions. MVdeltaC demonstrated a very efficient immuno-oncolytic activity. Based on these promising preclinical data, Oncovita plans to initiate a FIH trial in patients with solid tumors. The activity on CPI resistant tumors will be of particular interest to investigate. We have already improved the mode of production of genetically stable MVdeltaC.

A NOVEL CLASS OF T CELL-ACTIVATING ANTIBODY THAT SELECTIVELY TARGETS THE TCR B CHAIN TO PROMOTE ANTITUMOR ACTIVITY THROUGH ACTIVATION AND EXPANSION OF A NOVEL, POLYCLONAL EFFECTOR MEMORY T CELL SUBSET

1Andy Bayliffe*, 1Zhen Su, 1John Wherry, 1Jonathan Hsu, 1Madan Katraggada, 1Jacques Moisan, 1Gurkan Guntas, Karunya Srinivasan, Jessica Lowry, 1Rajesh Chopra, Roya Senvattalab, 1Wei Huang, 1Jian Tang, 1Marengo Therapeutics, Cambridge, MA, USA; 2University of Pennsylvania, Philadelphia, PA, USA; 3Apple Tree Partners, London, UK

Background Limitations with agents that enhance endogenous T cell responses to cancer, particularly in solid tumors, supports the study of alternative approaches. Directly targeting the variable (V) regions of the T cell receptor (TCR) is a novel approach to inducing T cell activation. STAR0602 is a bispecific antibody-fusion molecule that selectively activates and expands a subset of human αβ T cells expressing the germline-encoded Vb6 and Vb10 TCRs that are enriched in tumor infiltrating lymphocytes. STAR0602 simultaneously engages a novel, non-clonal mode of TCR activation with cytokine co-stimulation.

Methods The effects of STAR0602 on activation and expansion of primary human T cells was assessed in vitro by flow cytometry, homogeneous time-resolved fluorescence, TCRseq, and NanoString. A murine surrogate (mSTAR0602) was tested in murine syngeneic tumor models with tumor re-challenge and cellular depletion studies to assess potential for long-term protection and cell-specific activities, respectively. EMT6 tumors were excised for IHC staining and phenotyping of tumor-infiltrating lymphocytes (TILs) using flow cytometry and scRNAseq/TCRseq.

Results In vitro, STAR0602 induced TCR signalling and IL-2R pathway activation in human T cells that preceded expansion of Vb6/Vb10 T cell subsets to 80-90% of the T cell compartment. Compared to controls, 80-90% of STAR0602-stimulated human T cells adopted a novel, activated central memory (TCM) phenotype. In multiple syngeneic murine tumor models, mSTAR0602 monotherapy eradicated tumors, or led to substantial regressions (60-70% tumor growth inhibition) with long-term protection from tumor rechallenge. In vivo antitumor activity was dependent on the accumulation of Vb T cell subsets, and analysis of TILs showed expanded Vb T cells were almost exclusively polyclonal effector memory T cells (TEM) or TCM cells with minimal exhausted T cells or Tregs and were associated with a novel gene signature with upregulation of memory and effector programs, and downregulation of exhaustion pathways.

Conclusions STAR0602 is a first-in-class bi-specific fusion molecule that selectively binds and activates subsets of the germline TCR repertoire. In vitro, STAR0602 promotes a novel T cell phenotype with hallmarks of both effector and central memory cells, and in vivo mSTAR0602 demonstrates potent and durable single-agent anti-tumor activity in several solid tumor models that is dependent on expanded Vb T cells. The modulation of the tumor microenvironment (TME), striking increase in TCR diversity, and functional immune memory observed in murine models suggests that STAR0602 could remodel the adaptive immune response to solid tumors that are refractory to checkpoint inhibitor therapy, and thus represents a novel therapeutic strategy for patients.

CITRULLINATED GLUCOSE-REGULATED PROTEIN 78 IS A NOVEL CANDIDATE TARGET FOR CANCER IMMUNOTHERAPY

Victoria Brentville, Jia Chua, Peter Symonds, Anne Skinner, Ian Daniels, Katherine Cook*, Lindy Durrant. Scancell, Nottingham, UK

Background Post translational modification of proteins produces altered epitopes and can play a significant role in immune recognition. Citrullination is the modification of the positively charged arginine amino acid to a neutral charged citrulline residue. This modification is mediated by PAD enzymes and is increased during cellular stress (autophagy). Citrullination results in altered epitopes that can be presented upon MHC class II molecules for recognition by CD4 T cells. Citrullination also occurs in tumour cells as a result of continuous environmental stresses and increased autophagy. We have shown in animal models that the efficient stimulation of citrullinated epitope specific CD4 T cells results in dramatic elimination or regression of tumours. The ER chaperone glucose-regulated protein 78 (GRP78) is required for stress-induced autophagy and is directly linked to autophagosome formation. GRP78 is known to be highly expressed by many tumour types. In this study we investigated the potential of targeting citrullinated GRP78 for cancer therapy.

Methods In vivo experiments were performed with HLA-transgenic mice under an approved home office licence. Mice were immunised with citrullinated peptides in combination with CpG/MPLA adjuvant. Immune responses were determine using IFNγ ELISpot. Anti-tumour studies were carried out by implanting HLA-matched mouse tumour cells subcutaneously and immunising as above. Mass spectrometry analysis was performed to assess peptides presented on tumour cells. Blood samples from healthy individuals were obtained under ethical approval from the University of Nottingham. PBMC responses to citrullinated peptide were assessed using flow cytometry and proliferation assays.

Results Five peptides were selected for screening in HLA-transgenic mouse models. One citrullinated GRP78 peptide was identified that gives an CD4 T cell responses that is restricted through the HLA DP*0401 and HLA-DR*0101 alleles. In addition, this peptide is detected by mass spectrometry in B16 melanoma grown in vivo. Anti-tumour studies demonstrated that the citrulline modification specific CD4 responses to this epitope mediates efficient therapy of established B16 melanoma tumours (p<0.0001) in a HLA-transgenic HHDII/DP4 mouse model. Finally, the existence of a repertoire of responses to the citrullinated GRP78 peptide in healthy individuals has been demonstrated with 13/17 (76%) of health individuals showing a response to the peptide (p=0.0023).

Conclusions Together this data leads us to propose that citrullinated GRP78 is a candidate tumour antigen and that vaccination against citrullinated GRP78 may provide a promising approach for future tumour therapy.

A NEXT GENERATION DNA VACCINE CODING FOR THE IMMUNODOMINANT SEQUENCE OF ALPHA-ENOLASE WITH ENHANCED ABILITY TO INDUCE EFFECOTR T CELL RESPONSES TO CUREPancreatic CANCER

Silvia Brugiapaglia\*, Claudia Curcio, Daniele Giordano, Rosella Spadì, Emilia Dametto, Monica Benino, Alessandro Scaglioni, Antonio Amoroso, Paola Cappello, Francesco Novelli. University of Turin, Turin, Italy; A.O.U. Città della Scienza e della Salute di Torino, Turin, Italy

Background Pancreatic ductal adenocarcinoma (PDA) is one of the most aggressive malignancies with a 5-year survival rate of 11%.\(^1\) Only the 15% of patients have a resectable disease eligible for surgical resection followed by adjuvant chemotherapy to reduce the risk of relapse.\(^2\) The glycolytic enzyme alpha-Enolase (ENO1) has been identified as PDA associated antigen.\(^3\)\(^,\)\(^4\) A non-integrating plasmid DNA vaccine encoding for full-length human ENO1 (FL-ENO1 vaccine) was able to slow tumor progression, inducing an integrated immune response, in mice engineered to spontaneously develop PDA (KPC).\(^5\) However, in FL-ENO1 vaccinated mice myeloid derived suppressor cells and regulatory T cells arose again, leading eventually to tumor recurrence. To optimize the ENO1 vaccine, we focused the research on the identification of the most immunogenic long epitopes widely presented by HLA molecules.

Methods A library of 14 peptides covering the sequence of ENO1 was synthetized to screen healthy donors and PDA patients for their capacity to recognize fractions of ENO1 through the stimulation of T cells with ENO1 peptides. According to the proliferative response and the cytokine release, the most immunogenic sequences of ENO1 were identified and cloned into the pVax plasmid (ENO3PEP vaccine). KPC mice were vaccinated at 8 weeks and every two weeks for a total of four rounds and sacrificed at 18 weeks of age either with empty, FL-ENO1 or ENO3PEP vaccine. The presence of anti-ENO1 specific antibodies and the number of specific T cells secreting IFN-gamma in response to ENO1 were assessed respectively by ELISA and ELISpot. Pancreas tumoral areas were analyzed on hematoxylin and eosin-stained sections, while the immune infiltrate was characterized through immunohistochemistry.

Results Three portions of ENO1 emerged as immunodominant as T cells from healthy donors and PDA patients stimulated with the related peptides showed the highest proliferation index and ratio of secreted IFN-gamma/IL-10 both compared to those stimulated with full-length ENO1. In KPC mice, the ENO3PEP vaccine i) significantly reduced the pancreatic tumor lesions, ii) increased the production of anti-ENO1 antibodies, iii) enhanced the secretion of IFN-gamma by ENO1-stimulated T cells, iv) recruited more T cells at tumor site compared to FL-ENO1 vaccine.

Conclusions The ENO3PEP vaccine, coding for the most immunogenic sequences of ENO1, was able to efficiently delayed tumor progression, inducing a strong integrated humoral and cellular response, emerging as potential next generation DNA vaccine suitable for immunotherapy in virtually all PDA patients.

