TGFBR2, would render NK cells resistant to TGF-β mediated suppression.

Methods NK cells were expanded from CD3-PBMC starting material in the presence of 20 ng/mL IL-15 for 14 days. A variety of methods were performed to assess the effects of CRISPR-Cas12a gene editing on primary human NK cells including NGS to assess editing efficiency, flow cytometry, in vitro spheroid killing assays and an in vivo NSG tumor model. These methods were performed consistent with protocols widely accepted in the field.

Results Following editing optimization, we achieved greater than 80% in/dels at both targets in NK cells in both single and double gene knockout (KO, DKO) contexts. Using flow cytometry-based assays we demonstrated that TGFBR2 KO NK cells phosphorylated less SMAD2/3 relative to unedited control NK cells in response to TGF-β, while CISH KO NK cells showed enhanced pSTAT3 and pSTAT5 upon IL-15 stimulation. We next explored the ability of these single knockouts in controlling 3D SK-OV-3 ovarian tumor spheroids and PC-3 prostate tumor spheroids in vitro over 5 days of co-culture. Consistently, both single knockouts demonstrated improved cytotoxicity against tumor targets in the presence of exogenous TGF-β ($p<0.0001$ for both single KOs). Importantly, in both the SK-OV-3 and PC-3 tumor spheroid killing assays, DKO NK cells demonstrated superior rapid and sustained tumor killing compared to either single knockouts or unedited control NK cells ($n=7$ independent experiments, 4 unique NK cell donors, $p<0.0001$), demonstrating additive effects of simultaneously targeting both pathways. Relative to control NK cells, DKO NK cells had increased expression of CD107a, CD25, CD69, and NKp44 after exposure to tumor cells and produced higher concentrations of TNF-a and IFN-g ($p<0.01$). In an in vivo NSG mouse xenograft model, where SK-OV-3 cells are injected i.p. one week prior to i.p. NK cell infusion, DKO NK cells controlled tumor growth more effectively than unedited NK cells, resulting in lower tumor burden and an increase in median survival time.

Conclusions In summary, using CRISPR-Cas12a we demonstrated highly efficient gene editing of primary human NK cells at two unique targets designed to augment NK cell antitumor activity. Together, the increased overall effector function of CISH/TGFBR2 DKO primary human NK cells support their development as a potent allogeneic cell-based medicine for cancer. This potential medicine, termed EDIT-201, is being advanced to clinical study.

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146 ALLOANTIGEN-SPECIFIC TR1 CELLS DESIGNED TO PREVENT GVHD HAVE A DISTINCT MOLECULAR IDENTITY AND SUPPRESS THROUGH CTLA-4 AND PD-1

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Background Graft-vs-host-disease (GvHD) is a life-threatening complication of allogeneic hematopoietic stem cell transplantation (allo-HSCT), limiting the use of this potentially curative treatment for hematological malignancies. To address this, we have developed T-allo10 cell therapy, which is enriched with type 1 regulatory T (Tr1) cells. Tr1 cells are peripherally inducible, CD49b+LAG3+IL-10+FOXP3- regulatory T cells that can confer alloantigen-specific tolerance, making them an attractive alternative to existing GvHD therapies, which non-discriminately impair both GvHD and protective immunity. T-allo10 cells are currently being evaluated in a phase I clinical trial in patients with hematological malignancies undergoing allo-HSCT (NCT03198234). Herein, we aimed to confirm that Tr1 cells are the active ingredient responsible for the T-allo10 suppressive function, and reveal the underlying molecular signatures to elucidate the mechanisms of Tr1 cell-mediated suppression.

