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ADI-270: an armored allogeneic gamma delta T cell therapy designed to target CD70-expressing solid and hematologic malignancies

Kevin P Nishimoto, Gauri Lamture, Yvan Chanthery, Alexander G Teague, Yogendra Verma, Melinda Au, Morgan Smith-Boeck, Michael Salum, Pranav Murthy, Smitha R Y Gundurao, Ramandeep Kaur, Jie Zhang, Aruna Azameera, Jonathan T S Wong, Elizabeth B Speltz, Katherine M Wang, Amy Doan, Jyothi Sethuraman, Dishant Bhatwala, Ana Giner-Rubio, Pavan Puligujja, Helen Budworth, Christopher J Rold, Swapna Panuganti, Aya Jakobovits, Marissa Herrman, Arun Bhat, Shon Green, Blake T Aftab

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KPN and GL are joint first authors.

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Adicet Therapeutics Inc, Redwood City, California, USA

Correspondence to
Dr Kevin P Nishimoto; knishimoto@adicetbio.com

ABSTRACT

Background The tumor microenvironment (TME) poses challenges that limit the efficacy of conventional CAR-T cell therapies. Homing barriers, immunosuppressive factors, and target antigen heterogeneity can impair CAR-T cell functional activity within the TME. Alternative strategies have contemplated incorporating the use of gamma delta (γδ) T cells as a CAR-T cell approach to potentially overcome these limitations. γδ T cells possess both innate and adaptive immunity to facilitate broad tumor recognition, and their natural propensity for tissue tropism may allow for more effective tumor infiltration. Reported here is the preclinical characterization of ADI-270, an allogeneic γδ CAR-T cell product targeting CD70$^+$ cancers, engineered with a third-generation CAR based on the natural CD27 receptor. ADI-270 is also double-armored to mitigate the immunosuppressive effects of TGFβ and reduce the potential for allogeneic rejection.

Methods Vδ1 T cells engineered to express an anti-CD70 CAR and dominant negative TGFβ receptor II (dnTGFβRII) were expanded from healthy donor human PBMCs. The phenotype and functional characterization of ADI-270 were assessed with in vitro cell culture assays and in vivo tumor xenograft models.

Results ADI-270 exhibited high levels of in vitro cytotoxicity against a panel of cancer cell lines and displayed a favorable inflammatory cytokine profile compared with reference scFv-based anti-CD70 CAR αβ T cells. Cytotoxicity remained potent despite low CD70 expression observed in multiple solid and hematologic tumor cell models. When armored with dnTGFβRII, ADI-270 exhibited functional resilience to TGFβ-mediated inhibition of T cell effector activity. In addition, the incorporation of potent and sensitive CD70-targeting decreased T cell-mediated alloreactive killing against ADI-270 in vitro without evidence of fratricide. Finally, ADI-270 displayed robust tumor tropism and control of primary and secondary tumor challenges in xenograft mouse models.

Conclusions These results demonstrate the robust potency and capacity of ADI-270 to extend antitumor activity to cancers with heterogeneous antigen expression. The functional armoring incorporated into ADI-270 provides a mechanism to overcome the limitations of reduced efficacy and persistence within the TME. ADI-270 has the potential to target multiple CD70$^+$ cancers with initial clinical evaluation proceeding in relapsed/refractory clear cell renal cell carcinoma.

Trial registration number NCT06480565.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The heterogeneity of tumor antigen expression and the immunosuppressive tumor microenvironment pose significant challenges in treating solid tumors using conventional CAR-T cell therapy. Alternative cytotoxic immune cell effectors are being explored as sources for CAR-based cell therapies to improve clinical responses in patients with solid tumors.

WHAT THIS STUDY ADDS

⇒ This study highlights the preclinical characterization of ADI-270, a novel allogeneic CD27 receptor-based anti-CD70 CAR Vδ1 gamma delta (γδ) T cell product armored with dnTGFβRII that eliminates tumors expressing heterogeneous levels of CD70, mitigates the immunosuppressive effects of TGFβ, and reduces alloreactive host T cell rejection. The intrinsic antitumor properties of Vδ1 T cells and the functional armoring integrated into ADI-270 provide mechanisms to enhance the efficacy in solid tumors.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The development of ADI-270 positions γδ T cell-based platforms as a promising immunotherapeutic strategy for treating patients with solid tumors. ADI-270 has the potential to target multiple CD70$^+$ cancers, with initial clinical evaluation proceeding in relapsed/refractory clear cell renal cell carcinoma (NCT06480565).