REFERENCES

NEXT-GENERATION TCR BISPECIFICS (TCER®)
TARGETING PePTIDE-HLA ANTIGENS FOR THE TREATMENT OF PATIENTS WITH SOLID TUMORS

Sebastian Bunk*, 1Martin Hofmann, 1Gabriele Pszolla, 1Meike Hutt, 1Felix Unverdorben, 1Frank Schwoebel, 1Nadine Aschmeine, 1Claudia Wagner, 1Maike Jaworski, 1Christoph Schraeder, 1Heiko Schuster, 1Sarah Misel, 1Toni Weinschenk, 1Dominik Maure, 2Carsten Reinhardt. 1Immatics Biotechnologies GmbH, Tuebingen, Germany; 2Immatics N. V., Tuebingen, Germany

Background T cell engaging bispecifics have emerged as a promising therapeutic opportunity for patients with solid cancers. However, challenges related to target specificity and drug safety profiles remain and many efforts are being made to generate optimized molecules with improved pharmacodynamic characteristics while reducing T cell engager-associated toxicities. We have developed a pipeline of novel bispecific molecules comprising a T cell receptor (TCR) for giving access to intracellular tumor antigens presented as peptide-HLA molecules and a unique T cell recruiting antibody aiming at conferring a favorable safety profile.

Methods We designed a novel TCR-incorporating bispecific format, called T cell engaging receptor (TCER®). TCER® molecules targeting different peptide-HLA antigens and using different recruiting moieties were generated and assessed for preclinical characteristics such as in vitro efficacy, in vitro safety and anti-tumor responses in tumor xenograft models.

Results Based on comparative preclinical testing of different TCR bispecific formats and T cell recruiting antibodies, we have developed a next-generation bispecific (TCER®) consisting of a high-affinity TCR capable of targeting tumor-specific peptide antigens and a low-affinity T cell recruiter designed to maximize efficacy while minimizing toxicity. The TCER® format harbors an effector function-silenced Fc part for the extension of serum half-life and improved manufacturability. For the development of different TCER® candidates, TCRs with promising functional avidity and high target-specificity are identified from the human repertoire and matured via yeast surface display to enhance TCR stability and to increase TCR affinity towards the target-peptide by at least 1,000-fold while retaining the target-specific binding pattern. TCER® molecules built with the matured TCRs show in vitro activity at picomolar concentrations against tumor cell lines presenting the target peptide at similar copy numbers as found on patient tumors. Further, the TCER® molecules demonstrate consistent tumor regression including complete remissions in tumor xenograft models in mice and thereby also uncovered an essential role for the type of T cell recruiting antibody. For our clinical lead TCER® candidates we confirmed a favorable in vitro safety profile with a broad therapeutic window between tumor and normal cell reactivity against more than 20 different human normal tissue cell types.

Conclusions We have developed a next-generation, half-life extended TCR Bispecific format that in preclinical tests demonstrated higher potency than multiple other established formats. By incorporating an innovative T cell recruiter we aim to reduce the risk for toxicities, specifically CRS, in patients. For each TCER® candidate we generate a robust preclinical data package before entering clinical development.

NON-CLINICAL CHARACTERIZATION OF CYT-303 FLEX-NK™ ENGAGER ANTIBODY SUPPORTS CLINICAL EVALUATION

Liang Lin*, Vishal Khairnar, Harish Potu, Hao-Ming Chang, Elisabetta Burchi, Armin Rath, Stanley Frankel, Jean Kadouche, Daniel Teper, Wei Li, Antonio Arulanandam. Cytovia Therapeutics, Natick, MA, USA

**Background** CYT-303 is a multifunctional bispecific NK engager (NKE) targeting NK cell activating receptor Nkp46 and tumor antigen Glypican-3 (GPC3) expressed in HCC (hepatocellular carcinoma). Cytovia’s proprietary FLEX-NK™ platform utilizes a novel FLEX-linker and human IgG1 back bone to allow for simultaneous binding to targeted cancer cells and NK cells. We evaluated additional CYT-303 Fc effector functions and the impact of CYT-303 when added to peripheral blood NK cells (PBNK) in Hep3B tumor spheroid cytolysis and Hep3B tumor serial killing assays. CYT-303 pharmacokinetics and safety in non-human primates were also evaluated.

**Methods** CYT-303 Fc effector function against Hep3B tumors was evaluated for antibody dependent cellular phagocytosis (ADCP) using human macrophages differentiated from purified monocytes isolated from peripheral blood and for complement dependent cytotoxicity (CDC) in the presence of rabbit complement. Hep3B tumor spheroids were established in special U-bottom adhesive plates and tumor spheroid killing assays were conducted with PBNKs and CYT-303 using the Incucyte™ Live Cell Analysis System. Serial killing assays were conducted by repeatedly adding the same PBNK cells to fresh tumor cells and CYT-303 following each round of tumor killing. CYT-303 single dose range finding pharmacokinetics and safety and 4-week repeat dose safety studies were conducted in cynomolgus monkeys by intravenous infusion dosing at 6, 20 and 60 mg/kg doses.

**Results** CYT-303 showed dose dependent ADCP by human macrophages against Hep3B tumors that was maximal at 0.4 ug/ml. CYT-303 also showed maximal CDC against Hep3B tumors at 0.4 – 2 ug/ml concentrations. CYT-303 in the presence of freshly isolated PBNKs showed increased time dependent killing of Hep3B tumor spheroids that peaked at 2-3 days following initiation of killing. This killing was enhanced in the presence of CYT-303 in a dose dependent manner. Furthermore, PBNK serial killing of Hep3B tumors was also enhanced by CYT-303. In the CYT-303 single dose range finding pharmacokinetics study in cynomolgus monkeys the Cmax and AUC0-168h values increased with dose and increases were approximately dose-proportional. CYT-303, half-lives (T1/2) ranged from 39 to 47.6 hrs and exposures persisted up to 1-week. No evidence for any cytokine release was observed. In the 4-week repeat dose toxicity study no CYT-303 related toxicities were observed, enabling CYT-303 clinical development.

**Conclusions** CYT-303 demonstrated potent ADCP and CDC against Hep3B tumors as well as Hep3B tumor spheroid and serial killing activities in the presence of PBNKs. Preclinical pharmacokinetics and safety study results in cynomolgus monkeys support CYT-303 clinical development.

A Biparatopic Anti-HER2 Antibody Enabled with Conditional 4-1BB Agonism Induces Potent Anti-Tumor Efficacy

Liandi Chen, Weifeng Huang, Xiaoni Miao, Shaogang Peng, Chao Wang, Yao Yan, Chuan-Chu Chou*, Andy Tsun, Yi Luo.

Background HER2 is a well-established therapeutic target that is overexpressed in multiple cancers. Monoclonal antibodies (mAbs) targeting HER2 such as Herceptin (trastuzumab) and Perjeta (pertuzumab) have been used in the clinic for many years. Despite good outcomes, there remains an unmet medical need that requires the further development of novel agents for recurrent or metastatic patients. The combination of HER2 mAbs have shown synergistic activity with improved clinical benefit. Moreover, biparatopic antibodies that are composed of trastuzumab and pertuzumab binding domains have shown promising results in the clinic. 4-1BB is a potent stimulator of T cells and NK cells, and when activated, can improve effector and/or memory responses. However, inherent hepatotoxicity has been observed during the clinical development of 4-1BB agonists. PM1234 is a trispecific antibody that binds to two different epitopes of HER2 (ECD4 and ECD2), and the CRD4 domain of 4-1BB. PM1234 stimulates immune cells such as T cells via HER2-mediated cross-bridging and 4-1BB activation, which results in potent anti-tumor efficacy. Moreover, Fc-effector function was shown to be essential for the in vivo anti-tumor efficacy of PM1234.

Methods PM1234 was generated as a biparatopic heterodimeric (1+1) IgG-like antibody composed of both trastuzumab and pertuzumab binding domains with anti-4-1BB VHHs fused to the C-terminus of the Fc. The immunomodulatory functions of PM1234 were evaluated using luciferase reporter cell assays, PBMC/primary T cell activation assays, and human 4-1BB Ki mouse tumor models.

Results PM1234 displayed strong HER2 binding and signal inhibition activity due to its biparatopic binding nature. The binding mode of PM1234 may allow up to double the available HER2 binding domains that can facilitate 4-1BB cross-linking and activation and was thus more potent than non-biparatopic anti-HER2 x 4-1BB bispecifics (trastuzumab x 4-1BB and/or pertuzumab x 4-1BB). PM1234 retained Fc effector function towards HER2 but with negligible activity towards the 4-1BB-targeting arm. PM1234 showed more potent activity in in vivo CT26 and MC38 tumor models than the control molecules containing Fc-silencing mutations, the combination of trastuzumab and pertuzumab, and anti-HER2-ADC. Importantly, PM1234 induced immune memory and potent anti-tumor efficacy to both HER2+ primary tumors and distal tumors without HER2 expression.

Conclusions PM1234 exhibited potent anti-tumor activity with the induction of strong immunological memory to suppress both primary HER2+ tumors and distal tumors. The differentiation of PM1234 shows the next-generation potential of HER2-targeted therapies in this competitive space and provides an insight into further improvements for benefiting patients with HER2+ tumors.

Ethics Approval All mice were maintained under specified pathogen-free conditions, and all studies were approved by the Animal Care and Use Committee of HUST-Suzhou Institute for Brainsmatics.

