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Oncolytic adeno-immunotherapy improves allogeneic adoptive HER2.CAR-NK function against pancreatic ductal adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDAC) responds poorly to conventional treatments and immunotherapy. We previously developed a binary oncolytic/helper-dependent adenovirus system (CAd*Trio*) that facilitated oncolysis and expressed the immunomodulatory molecule interleukin-12 and a programmed death ligand 1 (PD-L1) blocking mini-antibody. Given that CAd*Trio* enhanced endogenous natural killer (NK) cell anti-tumor activity in humanized mice bearing PDAC tumors and that NK cells can be adoptively transferred to patients safely in the allogeneic setting, we hypothesized that a combination of CAd*Trio* and allogeneic NK cells expressing a HER2-specific chimeric antigen receptor (HER2.CAR-NK) would be an effective, entirely "off-the-shelf" treatment against PDAC. We found that CAd*Trio*-derived immunomodulatory molecules prolonged HER2.CAR-NK persistence at tumor sites, allowing long-term tumor growth control and improved survival in both humanized mice and a heterogeneous PDAC patient-derived xenografts (PDX) model. This effect was based on CAd*Trio*-derived transgene support that shifted HER2.CAR-NK gene expression to that resembling an NK memory-like phenotype. Additionally, this allogeneic combination therapy was tolerated in humanized mice. Together, these data suggest that CAd*Trio* and HER2.CAR-NK cell combination immunotherapy may be a novel and effective option for the treatment for immunologically "cold" PDAC tumors.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) with 5-year survival at less than 10% is projected to become the second deadliest cancer in the United States by 2025.[1] Even targeted therapies at best provide only months of overall survival benefit. High HER2 expression is associated with poorer prognosis in PDAC patients, and thus frequently targeted by several approaches.[2,3] Yet in a phase 2 clinical trial, PDAC patients with grade 3 HER2 expression treated with the HER2 monoclonal antibody trastuzumab did not improve progression free or overall survival even when combined with chemotherapy.[4] PDAC tumors are classified as immunologically "cold" tumors with little immune cell infiltration and activation within the immunosuppressive tumor microenvironment (TME).[1–3] Thus, immunotherapies against PDAC would likely benefit from approaches that address PDAC targets as well as the immunologically "cold" TME.[1–3]

To promote immune cell infiltration and anti-tumor activity, oncolytic adenoviruses have been deployed against several tumor types including PDAC. Oncolytic adenoviruses selectively replicate in malignant cells resulting in lysis, in turn initiating local and systemic inflammatory responses that promote immune cell infiltration to the tumor site and counteract immunosuppressive TME; an effect that has been validated in patients with different tumor types including PDAC.[5–9] Our laboratory developed a binary oncolytic/helper-dependent adenovirus system (CAdVEC) which facilitates oncolysis (OAd) and promotes the expression of multiple immunomodulatory molecules (HDAd), thus overcoming limitations of OAd single agents including a limited transgene capacity of approximately 2 kb. However, local CAdVEC treatment was insufficient to cure distant (untreated) tumors in preclinical models. We thus combined local CAdVEC and systemic chimeric antigen receptor (CAR)-T cell treatment and found superior anti-tumor effects in pre-clinical studies with different solid tumor models.[8,10–12] Based on our encouraging pre-clinical results, we initiated a first-in-human phase 1 clinical trial with CAdVEC (expressing human interleukin 12p70

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[IL-12p70], programmed death ligand 1 [PD-L1] blocking mini-antibody, and herpes simplex virus thymidine kinase [HSV-tk] safety switch) and autologous HER2 antigen-specific CAR-T cells for patients with HER2 positive solid tumors, including PDAC, at Baylor College of Medicine (NCT03740256). Thus far, all patients have been safely treated with this combination immunotherapy.

Our pre-clinical studies demonstrated that combination immunotherapy with local CAd*Trio* and systemically administered autologous HER2.CAR-T cells cures PDAC tumors in humanized mice. Additionally, local CAd*Trio* treatment activates systemic endogenous human natural killer (NK) cell activity, including NK cell cytotoxicity and proliferation by the CAd*Trio*-encoded transgenes.[10] This is consistent with clinical observations linking NK cell abundance and activity at tumor sites with favorable outcomes in PDAC patients.[13–15] However, endogenous NK activity in most cases is insufficient to cure disease. Fortunately, adoptive NK cell-based immunotherapies relying on the natural ability of NK cells to surveil tumor, expand anti-tumor responses and can be safely given in the allogeneic setting without graft-vs-host disease (GvHD) or cytokine-release syndrome (CRS).[13,16–20] Despite their advantages, adoptive NK cells underperform without support through receptor modification or cytokine supplementation.[18,21–24] Given our expertise in combining CAd*Trio* with engineered CAR-expressing immune cells both preclinically and in trials, we hypothesized that CAd*Trio* combined with adoptively transferred allogenic HER2.CAR-NK cells would be a tolerable and readily available treatment against PDAC. Here, we show combination CAd*Trio* and allogeneic HER2.CAR-NK immunotherapy controls PDAC growth in multiple PDAC models via CAd*Trio* transgene-mediated promotion of HER2.CAR-NK persistence and NK memory-like activity.

RESULTS

HER2.CAR constructs enhance NK cell cytotoxicity against PDAC *in vitro*

As innate immune cells, NK cells can kill tumors through endogenous receptors (Figure S1), and this anti-tumor activity can be further enhanced through the addition of a CAR, allowing NK cells to also target a specific tumor cell antigen.[16,17,22] To identify which of our available HER2.CAR construct was optimal for use in NK cells, we transduced healthy donor-derived NK cells with one of three HER2 constructs: a 1st generation CD3ζ.CAR and two 2nd generation CARs (41BBz and CD28z) found to have comparable transduction efficiencies (76.28% and 85.23% CAR+, Figure 1A).[25] HER2.CAR-NKs were then cocultured with GFP-labeled PDAC cell lines (E:T = 1:5) for 72 h in an IncuCyte to monitor cancer cell viability (Figure 1B). Both 2nd generation constructs conferred significantly increased killing across all three PDAC cell lines compared to non-transduced and 1st generation CAR-NKs. Furthermore, the 41BBz.HER2.CAR construct provided greater tumor killing than the CD28z.HER2.CAR construct under high-stress conditions (e.g., E:T = 1:20). Accordingly, 41BBz.HER2.CAR improved the killing capacity of NK cells derived from three healthy donors without affecting NK expansion (Figure 1C). Since the 41BBz.HER2.CAR provided superior tumor growth control *in vitro*, this construct was selected as the optimal HER2.CAR-NK for further studies, including in combination with CAd*Trio*.

HD*Trio* transgenes influence HER2.CAR-NK phenotype

Previously, we have shown that endogenous NK cell function is enhanced by HD*Trio* transgenes in a humanized mouse model of PDAC.[10] To evaluate the effect of HD*Trio* transgenes on HER2.CAR-NK function, we infected PDAC cell lines with an HDAd expressing IL-12p70 (HD-IL-12) only, the PD-L1 mini-antibody (HD-PDL1) only, or HD*Trio* (expressing both IL-12p70 and PD-L1 mini-antibody). We then cocultured the infected PDAC cells with HER2.CAR-NKs for 24 h and subsequently isolated HER2.CAR-NKs for RNA sequencing (RNA-seq) (Figure 2A). Expression of activation-related genes, including interferon (IFN)-γ and leukemia inhibitory factor (LIF), was increased in HER2.CAR-NKs cocultured with CFPAC-1 and PANC-1 24 h post-coculture compared to before coculture (Figure S2). These activation-related genes were further upregulated in the context of IL-12p70, and we also observed an increased expression of lymphocyte activation gene 3 (LAG-3). Although we could not detect a significant change in HER2.CAR-NK gene expression in the context of PD-L1 blocker alone, when co-expressed with IL-12p70 (HD*Trio*), we again observed the activation-promoting effects of IL-12p70 as well as reduced expression of immunosuppressive genes T cell immunoreceptor with Ig and ITIM domains (TIGIT), signal regulatory protein gamma (SIRPG), and increased expression of T-box transcription factor (TBX21 or T-bet). These initial data suggest IL-12 plus PD-L1 blocker influence gene expression changes that promote the development of an enduring phenotype reminiscent of, yet distinct from, an adaptive or memory-like NK cell.[26–29] Moreover, this phenotype was successfully replicated across multiple NK cell donors, highlighting the utility of CAd*Trio* in reducing donor-to-donor variability (Figure 2B). While some gene expression differences are not statistically significant, these trends indicate potential biological consequences that warrant further study.