BACKGROUND

Currently approved chimeric antigen receptor (CAR)-T cell therapies derived from autologous αβ T cells have shown remarkable clinical benefit in several hematologic malignancies.[1] However, in solid tumors, the efficacy of conventional CAR-T cell therapies remains limited due to several factors, including poor tumor infiltration, the immunosuppressive tumor microenvironment (TME), and heterogeneous target antigen expression.[2] To overcome these challenges, recent advances in CAR-T cell development have focused on strategies to enhance homing, functional potency, and persistence within the TME. Novel CAR constructs designed to optimize antigen engagement, armoring to mitigate the immunosuppressive environment, and the use of alternative cytotoxic immune cell effectors as sources of CAR cell therapies are among several approaches being explored to improve clinical responses in patients with solid tumors.[3 4]

Gamma delta (γδ) T cells have emerged as key players in cancer immunity with an evolutionarily distinct role in immune surveillance and tumor targeting.[5] Unlike αβ T cells, the activation of γδ T cells is not dependent on MHC-mediated peptide presentation, enabling broader tumor recognition through both innate and adaptive immune mechanisms.[6] γδ T cells represent less than 5% of total circulating T cells, but the Vδ1 γδ T cell subset is highly enriched in epithelial and mucosal tissues, common locations for many types of solid tumors.[5] Correlative studies in multiple cancer types have identified an association between tumor-infiltrating Vδ1 T cells and significant favorable clinical outcomes, further emphasizing the potential importance of their unique tissue homing and retention capacity.[5] Recently, γδ T cells were also implicated as the effector cells driving responses to immune checkpoint blockade in colorectal tumors with B2M loss, which evade conventional αβ T cell recognition.[7] Given the intrinsic antitumor potential and the MHC-independent antigen recognition by the γδ TCR, unedited γδ T cells can be administered safely as an allogeneic cell-based immunotherapy with minimal risk for graft vs host disease (GvHD).[5] Further engineering of γδ T cells to express a CAR has been shown to enhance intrinsic tumor targeting with superior cytotoxic potential compared with unmodified γδ T cells in numerous preclinical hematologic and solid tumor models.[5 8–11] Together, this has led to the development of ex vivo expansion methods that are coupled with CAR engineering of γδ T cells for clinical use.[8 12]

The presence of immunosuppressive factors within the TME can impact T cell effector functions, resulting in reduced antitumor responses.[13] The immunosuppressive cytokine transforming growth factor beta (TGFβ) can be highly expressed within the TME from tumor cells, stromal fibroblasts, and immune cell subsets.[14] TGFβ has been shown to reduce T cell activation and proliferation, key functions that are critical to mounting effective antitumor responses.[15] Functional deficiency of tumor-derived γδ T cells was partially found to be driven by the presence of TGFβ.[16] Strategies to limit the immunosuppressive effects of TGFβ have aimed to inhibit TGFβ-mediated signaling through the expression of an inactive TGFβ receptor II (TGFβRII) or gene-edited knockout (KO) of endogenous TGFβRII.[17 18] Studies have demonstrated that incorporation of dominant-negative TGFβRII (dnTGFβRII), a truncated form of the naturally occurring TGFβRII receptor lacking the intracellular domain necessary for downstream TGFβ signaling, into T cell-based therapies including CARs can mitigate the immunosuppressive effects of TGFβ.[17 19 20]

Identifying antigens restricted to cancer cells for CAR-targeting, which are not expressed in normal tissues, is necessary to minimize on-target off-tumor toxicity. CD70, a type II transmembrane protein belonging to the TNF family, is an appealing tumor-specific target due to its high expression in multiple solid and hematologic tumors.[21] CD70 plays a role in pro-tumorigenic mechanisms such as enhanced growth, metastasis, immune evasion, and immune suppression.[22 23] In normal tissue, CD70 is transiently expressed in a subset of activated T cells, B cells, NK cells, and mature dendritic cells,[23 24] which represents an acceptable on-target, off-tumor population. Consequently, this has led to the development of novel strategies aimed at targeting CD70-expressing tumors for improved treatment outcomes.