TARGETING IMMUNOSUPPRESSIVE MACROPHAGES AND TREGS BY REPURPOSING METABOLIC DRUGS

1Shipeng Chen*, 1Ana Milena Vizcaino, 2Yuzhen Gao, 1Baukje Nynke Hoogenboom, 1Toos Daemen, 3Cesar Oyarce. 1University Medical Center Groningen, University of Groningen, Groningen, Netherlands; 2Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China; 3Amsterdam University Medical Center, University of Amsterdam, Amsterdam, Netherlands

Background Accumulating evidence demonstrates that the immunosuppressive tumor microenvironment (TME) contributes to tumor progression and invasion, and hampers response to cancer therapies. Among the immune suppressive cells and mediators in the TME, regulatory T cells (Tregs) and M2-like tumor-associated macrophages are known to suppress tumorspecific CD8+ T cells activity and contribute to the development of an immunosuppressive TME. The differentiation/function of Tregs and the phenotype/activity of macrophages are related to their metabolism. Thus, we hypothesized that metabolic drugs could be repurposed to target these immune suppressive cells.

Methods Clinically relevant metabolic drugs were selected to target different metabolic pathways (glutaminolysis, fatty acid oxidation, and mitochondrial respiration) of human peripheral blood mononuclear cells (PBMCs)-derived Tregs/macrophages and murine bone-marrow-derived macrophages. The effect of the drugs on the differentiation and polarization of Tregs/macrophages was determined by flow cytometric analysis. And the cytotoxic activity of re-polarized macrophages was measured by co-culturing with tumor cells.

Results It was demonstrated that targeting fatty acid oxidation or mitochondria of M2-like macrophages, resulted in M2-to-M1 polarization with strong tumor-cytotoxic activity. Moreover, targeting mitochondria or glutaminolysis inhibited the differentiation of T cells to Tregs, reduced the number of the differentiated Tregs, and decreased the expression of the immunosuppressive marker without affecting the proliferation and activation of CD4+ and CD8+ conventional T cells.

Conclusions These results demonstrate that targeting the metabolism of Tregs and tumor-associated macrophages could reverse the immune suppressive tumor microenvironment into an environment that could support cancer immunotherapies. This study opens a new avenue to repurpose clinically available metabolic drugs for metabolic reprogramming of the tumor microenvironment.

CUE-102 SELECTIVELY ACTIVATES AND EXPANDS WT1-SPECIFIC T CELLS FOR THE TREATMENT OF PATIENTS WITH WT1+ MALIGNANCIES

Natasha Girgis*, Yu Christie, Zohra Merazga, Steven Hatfield, Alex Histed, Fan Zhao, Raymond Moniz, Kristin Yeung, Fulvio Diaz, Wynona Bautista, John Ross, Saso Cemerski, Anish Suri, Matteo Levisetti, Steven Quayle. Cue Biopharma, Boston, MA, USA

Background Wilms’ Tumor 1 (WT1) was ranked as the highest priority antigen for therapeutic targeting in an effort by the National Cancer Institute. Development of novel modalities targeting WT1 provide a significant opportunity to address high unmet medical need in WT1-positive malignancies, including AML, ovarian, endometrial, breast, lung, colorectal and pancreatic cancer. Leveraging the Immuno-STAT™ platform of targeted IL-2 therapies, and the ongoing development of CUE-101, CUE-102 is being developed as a novel therapeutic fusion protein to selectively activate tumor antigen-specific T cells to treat WT1-expressing cancers. CUE-102 consists of two human leukocyte antigen (HLA) molecules presenting a WT1 peptide, four affinity-attenuated human interleukin-2 (IL-2) molecules, and an effector attenuated human immunoglobulin G (IgG1) Fc domain.

Methods Cellular activity and specificity of CUE-102 were demonstrated in human PBMCs, while the in vivo activity of CUE-102 was assessed in HLA-A2 transgenic mice. HLA-A2/WT1-specific TCRs were validated and expressed in primary human CD8+ T cells. Antigen-specific cells were identified by flow cytometry using tetramer staining, activation markers and cytokine production.

Results Multiple in vitro assessments demonstrated that CUE-102 selectively binds, activates, and expands naturally occurring WT137-45-specific CD8+ T cells from PBMCs of healthy and cancer patient donors, consistent with its design. These CD8+ T cells exhibit polyfunctional and cytotoxic responses upon challenge with WT1-presenting target cells. In addition, significant functional attenuation of the IL-2 components of CUE-102 was shown, similar to preclinical results obtained with CUE-101. In vivo studies in HLA-A2 transgenic mice confirmed that CUE-102 elicits and expands polyfunctional WT1-specific CD8+ T cells from naïve and previously immunized mice without significantly altering the frequencies of other immune lineages. The WT1-specific CD8+ T cells expanded in vivo exhibit polyfunctional cytokine responses upon restimulation and selectively kill target cells presenting WT1 peptide in vivo. WT1-specific CD8+ T cells elicited in vivo by CUE-102 were detectable for >180 days following the last CUE-102 treatment, demonstrating the establishment of a long-term memory response to this tumor antigen.

Conclusions CUE-102 elicits selective expansion of WT1-specific cytotoxic CD8+ T cells both in vitro and in vivo. These results, together with its similarity to CUE-101, support its anticipated tolerability profile and potential for clinical efficacy in an ongoing Phase 1 clinical trial (NCT05360680).

Ethics Approval Studies using animals were conducted in accordance with guidelines established by the Smart Labs Institutional Animal Care and Use Committee under protocol 21SL09-0007.

INHIBITION OF ACID SENSING BY GPR65 NORMALISES GENE EXPRESSION IN MACROPHAGES, INCREASES IMMUNE CELL INFILTRATION IN TUMORS, AND RESTRAINS SUBCUTANEOUS MC38 GROWTH IN MICE

1Alastair Corbin,1 Stuart Hughes,1 Musa Quareeshy,1 Tobias Bopp,1 Barbara Cipriani,1 David Miller,1 Alan Naylor,2 Rupert Satchell,2 Sourav Sarkar,2 Gavin Knox,2 Toszka Bohn,1 Tom McCarthy,1 Pathios Therapeutics Ltd., Oxford, UK; 2Sygnature Discovery Ltd., Nottingham, UK; 3University Medical Centre Mainz, Mainz, Germany; 4Malvern Panalytical, Edinburgh, UK

Background High frequencies of Tumor Associated Macrophages (TAMs) are related to poor patient prognosis. The Tumor Microenvironment (TME) is characterised by resource scarcity, toxic metabolic by-products, and low pH, together creating an immunosuppressive environment which polarises TAMs towards a pro-tumorigenic state.

Methods We identified the proton-sensing G-Protein-Coupled Receptor 65 (GPR65) as a key determinant of low-pH-induced immunosuppression in human cancers, specifically via modulating TAM phenotype in response to the acidic TME. The importance of GPR65 in human cancers is highlighted by three key findings: (1) cancer patients homozygous for the hypomorphic I231L variant exhibit a pronounced survival benefit, (2) GPR65 and downstream pathway genes are highly expressed in innate immune cells from all human solid tumors when assessed by single cell RNA sequencing, and (3) low pH treatment of macrophages in vitro leads to a marked suppression of inflammatory genes and an upregulation of a tissue repair signature.

Results We have identified potent and selective small-molecule antagonists of human GPR65 that inhibit the low pH-induced accumulation of cAMP in recombinant cell systems and primary human macrophages with single-digit nanomolar potencies. These compounds dose-dependently prevent the low pH-driven suppression of inflammatory cytokine and chemokine genes and counteract the upregulation of pro-tumorigenic and tissue repair genes in both human and mouse macrophages.

Oral administration of our exemplar compound PTT-3213 in subcutaneous MC38 tumor-bearing mice caused gene expression changes consistent with those observed in primary macrophages in vitro, indicative of a dramatic impact on the TME. Weekly dosing of PTT-3213 significantly reduced MC38 Tumor Volume (TV) compared to vehicle (46%). This monotherapy activity was comparable to bi-weekly administration of anti-PD1, whilst combination of PTT-3213 and anti-PD-1 led to a more pronounced curtailment of TV vs vehicle-treated animals (61%). In accordance with the increased expression of chemokine genes, PTT-3213 monotherapy in MC38-bearing mice markedly elevated the frequency of tumor-infiltrating NK cells (up to 22-fold). There was also an increase in the CD8+ /CD4+ T cell ratio which attained statistical significance in combination with anti-PD-1.

Conclusions Taken together, we have identified GPR65 as a key innate immune checkpoint and therapeutic target in solid tumors and propose that macrophage conditioning via GPR65 inhibition may provide an efficacious strategy to counteract the immunosuppressive action of the acidic TME on TAMs in patients.

Ethics Approval Protocols or procedures involving the care and use of animals in studies in China were reviewed and approved by the Institutional Animal Care and Use Committee of Crown Bioscience. During studies, the care and use of animals was conducted in accordance with the regulation of the Association for Assessment and Accreditation of Laboratory Animal Care.

Studies involving the welfare and use of animals within the UK complied with the UK Animals Scientific Procedures Act 1986 (ASPA) in line with Directive 2010/63/EU of the European Parliament and Council of 22/September/2010 on the protection of animals used for scientific purposes and UK Home Office guidance on the implementation of the Act and applicable codes of practice for the care and housing of laboratory animals.

Background Metastatic castration-resistant prostate cancer (mCRPC) remains an incurable disease. Bispecific T cell engagers (TCEs) targeting prostate-specific membrane antigen (PSMA) and CD3 on T cells showed great clinical potential for the treatment of mCRPC. However, cytokine release syndrome (CRS) and poor pharmacokinetic (PK) profile hinder their further development. To overcome these challenges, Janux has developed JANX007, a tumor-activated T cell engager (TRACTr) with enhanced safety and PK properties. JANX007 is a humanized trispecific protein that contains PSMA- and CD3-binding domains, an albumin binding domain to extend circulating half-life, and a CD3 inhibitory peptide mask fused to the molecule through tumor protease cleavable linker. Only when tumor-resident proteases cleave the TRACTr and enable mask separation can the resulting active molecule bind CD3. This cleavage-dependent CD3 agonism can potentially limit systemic toxicity associated with broad T cell activation.