Combination immunotherapy is superior to single-agent HER2.CAR-NKs in NSG mouse models

To evaluate the anti-tumor capability of HER2.CAR-NKs against PDAC *in vivo*, immunodeficient NSG mice with subcutaneous CFPAC-1 or PANC-1 tumors received 1×10^7 viral particles (vp) of CAd*Trio* and then infused with 5×10^6 firefly luciferase-labeled HER2.CAR-NKs 3 days later (Figures 3A and S3). In both PDAC xenograft models, combination CAd*Trio* and HER2.CAR-NK treatment resulted in delayed tumor growth compared to single-agent treatments (Figure 3A). In comparison to mice bearing CFPAC-1 tumors, PANC-1-bearing mice receiving single-agent CAd*Trio* treatment demonstrated tumor growth control, likely reflecting differences in susceptibility via endogenous NK receptors. In both PDAC models, the combination of CAd*Trio* and HER2.CAR-NKs resulted in enhanced CAR-NK persistence (Figures 3B and S4) with firefly luciferase signal from adoptively transferred HER2.CAR-NKs continuously detected at the CFPAC-1 tumor site for

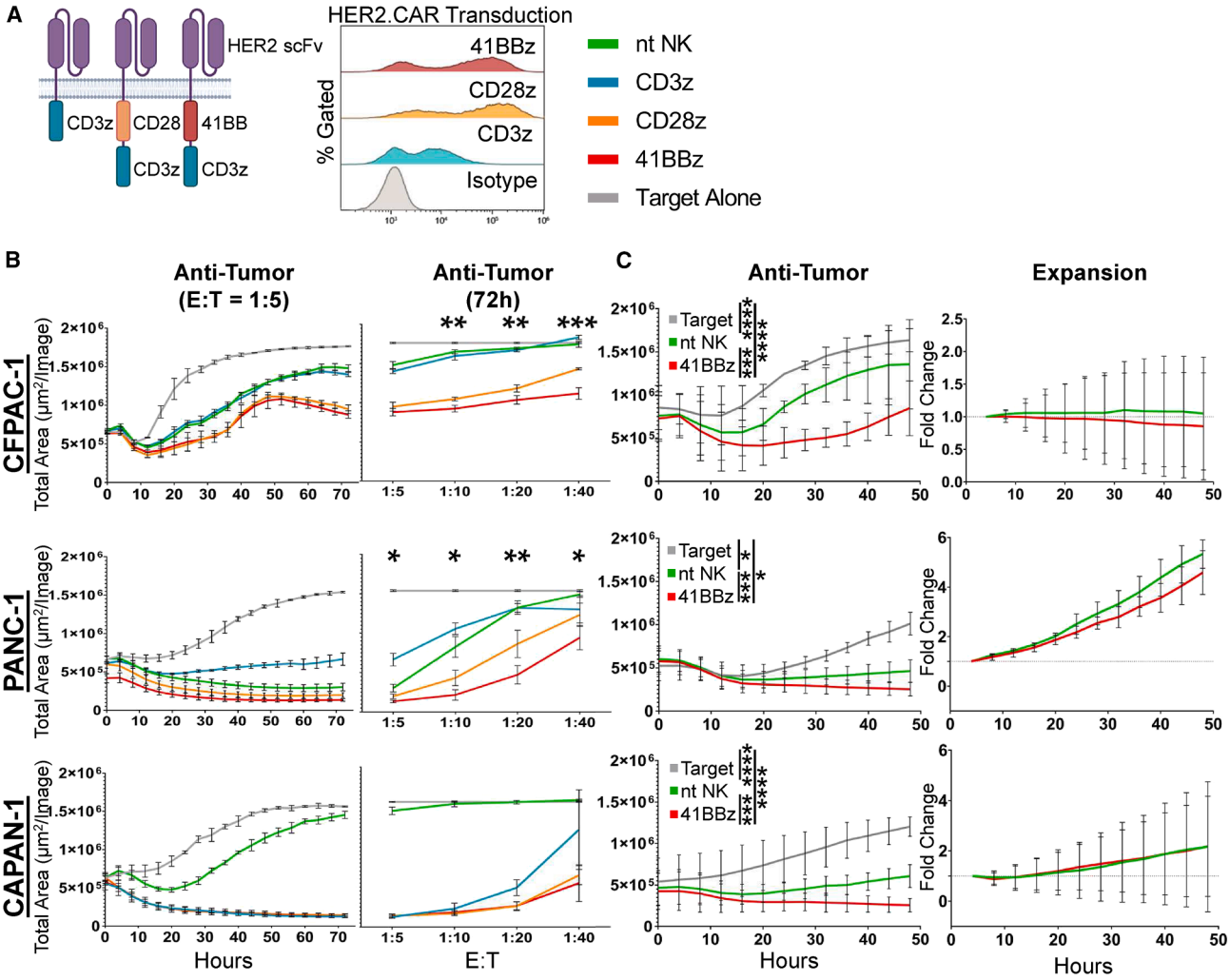

Figure 1. CAR modification improves NK cytotoxicity against PDAC

(A) PBMC-derived NK cells from a single healthy donor were transduced with one of three different HER2.CAR constructs (CD3zeta.HER2.CAR [CD3z], CD28-CD3zeta.HER2.CAR [CD28z], or 41BB-CD3zeta.HER2.CAR [41BBz]). CAR transduction efficiency was determined using flow cytometry 72 h post transduction. (B) To measure cytotoxicity, non-transduced (nt) NKs and CAR-NKs were then cocultured with PDAC cell lines expressing GFP at an E:T of 1:5 in an IncuCyte for 72 h and measured total area of GFP+ target cells (measure of viable target cells) ($n = 6$). Killing efficacy was evaluated under increasingly stressful E:T conditions by measuring total area of GFP+ target cells at the 72 h time point ($n = 6$). (C) The killing capacity of 41BBz.HER2.CAR-NK cells from multiple donors was evaluated by coculturing non-transduced or HER2.CAR-NKs from three healthy donors with GFP expressing target cells for 48 h (E:T, 1:20; $n = 4$–6/donor). To test 41BBz.HER2.CAR-NK expansion, non-GFP expressing PDAC cell lines were cocultured with either non-transduced NKs or HER2.CAR.41BBz-NKs expressing GFP (E:T, 1:20; $n = 4$–6/donor). Data represent fold change expansion of non-transduced NKs or HER2.CAR-NKs. Data are presented as means ± SD, and p values were determined by mixed-effects analysis with Tukey multiple comparisons. ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

up to 70 days post-infusion in 4/5 mice compared to 56 days for only 1/5 mice receiving HER2.CAR-NKs alone. Similarly, in mice with PANC-1 tumors, HER2.CAR-NK (firefly luciferase signal) could be detected through day 42 post-infusion when combined with CAd-*Trio* treatment, compared to just 14 days in the absence of CAd*Trio*-derived support. Since IL-15 is frequently used to supplement CAR-NK cells for improved persistence,[17] we compared HER2.CAR-NK function in the context of rhIL-15 with that of our combination treatment. The addition of IL-15 resulted in HER2.CAR-NK detection for up to 56 days post-infusion in CFPAC-1 tumor bearing mice and 28 days in PANC-1 tumor bearing mice (Figure S5). While IL-15 resulted in similarly extended HER2.CAR-NK cell persistence compared to the CAd*Trio* and HER2.CAR-NK combination treatment, IL-15-derived NK support did not result in similar tumor growth control to the combination treatment. These findings show that locally expressed CAd*Trio*-derived transgenes promoted HER2.CAR-NK anti-tumor activity at the tumor site better than systemic IL-15. This suggests that enhanced tumor control and