Methods T-allo10 cells were generated in a co-culture of healthy host or patient tolerogenic dendritic cells (DC-10) with allogeneic healthy donor CD4+ T cells, then tested for Tr1 phenotype, anergy, suppression and cytokine production. Sorted T-allo10-derived Tr1 cells and non-Tr1 cells, as well as control effector T cells (Teff) and parental CD4+ T cells, were analyzed by TCR- and RNA-seq. Protein expression for key differentially expressed genes were validated, and the functional roles for IL-10, CTLA-4 and PD-1 in T-allo10-mediated suppression were confirmed in a suppression assay.

Results We show that the T-allo10 cell product is: i) enriched for Tr1 cells, ii) anergic in response to alloantigen re-challenge, but not to non-specific stimuli or 3rd party antigens, and iii) suppresses host-reactive T cells, but not T cell responses to other antigens. Furthermore, T-allo10-derived, isolated Tr1 cells had a restricted TCR repertoire, suggesting they clonally expand in response to alloantigens. T-allo10-derived Tr1 cells have a distinct signature compared to non-Tr1 cells, and, in addition to IL-10, express high levels of CTLA-4 and PD-1 (but not FOXP3). Notably, blockade of CTLA-4 and the PD-1 pathway completely abolishes T-allo10-mediated suppression of T cell responses.

Conclusions Our data shows that Tr1 cells are the active, suppressive, and antigen-specific ingredient of T-allo10 cells. Furthermore, while the role of IL-10 in Tr1 cell-mediated suppression is well known, we uncover that Tr1 suppress in addition through CTLA-4 and PD-1. Collectively, these intriguing findings underscore the importance of CTLA-4 and PD-1 pathways in conferring cell-mediated immunological tolerance. Further, they define the key characteristics and modes of action of antigen-specific Tr1 cells, providing crucial information for the ongoing T-all10 trial and future design of novel Tr1 cell-based therapies.

Ethics Approval The patient study was approved by Administrative Panels on Human Subjects in Medical Research, Stanford University, Tallo10 eProtocol # 38734. Healthy donor samples were purchased as deidentified human blood products from the Stanford Blood Center, and are thus exempt.

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147 CD155 BLOCKADE BOOSTS ALLOREACTIVE NATURAL KILLER CELL ANTITUMOR EFFECTS AGAINST OSTEOSARCOMA

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Background Pediatric patients with relapsed and refractory osteosarcoma have poor prognoses with few treatment options. Allogeneic bone marrow transplant (BMT) has not yet shown a graft-versus-tumor (GVT) effect for osteosarcoma. Natural killer (NK) cells demonstrate antitumor activity against osteosarcoma, but adoptively transferred NK cells have limited

proliferation, cytotoxicity, and persistence in vivo. To enhance an NK-specific GVT effect, we propose blocking the poliovirus receptor CD155 checkpoint molecule, which is overexpressed on osteosarcoma and can engage both activating and inhibitory receptors on NK cells. The impact of CD155 blockade on GVT and graft-versus-host-disease (GVHD) is unknown.

Methods NK cells from C57BL/6 (B6) mice were expanded with recombinant IL-15/IL-15R and analyzed by flow cytometry. Cytotoxicity assays were performed with IL-15 expanded B6 NK cells and mKate2-expressing K7M2 murine osteosarcoma at a 1:1 ratio with blockade of CD155 and CD155 ligands. To test efficacy of NK cell infusion and CD155 blockade after allogeneic BMT, BALB/c mice were lethally irradiated, transplanted with allogeneic B6 bone marrow, and challenged with luciferase-expressing K7M2 on day 0. At day 7, mice received IL-15 expanded B6 NK cells intravenously with either anti-IgG control or anti-CD155 antibody intraperitoneally and IL-2 subcutaneously on days 7 and 11. Mice were monitored for tumor growth by bioluminescence, and toxicity by GVHD using weight loss and clinical scores.