Methods Peptide masks against the CD3 binding domain were identified via phage display. Mask efficiency was evaluated using human CD3 ELISAs. Masking and cleavable linker stability was characterized in human (healthy and mCRPC donor) and cynomolgus monkey serum. JANX007-induced cleavage-dependent activation of T cells was evaluated in human PBMC/prostate tumor cell in vitro co-culture assays. The pharmacokinetic and safety profile of JANX007 was evaluated in non-human primate (NHP) studies.

Results Engagement of CD3 target by JANX007 was shown to be cleavage dependent where masking reduced CD3 binding by >600x. In vitro, JANX007 exhibited a >500x decrease in potency to activate T cells and induce T cell-mediated tumor cell killing relative to non-masked TCE. JANX007 was highly stable in healthy and mCRPC human donor serum, with ≤1% cleavage per day. While proteolytic cleavage of JANX007 in the tumor microenvironment is expected to drive anti-tumor activity, the maintenance of masking in the blood compartment is expected to mitigate the safety risks associated with potential off-tumor toxicity and CRS. JANX007 was found to be highly stable in NHPs with minimally detectable cleavage. The lack of TCE accumulation in NHPs mitigated on-target healthy tissue toxicities and minimized CRS. Clinical chemistry, hematology, and pathology data package support No-Observed-Adverse-Effect-Level (NOAEL) ≥1.5 mg/kg/dose. Finally, the cleavable albumin-binding domain extended the circulating half-life of JANX007 to ~120h in NHPs, relative to the 2h half-life of non-masked TCE, supporting its projected once-weekly clinical dosing.

Conclusions Cleavage-dependent activity, half-life extended PK, the potential for superior safety and manufacturability properties of JANX007 support its further development as an attractive mCRPC therapeutic.

Acknowledgements We acknowledge Marque Todd for providing insightful comments and help with the interpretation of NHP safety studies.

Ethics Approval All animal experiments were approved by the Institutional Animal Use and Care Committee of the institutions conducting the studies and in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare.

A BIFUNCTIONAL TUMOR ACTIVATED IMMUNOMODULATOR (TRACIr) TARGETING PD-L1 AND CD28 IS A POTENT ENHANCER OF T CELL-MEDIATED ANTI-TUMOR ACTIVITY

Janux Therapeutics, San Diego, CA, USA

Background While PD-(L)1 blocking antibodies have demonstrated unprecedented clinical response rates, most patients fail to respond. Preclinical studies have shown that CD28 costimulatory pathway is essential for effective PD-(L)1 therapy. However, the first phase 1 clinical trial of the CD28 agonistic antibody TGN1412 failed due to an unexpected and rapid systemic proinflammatory cytokine response. To overcome the limitation of PD-(L)1 blockade, toxicity of systemic CD28 agonism, and potential healthy tissue toxicity, we engineered a Tumor Activated Immunomodulator (TRACIr). The TRACIr is a tri-specific protein that contains PD-L1- and CD28-binding domains, an albumin-binding domain that extends circulating half-life, and an inhibitory peptide mask bound to the CD28-binding domain via a tumor protease cleavable linker. Only when tumor-resident proteases cleave the TRACIr and enable mask separation can the resulting active molecule bind CD28. This cleavage-dependent CD28 agonism can potentially limit systemic toxicity while enhancing the activity of T cells in the tumor.

Methods Peptide masks against the CD28 binding domain were identified via phage display. Mask efficiency was evaluated using CD28-specific ELISAs. The functional engagement of TRACIr binding arms was evaluated in bioluminescent PD-L1/CD28 cell reporter assays. TRACIr-induced T cell activation was evaluated in human PBMC/tumor cell co-culture assays. Functional activity was confirmed in human renal (RCC) and non-small cell lung cancer (NSCLC) patient-derived TILs. In a mouse model of triple-negative breast cancer (TNBC; MDA-MB231), the cleavage-dependent antitumor activity of TRACIr was demonstrated in combination with CD3 stimulation. The pharmacokinetic and safety profile of TRACIr was evaluated in non-human primate studies.

Results Non-masked PDL1xCD28 bispecific molecule exhibited potent binding to PD-L1 (1 nM KD) and CD28 (3 nM KD). While the presence of the mask decreased binding to CD28 by >1,000x, PD-L1 binding remained unaffected. PD-L1 blocking activity was comparable with atezolizumab, avelumab, nivolumab, and pembrolizumab. In contrast, CD28 agonistic activity was significantly compromised by the presence of the mask. Moreover, the TRACIr enhanced activation of peripheral blood T cells and TILs was signal 1- and cleavage-dependent and superior in magnitude compared to anti-PD-(L)1 and anti-CD28 monoclonal antibodies. In the TNBC tumor model, TRACIr dramatically enhanced the antitumor activity of a CD3 targeted bispecific antibody in a cleavage-dependent manner. Finally, TRACIr was well tolerated in NHPs at high doses and exhibited half-life extended pharmacokinetics.

Conclusions Preclinical activity and safety profiles of PDL1xCD28 TRACIr support its further development as an attractive bifunctional T cell modulator.

Acknowledgements We acknowledge Marque Todd for providing insightful comments and help with interpretation of NHP safety studies.

Ethics Approval All animal experiments were approved by the Institutional Animal Use and Care Committee of the institutions conducting the studies and in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare.

Background General controlled nonderepressible 2 (GCN2) is a central kinase in the integrated stress response (ISR) that responds to amino acid deprivation. Cancer cells can utilize the ISR for survival, but prolonged or hyperactivation of the ISR reduces proliferation and induces apoptosis. We are developing HC-7366, a First-in-Class, First-in-Human GCN2 modulator that activates GCN2, resulting in anti-tumor activity. HC-7366, currently in a phase 1 trial (NCT05121948), has demonstrated robust efficacy in multiple pre-clinical solid tumor and AML models.

Myeloid-derived suppressor cells (MDSC) inhibit anti-tumor T cell immunity and promote metastatic spread. Immature myeloid cells are also marked by ISR activation. We hypothesized that HC-7366 treatment could further activate the ISR in MDSCs, leading to cell death or reduced suppressive function and improving anti-tumor immunity. To test this, we used the 4T1 murine breast cancer model, which is characterized by expansion of MDSCs that facilitate lung metastasis.

Methods 4T1 cells were orthotopically transplanted into BALB/c mice. Tumor volume was monitored, and tissues or blood were collected at various timepoints for flow cytometry, IHC, or JESS analysis. Mouse bone marrow derived MDSCs were cultured with T-cells in the presence of HC-7366 in vitro, and anti-proliferative function was evaluated.

Results HC-7366 treatment showed consistent anti-metastatic efficacy, reducing lung metastases by an average of ~75% across multiple studies. Primary tumors and metastases in treated mice demonstrated GCN2 pathway activation by increases in downstream signaling proteins, including the amino acid biosynthesis proteins ASNS and PSAT1. Anti-tumor efficacy was correlated with significantly decreased Ly6G+ PMN-MDSC frequency in the lungs, spleen, and blood. Additionally, significantly increased expression of the activation markers CD86 and MHCII was observed on PMN-MDSC in both lungs and spleen. Lungs also showed significantly increased T-cell and NK-cell infiltration, activation, and proliferation as measured by increased expression of IL-2, Ki67, T-bet, and Granzyme B. HC-7366 treatment also significantly reduced the S100A8/A9 calcium binding proteins in CD11b+ cells in both metastatic and normal lung tissue, which have been implicated in facilitating MDSC recruitment and proliferation. Reductions in S100A8/A9 were also detectable in PBMCs isolated from peripheral blood and plasma. In vitro suppression assays of bone marrow derived MDSCs co-cultured with T-cells in the presence of HC-7366 showed reduced Arginase 1 expression and T-cell inhibition.

Conclusions Collectively, these data demonstrate the anti-metastatic efficacy of HC-7366 and its inhibitory effects on MDSCs, outlining its potential as a monotherapy and in combination with other immunotherapeutics to treat MDSC-enriched metastatic cancers.

Ethics Approval All in vivo experimental procedures were performed in accordance with the NIH Guide for Care and Use of Animals and were approved by the Institutional Animal Care and Use Committee of University of Minnesota. IACUC protocol: 2009A38458

A NOVEL HUMANIZED T CELL-ENGAGING CD24xCD3 BISPICIFIC ANTIBODY FOR CD24-POSITIVE SOLID TUMORS

Madelyn Espina-Cotton*, Hong-fen Guo, Nai-Kong Cheung. Memorial Sloan Kettering Cancer Center, New York, NY, USA

Background CD24 is a small, heavily-glycosylated glycosyl-phosphatidylinositol (GPI)-anchored protein. It is overexpressed in a variety of adult solid tumors, including breast, ovarian, pancreatic, and colorectal cancers, as well as pediatric cancers such as neuroblastoma, Wilms tumor, and desmoplastic small round cell tumor (DSRCT). In normal tissue, CD24 is expressed transiently during development but is largely absent from mature, differentiated cells. Functionally, CD24 serves as a “don’t eat me” signal to macrophages, binding to Siglec-10 and preventing phagocytosis. It is known as a marker of cancer cell stemness and dysregulates numerous signaling pathways involved in proliferation, invasion, and metastasis. The presence of CD24 has been strongly correlated to poor clinical outcome for multiple tumor types. For these reasons, CD24 is a strong candidate for targeting with T cell-engaging bispecific antibodies (BsAbs).