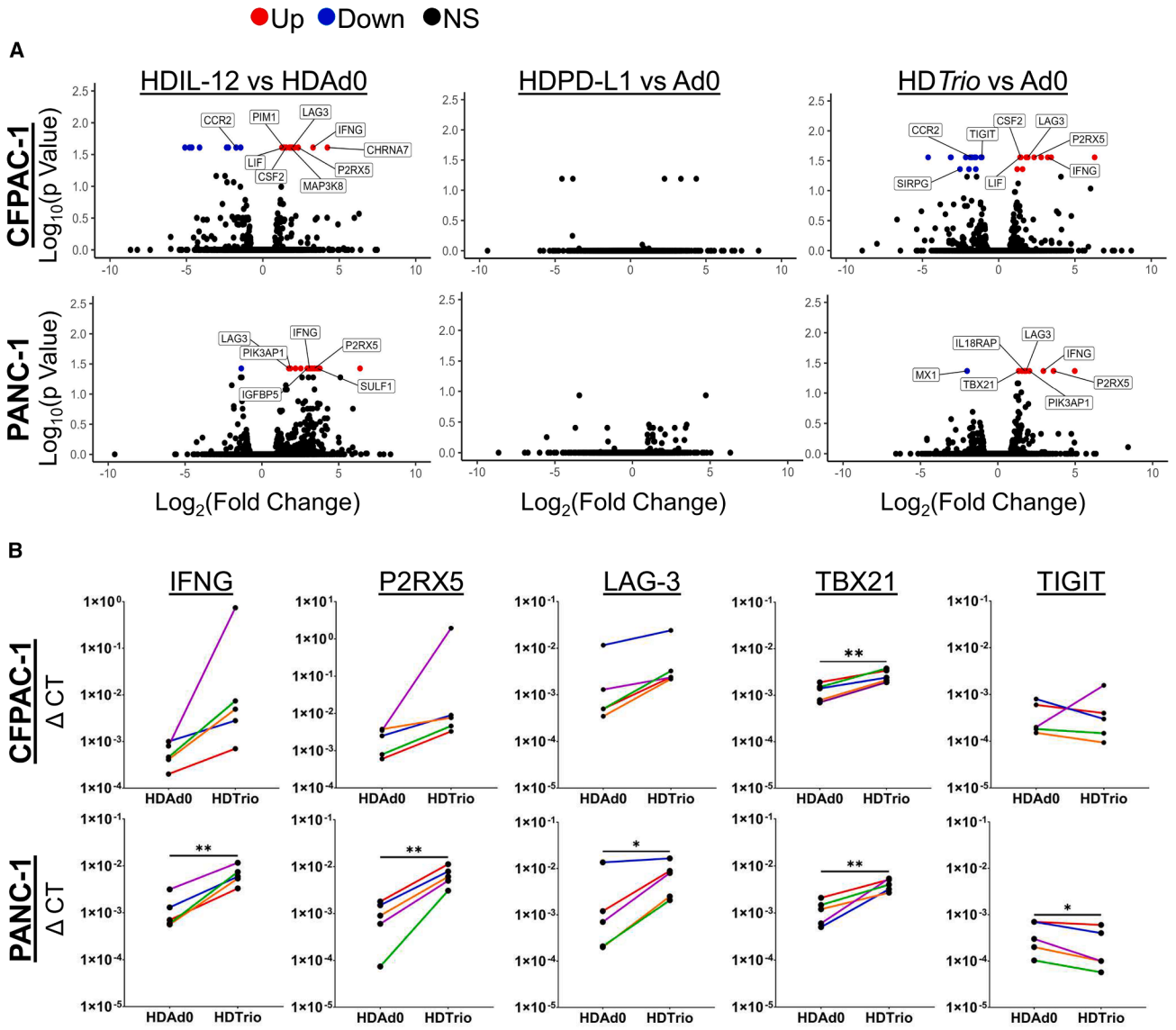

Figure 2. CAd*Trio*-derived transgenes enhance HER2.CAR-NK activation and promote development

(A) Total RNA was extracted from HER2.CAR-NKs after 24 h coculture with CFPAC-1 or PANC-1 infected with HD viruses: HDAd0 (no transgene), HDIL-12, HDPDL1, or HD*Trio*. Data represent 2-fold gene expression changes in HER2.CAR-NKs. Genes with increased expression are shown in *red* and decreased expression are shown in *blue*, while *black* are genes not substantially changed between comparators. (B) Total RNA was collected from HER2.CAR-NKs after coculture with CFPAC-1 cells infected with either HDAd0 or HD*Trio* for 24 h and converted to cDNA for PCR analysis (donor $n = 5$). Genes were quantified and normalized to human β-actin. p values were determined using two-tailed t test: IFNG t = 1.023; P2RX5 t = 1.011; LAG3 t = 1.876; TBX21 t = 6.719; TIGIT t = 0.3561; all df = 4. ns, not significant, $^{*}p < 0.05$ and $^{**}p < 0.01$.

improved survival in both PDAC models (Figure 3C) likely results from a combination of CAd*Trio*-mediated oncolysis and locally released factors that work in concert to improve HER2.CAR-NK anti-tumor activity.

Combination immunotherapy is both effective and well-tolerated in humanized mice

Given the superior anti-tumor activity of the combination treatment in immunodeficient xenograft models and our extensive experience with humanized mouse models,[10,30,31] we next tested the efficacy and tolerability of CAd*Trio* and allogeneic HER2.CAR-NK cell combination immunotherapy in humanized mice, well-suited to test expanded immune activation and toxicity. After humanization and establishment of tumors (100 mm^3), we treated mice with 1×10^7 vp of CAd*Trio* followed by 1×10^6 HER2.CAR-NKs 3 days later and a second HER2.CAR-NK infusion 4 weeks later. Control mice received no treatment, and others received CAd*Trio* alone or CAR-NK alone (Figure 4A). Although neither