Results Compared to unexpanded murine NK cells, IL-15 expanded NK cells (n = 6) show increased expression of NKG2D (65.33 ± 10.77% NKG2D+, p = 0.0077; 1030 ± 177.0 MFI, p = 0.0101) and an increased ratio of the CD155 activating (CD226) to inhibitory (TIGIT) ligand expression (11.71 ± 4.121, p = 0.0362). In cytotoxicity assays with IL-15 expanded allogeneic murine NK cells (n = 3 replicates), CD155 blockade enhances K7M2 osteosarcoma lysis (60.62 ± 3.19%, p = 0.0189) compared to IgG control (29.01 ± 7.66%). CD226 blockade decreased tumor killing (10.62 ± 8.51%, p = 0.0053) compared to CD155 blockade. In vivo allogeneic murine NK cell infusion and anti-CD155 antibody treatment after allogeneic BMT decreased tumor area under the curve by 44.3% compared to IgG control, without exacerbating GVHD.

Conclusions These findings demonstrate that blockade of CD155 enhances an allogeneic NK cell-specific GVT effect for osteosarcoma treatment without exacerbating GVHD. CD155 blockade has the potential to improve usage of allogeneic BMT and NK cell adoptive immunotherapy as a combination treatment for osteosarcoma, and perhaps other pediatric sarcomas.

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148 IDENTIFICATION OF PROSTATE-RESTRICTED EPITHELIAL ANTIGENS FOR TRANSGENIC T CELL ADOPTIVE THERAPY AGAINST PROSTATE CANCER

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Background In advanced prostate cancer (PCa), progression to castration-resistant PCa (CRPC) is inevitable and novel therapies for CRPC are needed. Adoptive transfer of T cells targeting tumor antigens is a promising approach in the cancer field. Unfortunately, identifying antigens expressed exclusively in prostate tumor cells has been challenging. Since the prostate is not an essential organ, we alternatively selected prostate-restricted epithelial antigens (PREAs) expressed in both malignant and normal prostate tissue for transgenic T cell studies.

Methods RNA-seq data sets identifying genes enriched in PCa were cross-referenced with the NIH Genotype-Expression database to identify PREAs. Using a novel molecular immunology approach, select PREAs and major histocompatibility complex class I (MHC-I) molecules were co-expressed in HEK293F cells, from which MHC–peptide complexes were efficiently isolated. Peptides were eluted and sequenced by mass spectrometry. Peptide–MHC binding was validated with a T2 stabilization assay and peptide immunodominance was determined using an interferon-γ (IFN-γ) ELISpot assay following stimulation of healthy HLA-A2+ peripheral blood mononuclear cells (PBMC) with peptide pools. Following peptide stimulation, CD8+ T cells with peptide-specific T cell receptors (TCR) were enriched by peptide–MHC-I dextramer labeling and fluorescence activated cell sorting for single cell TCR α/β chain sequencing.

Results We identified 11 A2+ peptides (8 previously unpublished) from prostatic acid phosphatase (ACPP), solute carrier family 45 member 3 (SLC45A3), and NK3 homeobox 1 (NKX3.1) that bound to HLA-A2 with varying affinities. Extended culture stimulation of PBMC with peptide pools from each PREA, compared to the standard overnight culture, revealed a greater number of IFN-γ producing cells overall and a greater breadth of response across all the peptides. Antigen specific CD8+ T cells were detectable at low frequencies in both male and female healthy PBMC for 7 of the 11 peptides. Dextramer-sorted antigen-specific cells were used for single-cell paired TCR αβ sequencing and transgenic T cell development.

Conclusions Through this work we identified HLA-A2-presented antigenic peptides from the PREAs ACPP, SLC45A3, and NKX3.1 that can induce the expansion of IFN-γ producing CD8+ T cells. Through peptide–MHC-I dextramer labeling, we isolated PREA-specific CD8+ T cells and characterized TCR αβ sequences with potential anti-tumor functionality. Our results highlight a rapid and directed platform for the development of MHC-I-restricted transgenic CD8+ T cells targeting lineage-specific proteins expressed in prostate epithelia for adoptive therapy of advanced PCa.

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