Methods We used immunohistochemical (IHC) staining on patient specimens and flow cytometry on cancer cell lines to evaluate CD24 expression in a variety of tumor types. We then generated a panel of CD24xCD3 BsAbs of different formats including a humanized IgG-[L]-scFv BsAb and assessed purity and stability using high-performance liquid chromatography. To evaluate the ability of the BsAbs to engage T cells against CD24-expressing tumor cell lines, we performed standard chromium release assays. We used immunocompromised mice bearing luciferase-transduced intraperitoneal xenografts to test the ability of the BsAbs to redirect T cells to CD24-expressing tumor cells in vivo, and monitored tumor growth using in vivo bioluminescent imaging.

Results CD24 was found to be strongly expressed by DSRCT, NB, rhabdomyosarcoma, Ewing sarcoma, mesothelioma, liver cancer, breast cancer, ovarian cancer, and pancreatic cancer. CD24xCD3 BsAbs had high purity (<90%) and were stable for several weeks at 40 C. The humanized CD24xCD3 IgG-[L]-scFv BsAb retained binding ability, indicating that the humanization process did not affect its affinity for CD24 on tumor cells. All the CD24xCD3 BsAbs induced T cell-mediated cytotoxicity against DSRCT cell lines in vitro. However, as we have shown previously with BsAbs targeting other tumor antigens, the IgG-[L]-scFv format is superior in vivo, completely ablating established intraperitoneal DSRCT and ovarian cancer xenografts.

Conclusions CD24 is expressed on a variety of solid tumors and is a viable target for T cell-engaging BsAbs. A novel humanized CD24xCD3 BsAb built on an IgG-[L]-scFv platform is effective at clearing disseminated intraperitoneal CD24-positive xenograft tumors. This strategy warrants further study and could eventually be tested in human trials for patients with advanced tumors.

REFERENCES
ULTRA-PH SENSITIVE NANOPARTICLES INCREASE THERAPEUTIC INDEX OF IL-2-FC

Qi Gang, 1Sang Huang, 1Raymundo Pantjoja, 1Zhichen Sun, 1Wei Li, 1Kathy Torres, 1Jonathan Wilhelm, 1Zinzong Chen, 1Tian Zhao, 1Ruslan Han, 1Jinming Gao. 1University of Texas Southwestern Medical Center, Dallas, TX, USA; 2OncoNano Medicine Inc, Southlake, TX, USA

Background Aldesleukin (IL-2) is clinically approved for the treatment of melanoma and renal cancer, but its use is restricted by short half-life and dose limiting toxicities. Protein engineering and produg approaches are under extensive investigation but with limited success. 1 Fc fusion of biivalent IL-2 (IL-2-Fc) increases the half-life and efficacy of IL-2 when administered intravenously, but also elevates toxicity compared to IL-2. We developed ON-BOARD, an ultra-pH sensitive nanoparticle technology for tumor-targeted delivery of drug payloads, including biologics to acidic tumor microenvironment (TME). 2, 3 The clinical safety and feasibility of ON-BOARD has been demonstrated by the effective delivery of fluorophores to solid tumors for imaging of multiple cancer types in Phase II clinical trials by pegilasstatane. 4 Herein we report ONM-405, an ON-BOARD-encapsulated IL-2-Fc, which is designed to mask toxicity in normal tissues after systemic administration, while achieving pH-activable release of IL-2-Fc at the tumor site against solid cancers.

Methods ONM-405 was formulated through non-covalent self-assembly. pH-dependent protein release was analyzed by fast protein liquid chromatography. Anti-tumor efficacy was evaluated by tumor growth inhibition and survival in murine cancer models. Toxicity was evaluated through body weight loss, cytokine release syndrome, lung edema and major organ histology in mice and cynomolgus macaques.

Results ONM-405 shows pH-dependent protection and release of IL-2-Fc. Intravenous injection of ONM-405 in mice demonstrates dramatically reduced toxicity and similar antitumor efficacy compared to IL-2-Fc (figure 1). The maximum tolerated dose (MTD) of IL-2-Fc was determined as 2.1 mg/kg, Q2D×3. Using the same IL-2-Fc equivalent dose, mice treated with ONM-405 show no body weight loss, >100-fold reduction in systemic IFN-γ compared to unencapsulated IL-2-Fc, and undetectable lung edema. At 9-fold higher doses over the MTD of IL-2-Fc (i.e., equivalent IL-2-Fc dose of 18.9 mg/kg), ONM-405 only displayed minor, reversible adverse responses such as temporary body weight loss. In tumor-bearing mice, ONM-405 inhibited tumor growth at 0.14 mg/kg in immune hot MC38 tumors and 0.7 mg/kg in cold B16F10 tumors, which are comparable to IL-2-Fc. At a dose of 6.3 mg/kg (3-fold higher than MTD of IL-2-Fc), ONM-405 achieved complete response in 5/7 MC38-bearing mice. ONM-405 has significantly widened therapeutic window (>135-fold, 0.14-18.9 mg/kg) compared to IL-2-Fc (15-fold, 0.14-2.1 mg/kg). The enhanced safety profile of ONM-405 is further validated in non-human primates.

Conclusions ON-BOARD technology offers an effective tumor-directed on/off switch achieving antitumor efficacy in solid tumors in response to the acidic TME, while minimizing systemic toxicity of IL-2-Fc in normal tissues.

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REFERENCES

Abstract 1329 Figure 1 ON-BOARD polymer encapsulation achieves anti-tumor efficacy while significantly reducing the toxicity of IL-2-Fc. (A) Tumor growth curve of B16F10 tumors treated with IL-2-Fc and UPS encapsulated IL-2-Fc (ONM-405) intravenously. (B) Dose escalation studies of ONM-405 in MC38 tumor models after intravenous injection. Significant tumor growth inhibition was observed, and 5/7 complete responses was achieved at 6.3 mg/kg IL-2-Fc equivalent dose. CR: complete remission. Arrows indicate the dosing time in A and B. C, Change of body weight over 6-day treatment at 2.1 mg/kg IL-2-Fc dose. D, Change of body weight over 6-day treatment at 2.1 mg/kg IL-2-Fc dose. E, Change of body weight over 6-day treatment at 2.1 mg/kg IL-2-Fc equivalent dose. F, Change of body weight over 6-day treatment at 2.1 mg/kg IL-2-Fc equivalent dose. Statistical significance was analyzed by two-way or one-way ANOVA.

***P<0.001, ****P<0.0001, ns: not significant.

CHARACTERISING THE IMMUNOTHERAPEUTIC CAPABILITIES OF BACTERIAL OUTER-MEMBRANE VESICLES

Jack Firth, Jingjing Sun, Jiandong Huang, Kenneth Gustafsson, Mona Bajaj-Elliott.
UCI, London, UK; University of Hong Kong, Hong Kong, Hong Kong

Background Outer-membrane vesicles (OMVs) are highly immunogenic particles shed by Gram-negative bacteria, utilised for a variety of functions from nutrient acquisition to antibiotic resistance. Their potent immunogenicity, in combination with an inability to replicate, has led to these vesicles being developed as a novel class of vaccines. Increasing evidence also suggests that the innate immune activation stimulated by OMVs can facilitate the recognition and destruction of malignant cells, inducing a sustained elimination of tumours in various animal models. At present however, the mechanistic pathways underlying the anti-tumour response remains poorly understood. Herein, we sought to investigate OMV-mediated immune interactions, elucidating key cells able to be leveraged in the context of a potential immunotherapy.

Methods OMVs were isolated from a hypervesiculating E.coli K-12 MG1655 strain expressing penta-acylated LPS, achieved via pal and lpxM deletions. Co-cultures were performed using peripheral blood mononuclear cells (PBMCs) from healthy donors, with cytokine and cell marker expression determined using ELISA and flow cytometry. Negatively-isolated OMV-activated lymphocytes were co-cultured with various cancer cell lines, and cytotoxicity investigated using the MTS assay and flow cytometry.

Results Our findings demonstrate that MG1655 Δpal ΔlpxM OMVs induce a broad increase in activation markers on NK cells, αβ T cells and γδ T cells. We observed a concordant release of IFN-γ and granzyme B, suggesting the cells exhibit a cytotoxic phenotype upon OMV stimulation. γδ T cells were found to be the predominant cell type to proliferate, expanding from 3% to 40% of the total lymphocyte population. Noticeably, the majority of γδ T cells were of the Vγ9Vδ2 type, which possess the ability to respond to both bacterial metabolites as well as stress markers present on malignant cells. Since Vγ9Vδ2 T cells present an MHC-independent innate-like activation mechanism, they are well positioned to respond to OMV stimulation whilst maintaining oncolytic capabilities. Indeed, we observe robust cytolytic activity of Vγ9Vδ2 T cells against both breast cancer and leukaemia cell lines (SkBr3 and Nalm6 respectively) after OMV-mediated expansion. These data therefore identify Vγ9Vδ2 T cells as able to directly respond to OMV-stimulus whilst maintaining anti-tumour capabilities.

Conclusions Our findings support the hypothesis that Vγ9Vδ2 T cells are a crucial component of the OMV-mediated anti-tumour immune response, cells that may be used to improve future immunotherapies.

REFERENCE


Ethics Approval Human PBMC were purchased from Cambridge Bioscience. According to their site, Cambridge Bioscience source human material in partnership with Research Donors, an HTA-licensed clinic, based in London, dedicated to the collection and processing of human blood and fresh leukopaks for research purposes. Research Donors is ISO 9001 2015 certified with Research Ethics (REC) approval as a Research Tissue bank, and participates in the UK NEQAS QA scheme.