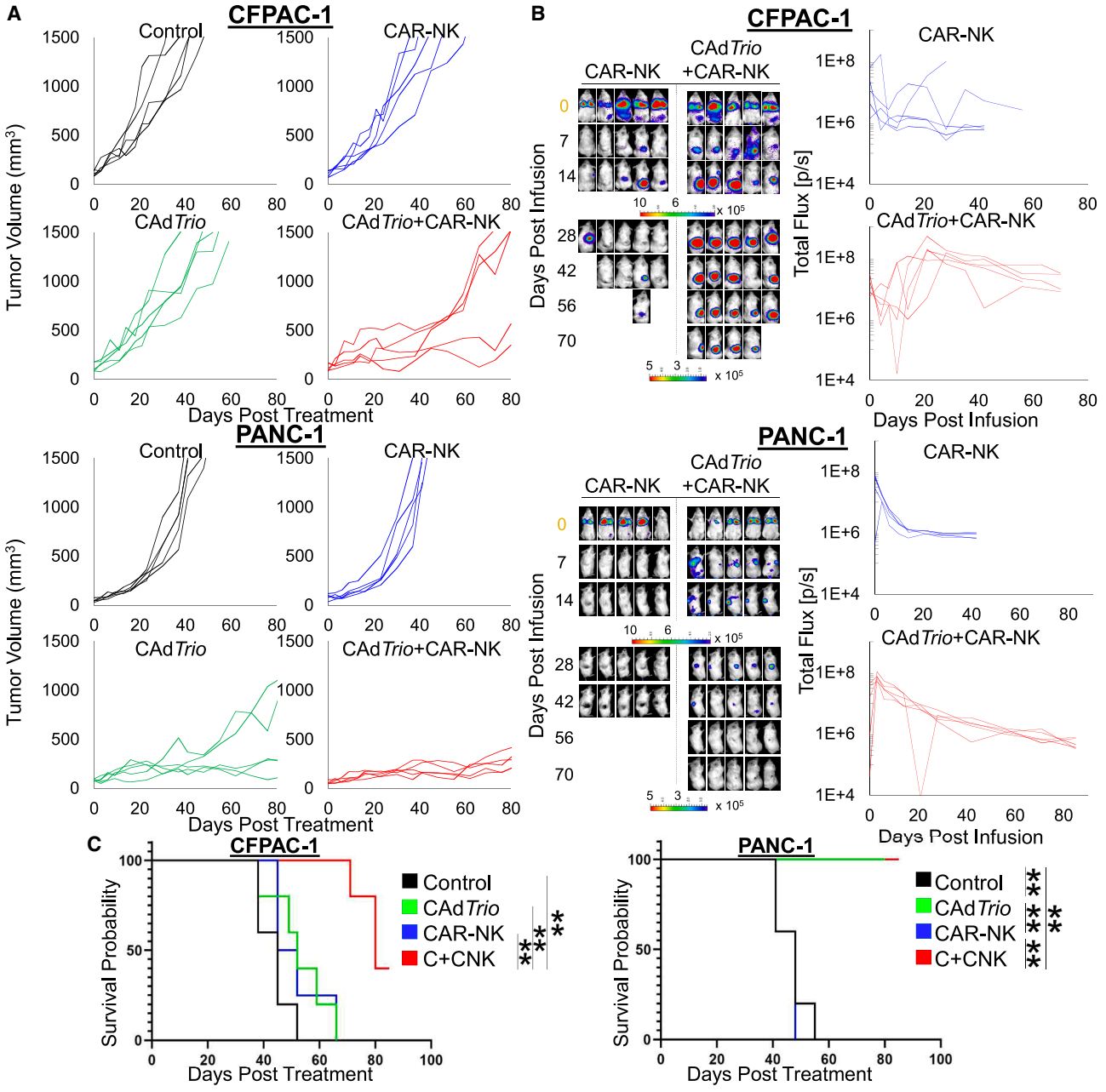

Figure 3. CAd*Trio* combined with CAR-NK cells is superior to single agent HER2.CAR-NK therapy in NSG mouse

(A) CFPAC-1 or PANC-1 cells were transplanted into the right flank of NSG mice (control [untreated], HER2.CAR-NK alone, CAd*Trio* alone, or CAd*Trio*+HER2.CAR-NK: $n = 5$). A total of 1×10^7 vp of CAd*Trio* (OAd:HD = 1:1) were injected intratumorally, then 5×10^6 HER2.CAR-NKs expressing GFP were administered via the tail vein 3 days after CAd*Trio* injection. Tumor volumes were monitored by caliper measurement at indicated time points, and data from individual animals are shown. (B) HER2.CAR-NK bioluminescence was monitored at indicated time points, and data from individual animals are shown. (C) Kaplan-Meier survival curve after CAd*Trio* administration ($n = 5$). p values were determined using the log rank Mantel-Cox test (dF = 3). ns, not significant, $^*p < 0.05$ and $^{**}p < 0.01$. Abbreviations are as follows: s.c., subcutaneous; i.t., intratumoral; i.v., intravenous.

HER2.CAR-NKs alone nor CAd*Trio* alone conferred tumor control in CFPAC-1 tumor-bearing humanized mice, combination CAd*Trio* and HER2.CAR-NK treatment resulted in prolonged tumor control. In PANC-1 tumor-bearing mice, HER2.CAR-NK and CAd*Trio* single treatments controlled tumor growth compared to untreated animals, but tumors in mice treated with

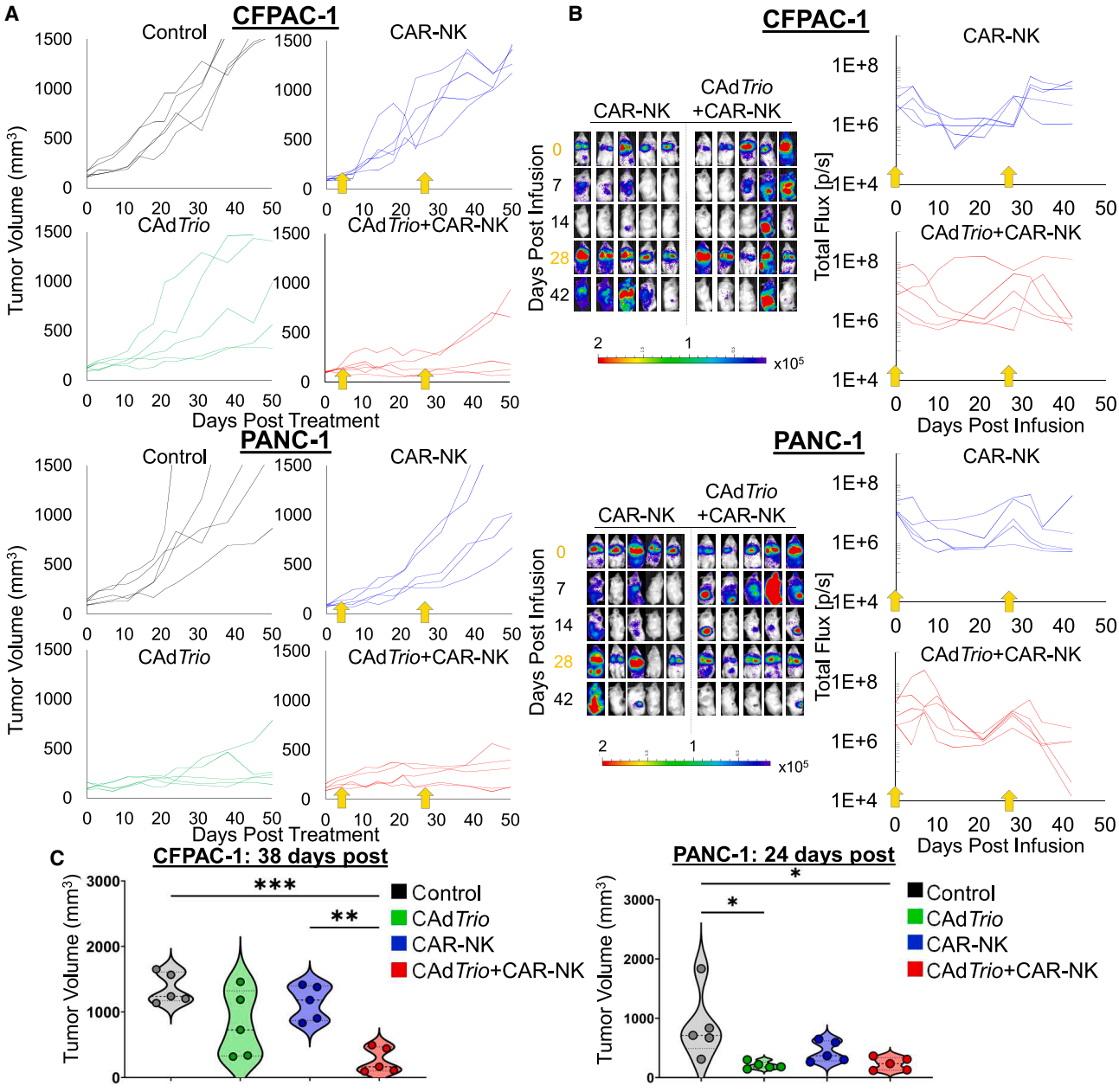

Figure 4. CAd*Trio* and CAR-NK cell combination immunotherapy is both effective and well tolerated in humanized mouse models

(A) CFPAC-1 or PANC-1 cells were transplanted into the right flank of humanized mice (control [untreated], HER2.CAR-NK alone, CAd*Trio* alone, or CAd*Trio*+HER2.CAR-NK) ($n = 5$). A total of 1×10^7 vp of CAd*Trio* (OAd:HD = 1:1) were injected intratumorally, then 1×10^6 HER2.CAR-NKs expressing GFP were systemically administered 3 days after CAd*Trio* injection, and then again 4 weeks later (yellow arrows). Tumor volumes were monitored by caliper measurement at indicated time points, and data from individual animals are shown. (B) HER2.CAR-NK bioluminescence was monitored at indicated time points, and data from individual animals are shown. (C) Tumor volumes at first endpoint event (CFPAC-1 day 38 [$n = 5$], PANC-1 day 24 [$n = 5$]). p values were determined by one-way ANOVA with Tukey multiple comparisons. ns, not significant, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. Abbreviations are as follows: s.c., subcutaneous; i.t., intratumoral; i.v., intravenous.

HER2.CAR-NKs eventually progressed. In contrast, CAd*Trio* single treatment and combination CAd*Trio* and HER2.CAR-NK treatment resulted in sustained tumor control, highlighting the efficacy of CAd*Trio* in treating PANC-1 tumors. Interestingly, HER2.CAR-NK-based luciferase signal in humanized mice depreciated below detection thresholds in most animals 14 days after infusion in both CFPAC-1 and the CAd*Trio*-sensitive PANC-1 models, suggesting a differential expansion potential in the

context of a functional immune system compared to immunodeficient mice (Figure 4B).