Background Prostaglandin E2 (PGE2) is a bioactive lipid produced by tumor cells that drives disease progression through stimulating tumor proliferation, enhancing angiogenesis and suppressing immune function in the TME. PGE2 is also a mediator of adaptive resistance to immune checkpoint inhibitor therapy via the upregulation of cyclooxygenase-2 (COX-2). While the role of PGE2 signaling in cancer is clear, how best to inhibit PGE2 for cancer treatment remains under investigation. Inhibition of COX-1 and/or COX-2 has shown promising results in observational studies and meta-analyses, but inconsistent results in prospective studies. While COX-2 and single EP inhibitors continue to be developed, the nature of PGE2 signaling supports our rationale to inhibit PGE2 by dual antagonism of the pro-tumor EP2/EP4 receptors, while sparing the pro-immune EP1/EP3 receptors. To our knowledge, TPST-1495 is the first clinical-stage dual inhibitor of both the EP2 and EP4 receptors.

Methods We utilized in vitro murine and human whole blood assays to isolate individual effects of EP inhibitors, as well as multiple syngeneic, xenograft, and GEM models to elucidate the effects of PGE2 pathway antagonism in vivo.

Results In mouse and human blood assays, dual blockade of EP2 and EP4 receptors with TPST-1495 reversed PGE2-mediated suppression of T cells and monocytes, while single receptor antagonists were unable to block suppression at higher PGE2 concentrations. In vivo, TPST-1495 monotherapy significantly reduced tumor outgrowth in five of seven syngeneic, xenograft and genetically engineered mouse models. CT26-bearing mice treated with TPST-1495 showed significant increases immune cell infiltration by CD8+, CD4+ and NK cells, and increased M1:M2 ratio among macrophages. APC-min/+ mice treated with TPST-1495 displayed almost complete reduction in tumor burden, which was not observed with other PGE2 pathway inhibitors, and increased in immune cell presence as demonstrated by histopathology. Transcriptional analysis of resected tumors demonstrated an increase in interferon gamma signature, as well as an increase in a gene profile associated with PGE2 inhibition.

Conclusions These results demonstrate the redundancy of EP2 and EP4 receptor signaling and the requirement for EP2 and EP4 to be blocked to achieve full therapeutic effect of PGE2 inhibition in tumors. The data further define the simultaneous effect of TPST-1495 on immune and non-immune compartments that lead to tumor regression. TPST-1495 is currently being evaluated in an ongoing Phase 1 first-in-human study (NCT04344795) to characterize PK, PD, safety, and to identify a recommended phase 2 dose for expansion cohorts in key indications and biomarker-selected patients.

REFERENCES


Ethics Approval All murine studies were performed in accordance with human animal protocols guided by an IACUC.

ANTI-CD161 ANTIBODY IMT-009 IS A NOVEL IMMUNOTHERAPEUTIC AGENT THAT REINVIGORATES T AND NK CELL FUNCTION AND ANTI-TUMOR EFFICACY THROUGH BLOCKING INTERACTION OF CD161 WITH ITS LIGAND CLEC2D

Alexandria Fusco, Elizabeth Scanlon, Franco Irvine, Flavian Brown, Jeffrey Colbert, Andy Tu, Stephanie Gaerlan, Kelly Nichols, Teresse de Rham, Matthew Huggins, Kendall Dionne, Ming Tang, Heather Flick, Alkon Tisdale, Seng-Lai Tan, Shruti Malu*. Immunitas Therapeutics, Waltham, MA, USA

Background The CLEC2D/CD161 axis is a novel ligand-receptor pathway for immunotherapeutic intervention. IMT-009 is a monoclonal, aglycosylated human IgG1 antibody directed against CD161, a C-type lectin-like receptor, which is broadly expressed on NK cells and subsets of both CD4+ and CD8+ T cells [Mathewson et al. 2021]. Its cognate ligand, CLEC2D (LLT1), is expressed on the surface of both malignant cells and immune cells, including activated B cells and myeloid cells.

Methods Functional inhibition of CD161 by IMT-009 was demonstrated by using several in vitro pharmacological and cellular assays which assessed NK cell degranulation, cytokine production and cellular cytotoxicity towards tumor targets, as well as T cell receptor signaling and polyfunctionality using primary antigen-specific human T cells. To prioritize indications that will likely benefit from CD161 blockade therapy, multiplexed immunofluorescence analysis of over 30 solid tumor types was performed.

Results IMT-009 binds CD161 with high affinity and selectivity, blocking its interaction with CLEC2D at an IC50 of 0.94 nM. In presence of CLEC2D-expressing target cells K562, NK cell degranulation, cytokine production and cellular cytotoxicity towards tumor targets is highly suppressed; IMT-009 can overcome this inhibition with an EC50 of 0.2 nM. Similarly, IMT-009 reversed CLEC2D-mediated inhibition and restored T cell receptor signaling and cytokine production in a Jurkat cell reporter system (EC50 = 3.5 nM), as well as enhanced polyfunctionality of primary antigen-specific human T cells, including secretion of TNF-α, IL2, and IFNγ (EC50 = 0.2 nM, 0.4 nM, and 1.4 nM, respectively), and direct T cell mediated cytotoxicity. IMT-009 also released CD161-mediated suppression on effector memory CD161+ CD4+ T cells, resulting in an increased frequency of IFN-γ+ cells and an increase in their proliferation indicative of a stronger recall response to antigen. Finally, multiplexed immunofluorescence data of over 30 solid tumor types showed the highest density of CLEC2D+ and CD161+ cells in the following indications: NSCLC-squamous cell carcinoma, NSCLC- adenocarcinoma, Head and Neck squamous cell carcinoma (HNSCC), Triple negative breast cancer (TNBC), Cutaneous squamous cell carcinoma and Colorectal carcinoma.

Conclusions These results support the development of IMT-009 as a novel cancer immunotherapy for application in several solid tumor indications.

REFERENCES

Background Interruption of the programmed cell death-1 (PD-1) inhibitory pathway by binding PD-1 or its ligand PD-L1 is an effective treatment for various cancers,\(^1\) although resistance is common.\(^2\) PD-1 has a second ligand, PD-L2, that can be expressed by a variety of immunosuppressive stromal cells, endothelial cells, and tumor cells.\(^3\) IMGS-001 is a dual specific monoclonal antibody designed to bind PD-L1 and PD-L2 and block their engagement with PD-1. The Fc region is engineered to induce robust cell-mediated cytotoxicity, enabling depletion of PD-L1\(^+\) and PD-L2\(^+\) immunosuppressive cells throughout the tumor microenvironment. Here we describe the development of IMGS-001, including potency, specificity, cytokine release potential, pharmacokinetics (PK), and repeat-dose toxicity.

Methods Affinities were measured with the Octet system. Reporter cell assays assessed PD-1 pathway blockade, antibody-dependent cellular cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis (ADCP). Specificity was evaluated by Retrogenix microarray technology. Potential for spontaneous cytokine release was measured by co-culturing with healthy donor peripheral blood mononuclear cells. PK was measured in mice and in cynomolgus monkeys. In a GLP toxicity study, IMGS-001 was dosed weekly over 4 weeks at 10, 50, or 100 mg/kg with a 4-week recovery.

Results The affinity of IMGS-001 to monomeric PD-L1 and PD-L2 is 7.62 nM and 1.90 nM, respectively. Dimer affinities are 1.28 nM and 600 pM. It has an EC\(_{50}\) of 0.3-1.1 nM in a PD-1 blockade assay, the same range as pembrolizumab and avelumab. IMGS-001 has an EC\(_{50}\) of <0.5 nM in ADCC and ADCP assays. Specificity screening showed no relevant off-target binding and there was no evidence of specific cytokine release. Mouse PK showed drug exposure of \(\sim 1.0 \times 10^4\) mg-hr/ml at the efficacious dose. Half-life was 3.2 days in mice, and 3.7 days in cynos. Repeat-dose toxicity showed mild to moderate hematological and pathological changes, all of which had evidence of reversal within the recovery period.

Conclusions These data indicate that IMGS-001 binds PD-L1 and PD-L2 and functions per its design. It shows no biologically relevant off target effects, was administered up 100 mg/kg without toxicity, and has a viable PK profile for human administration. Its mechanisms of elimination of immunosuppressive cells with PD-1 pathway blockade could benefit patients that are resistant to existing PD-(L)1 drugs by restoring immune driven anti-tumor activity. IMGS-001 is poised to enter clinical trial in immune excluded tumors by the end of 2022.

REFERENCES
AVA-ADR-001 SUPPRESSES TUMOR GROWTH AND INDUCES ANTI-TUMOR IMMUNITY BY SELECTIVELY INHIBITING ADAR1 P150

Arun B Papaiah, Avijit Goswami, Sandeep Goyal, Kawaljit Singh, Princy Khurana, Aditya Kulkarni*. Avammune Therapeutics Inc., Levittown, PA, USA

Background Adenosine deaminase, RNA specific (ADAR1), catalyzes the hydrolytic deamination of adenosine (A) to inosine (I) in double-stranded (ds) RNAs. There are 2 isoforms of ADAR1 (p110 in the nucleus; p150 in cytoplasm) and both modify dsRNA in coding and non-coding regions. The ADAR1 p150 isoform is expressed from an interferon (IFN)-response promoter and has a Z-DNA/Z-RNA binding domain at the N-terminus. ADAR1 p150 edits 3'-untranslated region dsRNAs comprising of inverted Alu repeats and thereby suppresses MDA5-MAVS-IFN signaling. ADAR1 is commonly overexpressed in multiple myeloma, breast, lung, liver, skin and esophageal cancer where it promotes cancer progression. Inhibition of ADAR1 has promising anti-tumor efficacy as monotherapy and in combination with checkpoint inhibitors, radiotherapy and chemotherapeutic modalities. Herein, we outline the discovery of a potential first-in-class ADAR1 inhibitor for cancer immunotherapy.