Peripheral cytokine analysis of mice with CFPAC-1 tumors revealed a biological trend in elevated levels of pro-inflammatory cytokines IFN-γ, IL-6, IL-12, and tumor necrosis factor alpha in CAd*Trio*-treated mice 1 week after either single or combination treatment with HER2.CAR-NK (Figure S6), suggesting that CAd*Trio*-mediated local immune stimulation is important for adoptively transferred CAR-modified cell infiltration and functionality. While not statistically significant, the differences in these peripheral cytokine values is a promising indicator that local CAd*Trio* treatment initiates systemic inflammatory signaling which aids in adoptive HER2.CAR-NK and endogenous lymphocyte trafficking to the tumor; a phenomenon we have described previously.[10] Humanized mice with PANC-1 tumors treated with CAd*Trio* with or without HER2.CAR-NKs displayed a biological trend of increased pro-inflammatory cytokine levels 1 week after treatment compared to control mice and mice treated with HER2.CAR-NKs alone. Critically, despite the increase in peripheral cytokine concentrations, animal body weights and overall appearance in both models were generally stable across all treatment groups (Figure S7), suggesting these immunotherapeutic treatments, particularly the allogeneic CAR-NK cells, were well tolerated (Figure 4C). Together, these data indicate that combination CAd*Trio* and allogeneic HER2.CAR-NK cells is both effective and well tolerated in the context of an active human immune system.

Combination immunotherapy controls heterogeneous PDX tumor growth and improves animal survival

Heterogeneous tumor antigen expression is a well-documented challenge for CAR-T cell approaches, particularly in solid tumors, including PDAC.[18] Adoptive NK cells are a promising therapeutic approach to overcome this issue since these cells utilize multiple endogenous ligand/receptor interactions and are not reliant on the stable expression of any single antigen to facilitate anti-tumor activity.[17,22] To test our combination immunotherapeutic approach against heterogeneous PDAC tumors, we transplanted PDAC patient-derived xenografts (PDX) into the flanks of NSG mice. After tumors reached 100 mm^3, we treated mice with an intra-tumoral injection of 1×10^7 vp CAd*Trio* and 5×10^6 HER2.CAR-NKs systemically infused three days later, or either treatment alone (Figure 5A). Similar to the CFPAC-1 xenograft model (Figure 3), CAd*Trio* treatment alone or HER2.CAR-NKs alone demonstrated minimal tumor control. Again, CAd*Trio* and HER2.CAR-NK combination treatment consistently controlled initial PDX tumor growth with one animal achieving a complete response by day 52. We observed prolonged HER2.CAR-NK persistence in the presence of CAd*Trio* treatment (Figure 5B). Combination treatment led to improved survival of treated animals compared to single agent treatments (Figure 5C).

Immunotherapeutic approaches, including CAR modified lymphocytes, apply selective pressures to the tumor that results in immunotherapeutic resistance phenomena such as antigen escape.[18,22] To better understand how the PDX tumor escapes this combination immunotherapy, we harvested residual tumors to measure the expression of HER2 and NK ligand/receptor expression via flow cytometry at the end of experiment (Figure S8). While tumors treated with single agents showed similar levels of PD-L1 and HER2 expression to the untreated control tumor, the PD-L1 expression in the combination-treated tumor was marginally increased and HER2 expression was reduced. However, we observed a more dramatic shift in the expressions of two key determinants of NK cell function, CD112 and CD155.[27,32,33] The single agent-treated CAd*Trio* and HER2.CAR-NK tumors did not have any impact on the expression of CD112 and slightly increased the expression of CD155 compared to the untreated control tumor. Conversely, the combination-treated tumor analysis revealed an increase in CD112 expression and simultaneous decrease in CD155 expression.

To expand on these late-stage observations, we repeated this study and harvested all tumors 4 weeks after administration of HER2.CAR-NKs to measure NK ligand expression at the initiation of tumor recurrence (Figure S9). The expression levels of HER2, CD112, or CD155 were not significantly altered between any experimental groups, while PD-L1 expression was significantly elevated in the combination CAd*Trio* and HER2.CAR-NK treatment group compared to untreated control tumors; a biological trend that was reflected in single agent treatment groups. All treatment groups increased the expression ULBP1, an NKG2D ligand, with combination- and single-agent CAd*Trio*-treated tumors achieving statistical significance. We also observed increased expression of another NKG2D ligand MICA/B, specifically in the combination treatment group, indicating that CAd*Trio* function is working in concert with HER2.CAR-NK activity by increasing cellular stress ligands in PDAC PDX tumors.

Analysis of peripheral blood cytokines in the initial PDX study showed an abundance of IFN-γ in combination-treated mice compared to single agent treatments where none was detected and control (untreated) animals showed low levels (Figure 5D). Although peripheral IFN-γ levels in combination-treated mice reduced at day 21, mice in this treatment group survived long enough for sample collection at days 42 ($n = 5$) and 63 ($n = 3$), which showed prolonged IFN-γ expression. As expected, based on our previous studies,[10,11] mice treated with CAd*Trio*, either singly or in combination with HER2.CAR-NKs, showed elevated IL-12p70 at days 7 and 21 compared to HER.CAR-NK-treated and control mice due to the expression of IL-12p70 derived from CAd*Trio*. Interestingly, combination-treated animals had greater peripheral concentrations of IL-12p70 than CAd*Trio* alone both at day 7 and day 21. We continued to detect IL-12p70 in the surviving combination mice until day 63. Given the limited IL-12 levels in the blood of mice treated with CAd*Trio* alone, we suspected that HER2.CAR-NKs might be the source of the additional IL-12. To test if HD*Trio*-derived immunomodulation (IL-12 plus PD-L1 blocker) induced the expression of *IL-12* in HER2.CAR-NKs, we performed RT-qPCR on HER2.CAR-NKs after 24 h

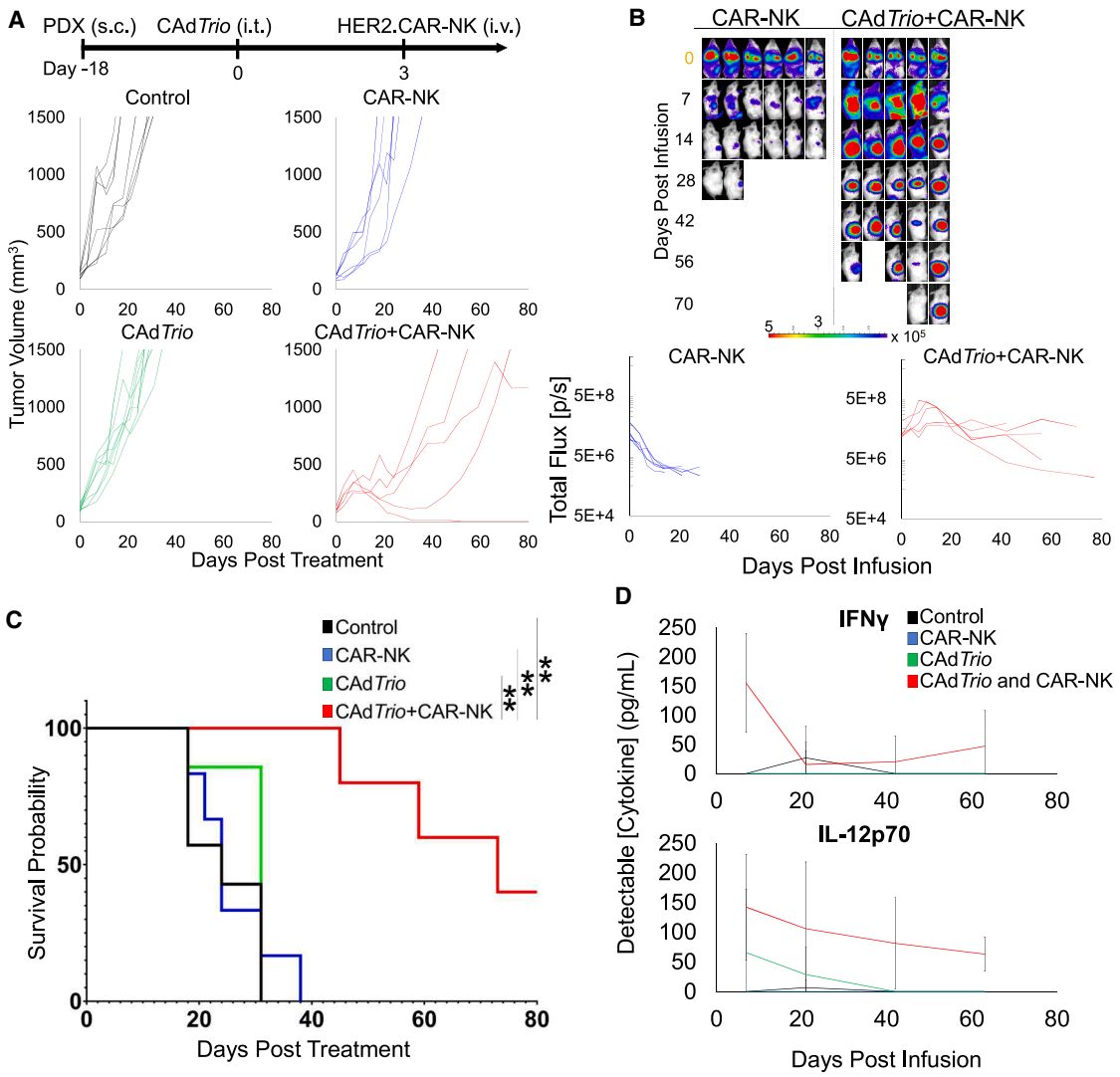

Figure 5. Combination immunotherapy controls heterogeneous PDX tumor growth and improves survival

(A) PDAC PDX tumors were subcutaneously transplanted into the right flank of NSG mice (control [untreated], HER2.CAR-NK alone, CAd*Trio* alone, or CAd*Trio*+HER2.CAR-NK; n = 5–8 animals). A total of 1 × 10^7 vp of CAd*Trio* (OAd:HD = 1:1) were injected intratumorally, and then 5 × 10^6 HER2.CAR-NKs expressing GFP were systemically administered 3 days after CAd*Trio* injection. Tumor volumes were monitored by caliper measurement and (B) HER2.CAR-NK bioluminescence at indicated time points. Data from individual mice are shown. (C) Kaplan-Meier survival curve after CAd*Trio* administration. (D) Serum was collected at days 7, 21, 42, and 63 post HER2.CAR-NK infusion to measure IFN-γ and IL-12p70. $p < 0.01$. p values were determined using the log rank Mantel-Cox test (dF = 3). ns, not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. Abbreviations are as follows: s.c., subcutaneous; i.t., intratumoral; i.v., intravenous.

coculture with HDAd0 (no transgene) or HD*Trio*-infected PDAC cells. We observed that HD*Trio* transgenes induced the expression of IL-12p35 and IL-12p40 mRNA in HER2.CAR-NKs derived from 5 different donors (Figure S10), indicating that regardless of donor, HER2.CAR-NK cells respond to HD*Trio* transgene products by producing more pro-inflammatory cytokine. Together, these data indicate that CAd*Trio* and HER2.CAR-NK combination treatment controls heterogeneous tumor cell growth by prolonging HER2.CAR-NK cell anti-tumor function through CAd*Trio*-derived transgenes.

DISCUSSION

Overall, we present evidence from multiple PDAC models that the combination immunotherapy CAd*Trio* and allogeneic HER2.CAR-NKs is a reliably superior treatment against immunotherapy-resistant, immunologically "cold" PDAC tumors. While CAd*Trio* treatment alone was shown to be effective in most PANC-1-bearing NSG and humanized mice, the combination of CAd*Trio* and HER2.CAR-NK immunotherapy consistently controlled tumor growth due to CAd*Trio*-derived transgenes enabling HER2.CAR-NK persistence at the tumor site and prolonged anti-tumor activity

in xenograft models. The stimulatory action of IL-12p70 combined with immune checkpoint blockade through the PD-L1 mini-antibody promoted an NK cell phenotype associated with a memory-like NK cell[34–36]; an observation which was successfully replicated in CAR-NK cells derived from multiple donors. Additionally, results from two clinically relevant models exhibited that (1) CAd*Trio* and allogeneic HER2.CAR-NK combination immunotherapy is both effective and tolerable in humanized mice and (2) this combination approach can control heterogeneous PDAC tumor growth in a PDAC PDX model.

We have previously shown that CAd*Trio* is an effective multifaceted approach for addressing solid tumors, including PDAC.[10] Since local CAd*Trio* treatment alone is insufficient to consistently eliminate distant (untreated) tumor in both pre-clinical and clinical studies,[10,30] we combined the CAdVEC platform with adoptive CAR-T cell therapy and demonstrated durable responses in pre-clinical studies which successfully translated into the clinic (NCT03740256). CAd*Trio* was initially devised to complement T cell activity (including adoptive CAR-T cells) through oncolytic activity to enhance inflammation and tumor antigen spread, supplemented IL-12 protein in the TME, and sustained anti-tumor activity through anti-PD-L1 immune checkpoint blockade. However, the TME is composed of a mix of cell types that have redundant and independent actions against the tumor. With this in mind, we found that CAd*Trio* mechanisms of action (e.g., inflammation, oncolysis, and immunomodulatory transgenes) affect multiple cell types within the TME, including improvements to endogenous NK cell function.[10] Still, adoptive T cell therapy possesses its own limitations, such as the need for autologous products and the associated exorbitant cost tied to complex manufacturing procedures.[18,37,38] Allogeneic CAR-T cells require further modifications to circumvent GvHD and mitigate graft rejection.[37,39] Allogeneic NK cells, as readily available products derived from a variety of sources (e.g., healthy donor peripheral blood or cord blood), can be rapidly deployed to a patient who would otherwise wait while an autologous product is manufactured. Already, allogeneic NK cells have demonstrated a reliable safety record in clinical studies.[17,24,40] The next set of challenges for adoptive NK cell strategies involve improving persistence and cytolytic activity for solid tumor treatments.

The CAdVEC platform is particularly suited to enhance immune cell activity, as we have previously demonstrated using CAd*Trio* to strengthen adoptively transferred CAR-T cell functionality as well as endogenous immune cell, specifically NK cell, activity.[10] Specifically, we found that CAd*Trio*-components enhanced GNLY expression in endogenous NK cells of humanized mice.[10] Encouragingly similar to T cells, NK cells demonstrate improved anti-tumor activity, persistence, and IFN-γ production in response to IL-12,[41,42] and improved anti-tumor activity in the context of anti-PDL1 immune checkpoint blockade.[10,43] However, T and NK cell behavior differs in the context of IL-12. For example, T cell receptor signaling increases the expression of IL-12Rβ1, thus the TCR is critical for T cell sensitivity to IL-12.[44] Conversely, the expression of IL-12Rβ1 in NK cells is not restricted by a specific ligation; instead, responses to IL-12 are influenced by other cytokines (i.e., IL-2, IL-15, and/or IL-18).[42,44] Further contrasting NK and T cells, a key characteristic of NK cell biology is the ability to naturally survey and eliminate malignant cells via multiple endogenous activation receptors. Accordingly, CAR-NK cell cytotoxicity is not restricted solely to the expression of a CAR ligand. Therefore, we tested our combination immunotherapy with CAd*Trio* and CAR-NK cells in a heterogeneous PDAC PDX model that moderately expressed the CAR antigen HER2, as well as CD112 and CD155. These two proteins along with DNAX-accessory molecule 1 (DNAM-1) and TIGIT constitute as major actors in a dynamic axis of ligand/receptor interactions determining NK cell activation/inhibition and development.[20,32,33,45–48] CD112 or CD155 binding the NK activation receptor DNAM-1 facilitates degranulation; conversely, CD112 or CD155 binding TIGIT results in NK cell inhibition.[24] In our PDX model, single agent HER2.CAR-NKs were able to slow initial tumor growth in 50% of mice, while single agent CAd*Trio* was incapable of affecting any tumor growth control. With the added stimulation from CAd*Trio*, CAR-NKs slowed all initial tumor growth and even cured one of the animals. These data suggest that single agents provided limited pressure to cancer cells; however, combined treatments applied greater selective pressure, resulting in marked phenotypic alterations of tumor cells. Although viral infection can alter some NK activation signals on tumor cells,[49,50] we observed in our PDX model that CAd*Trio* alone did not significantly alter the expression of CD112 or CD155 (similar to a previous study[51]) nor did HER2.CAR-NK treatment alone. Instead, the combination treatment affected a dramatic reduction in CD155 and an increase in CD112 expression. Given our findings, future studies could explore the addition of a CD112 blockade molecule in our CAdVEC platform to encourage continued NK activity[21,48,52,53]; i.e., shifting NK cell signaling away from inhibition and anergy in favor of anti-tumor activity. Although anti-TIGIT strategies in combination with adoptive NK cell therapy have been tested previously,[27,54–56] our RNA-seq data showed that HD*Trio* transgenes reduce TIGIT expression. Instead, future studies could incorporate a form of LAG-3 blockade since our RNA-seq data also indicated increased LAG-3 expression in the context of HD*Trio*; potentially synergizing with the anti-PD-L1 molecule expressed by HD*Trio* in promoting NK cell activity.[53,57,58]