MethodsAVA-ADR-001 was identified through a high throughput p110 knockout cell-based assay. The ability of AVA-ADR-001 to induce interferons was confirmed in various cell lines like A549 p110 KO, HCT116 and B16F10. Finally, the anti-tumor efficacy of AVA-ADR-001 was evaluated in B16F10 syngeneic melanoma mice model as monotherapy and in combination with anti-PD1.

Results We have identified a first-in-class small molecule inhibitor of ADAR1, which shows significant IFN response in vitro in an MDA5 dependent manner. In vitro binding studies have confirmed direct binding of AVA-ADR-001 with the Z-domain of ADAR1 thus confirming its selectivity to the p150 isoform. AVA-ADR-001 demonstrates micromolar EC50 and anti-tumor efficacy against B16F10 melanoma syngeneic mouse model. 100 μg of AVA-ADR-001 treatment resulted in 45% tumor growth inhibition (TGI), 1.5x superior to Anti-PD1 treatment. Combining AVA-ADR-001 with Anti-PD1 demonstrated a synergistic effect 2x superior to Anti-PD1 alone. Additionally, several interferon stimulated genes like IFIH1, IFN-β and CXCL-10 were significantly upregulated in the tumor samples of the AVA-ADR-001 monotherapy and combination groups.

Conclusions To our knowledge no selective small molecule inhibitors of ADAR1 have been reported so far and AVA-ADR-001 is the first disclosure of such an inhibitor. AVA-ADR-001 is a potent and selective first-in-class ADAR1 inhibitor which has shown significant IFN induction in various cancer cell lines and in vivo in the tumor microenvironment resulting in substantial tumor growth inhibition as monotherapy and synergistically in combination with Anti-PD1. Considering the immune-suppressive and pro-metastatic role of ADAR1, AVA-ADR-001 serves as a promising starting point for novel ADAR1 inhibitors as therapeutic modalities in cancer immunotherapy.

AVA-NP-695 POTENTLY AND SELECTIVELY INHIBITS ENPP1 TO ACTIVATE STING PATHWAY AND ABROGATE TUMOR METASTASIS IN 4T1 BREAST CANCER SYNGENEIC MOUSE MODEL

Aditya Kulkarni*, Avijit Goswami, Sandeep Goyal, Princy Khurana, Arun Papaiah. Avammune Therapeutics Inc., Levittown, PA, USA

Background Innate immune modulators such as STING agonists have become attractive approaches to improve the efficacy of Immune Checkpoint Inhibitors (ICI) due to their ability to turn cold tumors hot. Owing to the modest clinical efficacy of STING agonists, there is a need for other approaches for activating the cGAS-STING pathway for cancer immunotherapy. One such approach is through the inhibition of the enzyme ENPP1, a negative regulator of the STING pathway which directly hydrolyses 2’3’-cGAMP. ENPP1 is overexpressed in several tumor cells like human astrocyte tumors and TNBC cells like 4T1 and MDA-MB-231, and plays a key role in tumor progression and block T cell infiltration in breast and lung cancer patients. ENPP1 not only abolishes the cGAS-STING mediated immune activation but also produces adenosine, an immune suppressor which promotes cell migration. AVA-NP-695 is a highly potent orally available ENPP1 inhibitor being developed for cancer immunotherapy.

Methods The inhibition potency of AVA-NP-695 was confirmed by enzymatic assays using various substrate like p-Nitrophenyl-5’-TMP, cGAMP and ATP. The efficacy of AVA-NP-695 was depicted in 4T1 Tumor bearing BALB/c mice as monotherapy and in combination with anti-PD-L1, Olaparib and Paclitaxel. Efficacy of AVA-NP-695 in combination with radiation (6.2Gy X 4) was also evaluated in ENPP1 overexpressing ANV5 tumors.

Results Herein, we demonstrate that AVA-NP-695, a selective and potent ENPP1 inhibitor showed no adverse effect at 1000mg/kg BID in 7 Day repeated dose in BALB/C mice, thereby demonstrating an excellent therapeutic window. Results from in vivo studies have shown superior tumor growth inhibition (TGI) and impact on tumor metastasis by AVA-NP-695 (6mg/kg BID) compared to Olaparib and Anti-PD1 in a syngeneic 4T1 breast cancer mouse model. Subsequently, combination of AVA-NP-695 with Anti-PD-L1, Olaparib and Paclitaxel and demonstrated encouraging combinatorial efficacy of AVA-NP-695 along with Paclitaxel. Monotherapeutic arm for Paclitaxel and AVA-NP-695 depicted 40% and 43% TGI respectively, however their combined treatment resulted in ~60% TGI. Additionally, the AVA-NP-695 treatment alone showed 50% enhanced mean survival time followed by 68%, 68% and 72% when given in combination with anti-PD-L1, Olaparib and Paclitaxel respectively. Finally, AVA-NP-695 showed complete tumor ablation of ANV5 ENPP1 overexpressed tumors when given in combination with radiation. Combination group showed significantly delayed recurrence compared to only radiotherapy.

Conclusions The potent anti-tumor efficacy of AVA-NP-695 both as monotherapy and combination along with its safety profile provides a strong rationale for the therapeutic potential of AVA-NP-695 against solids tumors, particularly breast cancer.

Background For peptide-based cancer vaccines, successful eradication of tumors relies on the effective and persistent delivery of antigenic peptides to antigen presenting cells (APCs) to prime potent antigen-specific, cytotoxic T cells. Peptide-based vaccines have historically been either; (1) water-based formulations, which provide short exposure of peptides to immune cells; or (2) oil-in-water emulsions that provide longer peptide exposure but can elicit dysfunctional and exhausted T cell phenotypes. By contrast, the DPX® technology is a non-aqueous, lipid-in-oil, immune-educating therapeutic delivery platform. Antigenic peptides formulated in DPX elicit a robust, targeted, and persistent tumor antigen-specific T cell response that for our lead DPX product, Maveropepimut-S, has translated into clinical benefit in multiple cancer indications, including DLBCL and ovarian cancer.

Methods Herein, we compare DPX to aqueous and emulsion-based formulations for the dynamics of immune cell recruitment to the site of injection (SOI), peptide antigen consumption, and trafficking by immune cells. Immune cell composition and antigen uptake at the SOI were assessed by multi-parameter flow cytometry and confocal microscopy using model peptide antigens administered in C57/Bl6 mice. Antigen-specific immune responses were assessed in draining lymph nodes and/or spleens by IFN-γ ELISPOT.

Results These data reveal that aqueous formulations were poorly able to retain lymphocytes at SOIs and consequently did not elicit a detectable IFN-γ ELISPOT response. Both formulations containing an oil component (DPX and emulsion) were superior in recruiting APCs cells to the SOIs and inducing antigen-specific immune responses. Significant increases in immune cell infiltration were detected as early as 2 days post DPX injection. Antigen uptake was confirmed using confocal microscopy. Both DPX and emulsion platforms induced a prompt increase in antigen presentation in the context of MHC. However, antigen presentation driven by the DPX platform had a distinct profile enriched in CD11b⁺CD11c⁺MHCII⁺ APCs co-expressing the CD80, CD86, and CD40 activation/costimulatory markers. The recruitment and activation of this subset was evident regardless of whether a peptide was present in DPX. By contrast, the emulsion incites a distinctly different CD11b⁺CD11c⁻ population. Interestingly, CD11b⁺CD11c⁻ cells have tendency to express higher number of peptide-MHC complexes per cell compared to the CD11b⁺CD11c⁻ population.

Conclusions Collectively these findings highlight quantitative, qualitative, and temporal differences in immune cell recruitment amongst three delivery platforms and show the unique character of the immune response triggered by the DPX platform typified by the recruitment of CD11b⁺CD11c⁻ APCs that have intrinsically higher capacity for antigen uptake, presentation, and activation.

Ethics Approval Experiments were conducted in accordance with ethics protocols approved by the University Committee on Laboratory Animals at Dalhousie University, Halifax, N.S., Canada.
PRECLINICAL EVALUATION OF STAR0602, A NOVEL, FIRST-IN-CLASS ANTI-TCR VB TARGETED BISPECIFIC ANTI-BODY WITH POTENT ANTI-TUMOR ACTIVITY FOR PD-1 REFRACTORY SOLID TUMORS

Zhen Su, James Gulley*, Jeffrey Schlom, Jeffrey Wherry, Ke Liu, Renee Donahue, Madan Katraggada, Rajesh Chopra, Jacques Moisan, Jonathan Hsu, Yeo-Ting Tsai, Marengo Therapeutics, Cambridge, MA, USA; NCI, Bethesda, MD, USA; NIH, Bethesda, MD, USA; Princess Margaret Cancer Center, Toronto, Canada; MGH, Boston, MA, USA; UPENN, Philadelphia, PA, USA; Marengo Therapeutics, London, UK

Background Despite recent advancements with immune checkpoint inhibitors (e.g., anti-PD1 inhibitors) many cancer patients develop treatment resistance, which supports the study of alternative approaches to induce potent and safe anti-tumor T cell responses. STAR0602 is a bifunctional antibody-fusion molecule that selectively activates and expands a sub-set of human αβ T cells expressing variable (V) b6 and b10 regions of the T cell receptor (TCR). STAR0602 simultaneously engages a novel, non-clonal mode of TCR activation with cytokine co-stimulation.

Methods The prevalence of STAR0602-targeted Vb T cells in tumor-infiltrating lymphocytes (TILs) from human tumor tissues was investigated by flow cytometry and by interrogating TIL TCRseq data from a large cancer database. The effects of STAR0602 on T cells from healthy donors and cancer patients were assessed in vitro by flow cytometry and NanoString. Using high tumor mutational burden (TMB) and anti-PD1-insensitive murine and human models, we investigated anti-tumor activity, mechanism of action, and an enrichment strategy for patient trials. Finally, the pharmacokinetics (PK) and pharmacodynamics (PD) of IV STAR0602 were investigated in Cynomolgus monkeys.