NK cells utilize a mixture of ligand/receptor interactions to survey for malignant and infected cells and affect cytotoxicity.[20] The addition of a CAR expands the ligation repertoire and improves cytotoxicity without limiting cytotoxic activity to CAR-ligand expression. A recent study demonstrated a CD70-targeting CD28z.CAR construct provided superior NK cell activity against various solid and liquid tumors compared to other constructs (including a 41BBz.CAR) both *in vitro* and *in vivo*.[59] However, in this study, we found CD28z.CAR cytotoxic benefit was inferior compared to a 41BBz.CAR construct, specifically under highly stressed conditions, suggesting that the benefit of endodomain is dependent on target antigen/tumor type. These observations support the

viewpoint that while T cell-based CAR constructs promote NK cell activation and subsequent cytotoxicity, it stands to reason that NK cells would most benefit from a CAR construct designed specifically for NK cells. Several preclinical studies have begun parsing the relative efficacy of various NK cell-derived CAR designs incorporating NK cell-derived motifs such as 2B4 or DAP10.[60,61] Even though T and NK cells share analogous signaling pathways, the consequences of activating these pathways in adoptive NK cells requires further characterization.

PI3K-AKT-mTOR pathway activation, for example through CD28z. CAR ligation and/or IL-15 receptor activation, facilitates NK cell proliferation.[18,61,62] Similar to other reports, our data reflected improved 41BBz.CAR-NK cell persistence *in vivo* when supported by exogenous rhIL-15 supplementation, but even with this cytokine support our HER2.CAR-NKs did not control tumor growth. We identified HD*Trio*-derived IL-12 as the factor supporting HER2. CAR-NK function as well as persistence. The activation through 41BB endodomain triggers the nuclear factor κB (NF-κB) pathway,[63,64] initiating an IL-12 generating positive-feedback loop which is further promoted by IL-12p70 derived from CAd*Trio* resulting in additive activation of STAT4 leading to IFN-γ and TBX21 expression.[65] HER2.CAR-NKs also receive pro-inflammatory signals at tumor site due to CAd*Trio* treatment (type I IFN and subsequent STAT1 activation) which, in conjunction with CAd-*Trio*-derived IL-12p70 and PD-L1 blockade, promote maturation associated a memory-like NK cell phenotype, resulting in improved anti-tumor function.[66] In a syngeneic immunocompetent model of glioblastoma, EGFR.CAR-NK cells immediately diminished after administration, but persistence improved when combined with an oncolytic HSV-1 expressing a human IL-15/IL-15Rα fusion protein, similar to our findings.[67] These data suggest that CAR-NKs require cytokine support in order to sustain effective anti-tumor responses against solid tumors.[16,46,60,61]

While HER2.CAR-NK persistence was improved because of HD*Trio*-derived IL-12p70, this persistence could be improved further by encoding additional cytokine stimulation. Current adoptive NK cell strategies frequently use IL-15 modifications to improve NK cell proliferative capacity through STAT5 signaling cascade and cell survival, which leads to a potent NK phenotype that possesses increased cytotoxic potential upon restimulation.[17,35,46,66,68,69] Activation by IL-18 initiates the MyD88 signaling cascade in NK cells leading to NF-κB and mitogen-activated protein kinase (MAPK) pathway activation, promoting Th1 responses.[70,71] Currently, preconditioned cytokine-induced memory-like NK cells (CIML NK[34,66,68,69]) generated by IL-12/15/18 stimulation, are undergoing clinical testing as cancer immunotherapeutic interventions for a variety of liquid and solid malignancies.[20,68,72] The key difference between these *ex vivo* generated CIML NKs and our proposed combination immunotherapy platform is that our platform would leverage the CAdVEC technology to generate this powerful phenotype *in situ* at the tumor site. Since CAd*Trio* has an additional 20 kb capacity, we could further improve CAR-NK cell persistence by locally expressing additional IL-15 and/or IL-18 to influence memory-like development *in situ*.

NK cells have been repeatedly shown to be amenable for allogeneic off-the-shelf therapeutic. However, these adoptive NK cell approaches against solid tumors are still limited due to poor persistence and restricted anti-tumor activity.[23,24,39,72] Our work in humanized mice demonstrates that the combined CAd*Trio* and allogeneic HER2.CAR-NK immunotherapy is both effective and tolerated. Critically, local CAd*Trio* treatment generates chemotaxis due to treatment-related inflammation and the highest concentration of CAd-*Trio* transgenes at the injected tumor site,[10,30] thus attenuating the development of adverse events associated with systemic recombinant cytokine administration or constitutive cytokine expression from adoptively transferred immune cells.[8,10,12,30,73,74] Accordingly, we demonstrated in this study that the cumulative benefits from CAd-*Trio* resulted in superior HER2.CAR-NK trafficking and tumor growth control compared to the support provided by exogenously derived rhIL-15.

While not expressly tested here, HLA mismatched donor and recipient studies are worth pursing to refine this platform for clinical translation as an "off-the-shelf" allogeneic product. To that point, CAd*Trio* in combination with adoptive autologous HER2.CAR-T cells is already in phase 1 clinical trial at Baylor College of Medicine (NCT03740256). Taken together with the growing progress of adoptive CAR-NK cell strategies in clinical studies,[16,22,75] we posit that an "off-the-shelf" combination of CAd*Trio* and allogeneic HER2.CAR-NK cell immunotherapy could translate into the clinical setting and may provide a means to rapidly deploy an immediately available treatment for immunosuppressive, immunotherapy-resistant tumors such as PDAC.

MATERIALS AND METHODS

Cell lines and adenoviral vectors

Human pancreatic lines CAPAN-1, CFPAC-1, and PANC-1 from ATCC (Manassas, VA) were cultured under recommended conditions and authenticated with short tandem repeat (STR) profiling by ATCC.

To generate cell lines expressing the fusion protein EGFP-ffLuc, we infected cells with retrovirus encoding EGFP-*ffLuc*.[11,12] EGFP-positive cells were sorted using an SH800 Cell Sorter (Sony) after 3 passages post infection of retrovirus. The human adenoviral vectors OAd5/3Ad2E1AΔ24 and HDAd*Trio* were previously developed and characterized in detail.[10,76]

Primary cells

Human peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus according to the manufacturer's instructions (Axis-Shield) from healthy donor whole blood (approved by the Baylor College of Medicine IRB Committee). $CD56^+$ cells were isolated from PBMCs via magnetic column selection (Miltenyi Biotec) and cocultured with irradiated (100 Gy) K562-mb15-41BB-L (E:T 1:10)

in G-Rex cell culture devices (Wilson Wolf) for 3 days in expansion media (Stem Cell Growth Medium [CellGenix] supplemented with 10% fetal bovine serum and 500 IU/mL IL-2). Expanded cells were then CD3 depleted via magnetic column selection (MojoSort) and resuspended in expansion media for 24 h. $CD56^+CD3^-$ cells were then transduced with SFG-based retroviral vectors encoding a HER2-directed CAR and then cultured for three days in expansion media.[10,25] For HER2.CAR-NKs expressing EGFP-*ffLuc*, $CD56^+CD3^-$ cells were first transduced with HER2-directed CAR, rested overnight, and then transduced with EGFP-*ffluc* and cultured for two days in expansion media. All HER2.CAR-NKs were used immediately after generation without cryopreservation. Retrovirus production and methodology have been described previously.[77]

Coculture experiments

For tumor-killing assays, GFPffLuc-expressing cancer cells were seeded in 48-well plates. Non-transduced NK cells or HER2.CAR-NKs were added 24 h later at the ratios described in figure legends. To measure effector cell proliferation, cancer cells were seeded in 48-well plates and non-transduced NK cells expressing firefly luciferase or HER2.CAR-NKs expressing firefly luciferase were added 24 h later at the ratios described in figure legends.