Results Presence of STAR0602-targeted Vb T cells were confirmed in tissue from a range of human tumors, and present as 10-12% of TILs. Stimulation of T cells with STAR0602 resulted in potent expansion with ~80% adopting a novel memory phenotype, and significant boosting of antigen-specific T cells. In human autologous tumor organoid models, STAR0602 induced potent expansion of TILs and killing of tumors, including several PD1 refractory tumors. Dose-related anti-tumor activity (100% cure rate with a murine surrogate (mSTAR0602)) in EMT6-bearing mice correlated with expansion of memory Vb CD8+ T cells. In Cynomolgus monkeys, IV STAR0602 induced robust expansion of Vb CD8+ T cells in blood, with limited cytokine release or expansion of Treg. These data were used to build a PK/PD model to simulate human pharmacology and design a first-in-human trial with an enriched patient population.

Conclusions STAR0602 is a first-in-class T cell activator that targets subsets of the germline TCR repertoire that are enriched in TILs. STAR0602 potently expands both naive and antigen-specific human T cells. In PD1 refractory human and murine tumor models with a high TMB, STAR0602 and mSTAR0602 induce potent anti-tumor activity as monotherapy, mediated by selective expansion of Vb CD8+ memory T cells. This pharmacology was translated into monkeys with IV dosed STAR0602 and supports the design of a novel Phase 1/2 precision-oncology trial with STAR0602 planned to commence in 2022.

Background Intratumoral regulatory T (Treg) cells promote an immunosuppressive tumor microenvironment, and their increased frequency correlates with poor clinical prognosis.1,4 Gene expression profiling has identified chemokine receptor 8 (CCR8) as being highly upregulated by intratumoral Treg cells compared to their lymphoid tissue and blood counterparts, as well as other immune cell types.5,6 Because of this restricted expression, using anti-CCR8 antibodies capable of inducing antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) to deplete intratumoral Treg cells represents an attractive therapeutic hypothesis for cancer treatment. Treatment of mouse cancer models with anti-CCR8 antibodies depleted intratumoral Treg cells and reduced tumor growth.7,8 Furthermore, anti-CCR8 treatment can synergize with anti-PD-1 treatment in anti-PD-1-susceptible and –resistant cancer models, highlighting the therapeutic potential of anti-CCR8 antibodies. SRF114 is a fully human, afucosylated anti-CCR8 antibody designed to preferentially deplete CCR8+ Treg cells within the tumor microenvironment. Here, we explore the ability of SRF114 to activate effector immune cells associated with ADCC and ADCP, deplete Treg cells, and reduce tumor growth in a mouse cancer model.

Methods Surface expression of CCR8 on human intratumoral and peripheral immune cells was characterized by flow cytometry. Human peripheral blood mononuclear cells (PBMCs) and dissociated tumor cells (DTCs) were cultured with SRF114 to examine immune cell activation and Treg cell depletion. The efficacy of SRF114 was assessed in MC38 tumor-bearing human CCR8 knock-in (hCCR8 KI) mice.

Results CCR8 surface expression was highest on intratumoral Treg cells compared with peripheral Treg cells and other immune cells. In PBMC cultures, Fc gamma receptor (FcγR)-expressing cells, including natural killer (NK) cells and monocytes, exhibited dose-dependent activation from SRF114 bound to CCR8+ cells. DTC cultures treated with SRF114 also displayed dose-dependent NK cell activation and selective Treg cell depletion, with marginal impacts on effector T cell populations. The potency of SRF114 to activate FcγR+ cells in DTCs was enhanced compared with that in PBMCs due to increased CCR8 expression on Treg cells. In a syngeneic tumor model, hCCR8 KI mice treated with SRF114 exhibited a significant reduction in tumor growth and depletion of intratumoral Treg cells, with minimal impact on their peripheral counterparts.

Conclusions SRF114 can induce ADCC and/or ADCP pathways to deplete CCR8+ Treg cells in vitro. Demonstrated depletion of intratumoral Treg cells and reduction of tumor growth in vivo support SRF114 as a therapeutic candidate to deplete intratumoral Treg cells and drive antitumor immunity in human cancer patients.

References
Background Regulatory T cells (TREG) inhibit immune responses in many solid cancers and are associated with worse prognosis when they infiltrate tumours. ALD2510 is a low-fucose IL-2-sparing anti-CD25 antibody designed for the selective depletion of TREG, allowing the boost of immune effector functions within the tumor micro-environment (TME) and making it a promising candidate for therapy of solid tumors.

Methods ALD2510 potency and mechanisms of action were investigated in TREG depletion, ADCC, ADCP and trogocytosis assays and in syngeneic or humanized animal models of cancer. Characterization of TREG subpopulations within the TME was achieved by flow and mass cytometry followed by t-sne analysis using biopsies from gynecological cancer patients. ALD2510 safety, tolerability and pharmacokinetic (PK) profile were determined in cynomolgus monkey, with doses up to 100mg/kg. ALD2510 manufacturability was assessed through fed-batch production and analytical characterization.

Results ALD2510 showed strong TREG depletion, ADCC and ADCP activities, while sparing CD4+ and CD8+ T cell compartments, demonstrating its selectivity for TREG and a mode of action involving multiple effector cell types. ALD2510 also promoted trogocytosis of induced TREG, suggesting that tumour-infiltrating neutrophils may contribute to its function. In vivo, as monotherapy, ALD2510 confirmed selective and potent TREG depletion together with significant tumor growth inhibition, which could be further improved when combined with checkpoint inhibitors, leading to complete tumor regressions.

Immunophenotyping of tumor biopsies and related PBMCs from gynecological cancer patients and further t-sne analysis allowed the identification of four TREG subsets. That with the highest CD25 expression was shown to be tumour specific and presented the most immunosuppressive phenotype, thus constituting a preferential target for ALD2510.

In a cynomolgus exploratory toxicology study, ALD2510 showed excellent safety and tolerability with doses up to 100mg/kg. PK and exposition parameters were found in line with those of typical humanized IgG1 in NHP.

The manufacturability of the ALD2510 CHO cell line was demonstrated through 10L fed-batch production, where productivity reached ~4g/L. Analytical characterization of purified ALD2510 materials revealed excellent purity and activity, together with very low levels of process-/cell-related impurities.

Conclusions ALD2510 is a next generation Fc-enhanced, TREG-selective and IL-2-sparing anti-CD25 mAb showing in vitro and in vivo efficacy, fully satisfactory safety and tolerability in NHP and excellent manufacturability. ALD2510 thus appears as a promising candidate for treatment of solid tumor patients, especially those suffering from gynecological malignancies, where highly immunosuppressive CD25high TREG were identified in the TME.
**Background** To address the limitations of antibody-based agonists of immune costimulatory receptors, we have developed a new class of modular synthetic drugs, termed Bicycle® tumor-targeted immune cell agonists (Bicycles). The first molecule of this class, BT7480, a Nectin-4-dependent CD137 (4-1BB) agonist, entered clinical trials in 2021 in patients with solid tumors associated with Nectin-4 expression. Compelling preclinical data characterizing BT7480 led us to develop a second Bicycle TICA™ molecule, BT7455, which is designed to deliver highly potent CD137 agonism to Ephrin receptor A2 (EphA2)-positive cancers. EphA2 is a receptor tyrosine kinase overexpressed in several human cancers and its high expression correlates with poor clinical prognosis in certain cancer types.

**Methods** BT7455 bioactivity was assessed in vitro using a CD137 reporter assay and by measuring proinflammatory cytokine production in human PBMC/tumor cell co-cultures. BT7455 in vitro pharmacological activity was evaluated in efficacy studies in syngeneic EphA2-positive mouse tumor models and pharmacodynamic studies using transcriptional profiling of the tumor immune microenvironment by NanoString.

**Results** BT7455 engages EphA2 and CD137 with high affinity resulting in picomolar potency in co-culture assays consisting of EphA2-expressing tumor cells and CD137-expressing Jurkat NF-kB-luciferase reporter cells. Moreover, BT7455 led to EphA2-dependent production of interleukin-2 (IL-2) and interferon gamma (IFNγ) in primary human PBMC/tumor cell co-culture assays. Treatment of MC38 tumors in immunocompetent mice with BT7455 with an intermittent dosing regimen led to robust anti-tumor activity, including complete responses. Gene expression profiling of BT7455-treated tumors revealed modulation of the tumor immune microenvironment, including a rapid increase in cytokine expression (both myeloid and T cell origin) and an increase in cytotoxic cell scores. The kinetics and extent of the immune microenvironment modulation differentiated BT7455 from both a checkpoint inhibitor (anti-mouse PD-1) as well as an anti-CD137 agonist antibody (Urelumab analogue). BT7455 treatment also led to the increase in checkpoint gene expression, suggesting that combination with checkpoint inhibitor therapy may be effective. BT7455 exhibits linear pharmacokinetics in non-human primates and appears well-tolerated at exposures more than the predicted efficacious exposure in humans without significant elevation of cytokines or liver enzymes.

**Conclusions** BT7455 is a highly potent EphA2 expression-dependent CD137 agonist with optimal target binding, pharmacologic, and pharmacokinetic properties that enable intermittent dosing for curative effect through modulation of the tumor immune microenvironment in syngeneic mouse models. BT7455 is currently being evaluated in IND-enabling safety studies.

**References**


**Ethics Approval** All the procedures related to animal handling, care and treatment in the studies were performed according to the guidelines approved by the Institutional Animal Care and Use Committee of WuXi AppTec (Beijing, China), following the guidance of the Association for Assessment and Accreditation of Laboratory Animal Care.