Residual live cells (EGFP positivity) were measured using an IncuCyte.

Flow cytometry

The following fluorochrome-conjugated monoclonal antibodies were used: anti-human CD3, CD4, CD8, PD-L1, HER2, CD56, TIGIT, CD155, CD226, CD112, HER2, recombinant human HER2-Fc chimera, and anti-Fc (for detection of HER2.CAR) (BD Bioscience, Beckman Coulter, BioLegend, and R&D systems). Cells were stained with these Abs or the appropriate isotype controls Abs for 30 min at 4°C. Live/dead discrimination was determined via exclusion of 7AAD positive cells (BD Pharmingen). Stained cells were analyzed using a Gallios flow cytometer (Beckman Coulter). Data were analyzed with Kaluza software (BD Bioscience) according to the manufacturer's instructions.

RNA extraction and RT-PCR

CFPAC-1 and PANC-1 cells were infected with helper-dependent adenoviruses for 2 days prior to the addition of HER2.CAR-NKs expressing ffluc. After 24 h coculture, the supernatant was collected, and then plates were gently washed with PBS, which was combined with supernatant. HER2.CAR-NKs were then isolated using CD56 magnetic column selection (Miltenyi Biotec), and then RNA was extracted from magnet contents using RNeasy Plus Mini kit (-QIAGEN). RNA samples were quantified using the NanoDrop 2000 (Thermo Fisher Scientific) and then submitted for RNA-seq (Azenta) or converted to cDNA using SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific). The levels of human pro-inflammatory cytokine/chemokine described in the results were quantified using CFX96 Real-Time PCR Detection System (Bio-Rad) and normalized with human β-actin. We obtained all primer sets from Bio-Rad.

RNA-seq alignment and analysis

Reads in FASTQ format were sorted and aligned to the GRCh38 human genome with STAR 2.7.10b. Differential expression analysis was performed with cufflinks 2.2.1 using the *cuffdiff* function with the mean-variance relationship estimated by treating the experimental conditions as replicates, providing a conservative estimate on the differentially expressed genes.

Animal experiments

The Baylor College of Medicine Institutional Animal Care and Use Committee approved all animal experiments.

For the xenograft subcutaneous models, 2×10^6 CFPAC-1 cells or PANC-1 cells were resuspended in a volume of 100 μL of PBS and injected into the right flank of NSG male or female mice (7–8 weeks old). Eighteen days post transplantation, a total of 1×10^7 vp of CAd (OAd:HDAd = 1:1) were injected at a volume of 20 μL into the tumor. The ratio of OAd to HDAd in the CAd system was optimized to effectively propagate transgene(s) encoded in the co-injected HDAd with lytic effects. Three days post injection of CAd*Trio*, mice received 5×10^6 HER2.CAR-NKs expressing *ffluc* intravenously. For mice receiving HER2.CAR-NKs in combination with exogenous IL-15, 21 days after transplant, mice received 5×10^6 HER2.CAR-NKs intravenously followed by 1 μg recombinant human interleukin-15 (R&D) resuspended in 100 μL PBS intraperitoneally, 3× per week until endpoint was reached. All mice receiving HER2.CAR-NKs were assessed using the In Vivo Imaging System (Xenogen).[12] The endpoint was established at a tumor volume $\geq$1,500 mm^3.

For the humanized mouse model, newborn (1–2 days from birth) female and male NSGSGM3 (NSGTGCMV-IL3, CSF2, KITLG Eav/mloySz; Jackson Laboratory) were sublethally irradiated (100 cGy) and intrahepatically injected with 5×10^4 human cord blood unit (CBU)-derived $CD34^+$ cells. CBUs were obtained from MD Anderson Stem Cell Center, and $CD34^+$ cells were isolated using $CD34^+$ cell isolation kit (Miltenyi Biotech Inc.). After confirming human $CD45^+$ cells in PBMCs of mice 8–9 weeks post injection, 2×10^6 CFPAC-1 or PANC-1 cells were resuspended in a volume of 100 μL of PBS and injected into the right flank of male and female humanized mice. Eighteen days post transplantation, a total of 1×10^7 vp of CAd*Trio* (OAd:HDAd = 1:1) were injected in a volume of 20 μL into the tumor. Three days post injection of CAd-*Trio*, mice received 1×10^6 allogeneic HER2.CAR-NKs expressing ffluc intravenously and assessed using the In Vivo Imaging System (Xenogen).[12] Mice received repeat doses of 1×10^6 allogeneic HER2.CAR-NKs expressing ffluc at 4 weeks after the initial dose of HER2.CAR-NK. All doses of HER2.CAR-NKs were generated from PBMCs isolated one week prior to infusion per our protocol outlined previously. The endpoint was established at a tumor volume $\geq$1,500 mm^3.

For the PDX subcutaneous model, frozen tumors were kindly provided by Dr. Qizhi Yao and transplanted into the right flank of NSG mice. Twenty-eight days post transplantation, a total of 1×10^7 vp of CAd*Trio* (OAd:HDAd = 1:1) were injected in a volume of 20 μL into the tumor. Three days post injection of CAd*Trio*, mice received 5×10^6 allogeneic HER2.CAR-NKs expressing ffluc intravenously and assessed using the In Vivo Imaging System (Xenogen).[12]

Cytokine analysis

Blood samples were collected at the time points specified in the results section and figure legends. For PDX the experiment, serum samples were prepared for flow cytometry with a BD Accuri C6 Plus Flow cytometer per manufacturer instructions. The beads used were Human IFN-γ Flex Set and Human IL-12p70 Flex Set (Catalog nos. 558269 and 558283). Subsequent analysis was performed using accompanying manufacturer software, version 1.0.264.21 build 20120423.264.21. Humanized mouse plasma samples were analyzed using Multiplex Cytokine Immunoassay (EMD Millipore).

Isolation of residual tumors

After rinsing the harvested tumors with PBS, tumors were minced and incubated in RPMI media containing human tumor dissociation reagents (Miltenyi Biotech Inc.) at 37°C for 1 h. Cells were passed through a 70-μm cell strainer (BD Pharmingen), and murine stroma cells were removed using a Mouse Cell Depletion kit (Miltenyi Biotech Inc.). Human cells were stained with the antibodies described in the results.

Statistics and reproducibility

Results are represented as mean of two or more independent experiments (biological replication). Data with three or more groups were analyzed by ordinary one-way ANOVA analysis. Wilcoxon matched pairs test was used to compare two groups of paired data. Data were analyzed with GraphPad Prism 9. Volcano plots were generated using R version 4.2.2 and ggplot2 version 3.4.2.

DATA AVAILABILITY

All data relevant to the study are included in the article or uploaded as supplementary information. All other data are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.S. Methodology, M.S., G.B., A.R.S., D.M., C.P., L.J., Q.C.Y., M.W., and N.C. Investigation, M.S., G.B., A.R.S., D.M., C.P., and L.J. Visualization, M.S., G.B., A.R.S., D.M., C.P., L.J., M.W., and N.C. Funding acquisition, M.S. and R.P. Supervision, M.S. Writing – original draft, G.B. Writing – review & editing, M.S. and A.R.S.

DECLARATION OF INTERESTS

M.S. received research funding from Tessa Therapeutic Ltd. and AstraZeneca. M.S. was a scientific consultant and C.P was a consultant for Tessa Therapeutic Ltd.

SUPPLEMENTAL INFORMATION

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