Described here is the preclinical development of ADI-270, a novel allogeneic CAR-T cell product targeting $CD70^+$ malignancies designed to overcome the challenges associated with the TME. ADI-270 is composed of ex vivo expanded Vδ1 T cells intended to leverage their natural immunity and tissue tropism for tumor killing. The Vδ1 T cells are engineered to express a third-generation CAR that uses the CD27 natural receptor for optimal targeting of CD70[25–27] and is armored with dnTGFβRII to mitigate the immunosuppressive effects of TGFβ. Furthermore, as an allogeneic cell product, the incorporation of the anti-CD70 CAR also serves to target activated $CD70^+$ alloreactive lymphocytes to reduce Host vs Graft (HvG) rejection.

METHODS AND MATERIALS

Cell lines

A498, ACHN, NCI-H1975, HUT-78, 769-P, Caki-2, MM1.S, and THP-1 cell lines were purchased from ATCC. Cells were cultured as per the manufacturer's instructions. Tumor cells were modified to stably express NucLight Near Infrared (NucNIR) or Orange (NucO) following transduction with Incucyte NucLight Lentivirus (Sartorius). The A498 cell line containing a CRISPR/CAS9 (IDT) KO of CD70 (gRNA-UAUAGCCUGGGGUCCUGCUG) (Synthego) was generated using the 4D-Nucleofector System (Lonza). All tumor cell lines, cell-based in vitro assays, and manufacturing processes were maintained at 37°C, 5% CO_2.

CAR constructs and retroviral vector production

The CAR sequence consisting of the full-length CD27 receptor (Uniprot P26842, AA 20-260), 4-1BB (Uniprot

Q07011, AA 214-255) and CD3ζ (Uniprot P20963, AA 52-164) signaling domains, P2A skip sequence (AA 1-22), and dnTGFβRII (Unprot P37173, AA 23-199) were constructed by cloning DNA fragments into a self-inactivating Moloney murine leukemia virus gammaretroviral transfer plasmid. Versions of the full-length CAR, without dnTGFβRII, were replaced with a truncated low-affinity nerve growth factor receptor (Uniprot P08138, AA 29-274). The gammaretroviral vector (γ-RV) encoding the CAR was produced as previously described.[8 12] Briefly, the γ-RV was generated by transient transfection of 293 T cells using calcium phosphate or polyethyleneimine. Vector supernatants were concentrated by ultracentrifugation or tangential flow filtration and then frozen.

Anti-CD70 Vδ1 CAR-T cell production

CAR Vδ1 T cells were generated as previously described.[8 12] Briefly, for small-scale production, healthy donor peripheral blood mononuclear cells (PBMCs) were plated into flasks coated with immobilized anti-Vδ1 antibody (Adicet Therapeutics) in complete culture media. PBMCs were transduced with the γ-RV encoding the anti-CD70 CAR construct, expanded, and αβ T cells were depleted using the EasySep Human TCR αβ Depletion Kit (StemCell Technologies) prior to cryopreservation. For large-scale production, after transduction, cells were expanded in a Xuri perfusion bioreactor (Cytiva) with complete culture media. Following expansion, αβ T cells were depleted using the CliniMACS Plus (Miltenyi), further cultured in spinner flasks (Corning), and subsequently cryopreserved.

Anti-CD70 CAR αβ T cell production

T cells were enriched from human PBMCs using the EasySep Human T Cell Enrichment Kit (STEMCELL Technologies). The isolated T cells were activated with TransAct (Miltenyi) and cultured in X-VIVO15 containing 10% FBS and IL-2 (100 IU/mL, PeproTech). CRISPR/Cas9 KO of TRAC and CD70 was performed with gRNA-UCUCUCAGCUGGUACACGGC (TRAC) and/or gRNA-UAUAGCCUGGGGUCCUGCUG (CD70) using the 4D-Nucleofector System. T cells were transduced with the γ-RV encoding anti-CD70 CAR sequences derived from US20190233528A1 and WO2021095011A1 patents. Following transduction, cells were expanded, and αβ TCR^+ T cells were depleted prior to cryopreservation in CryoStor (Biolife Solutions).

Flow cytometry

Antibodies binding against human Vδ1 (Adicet Therapeutics or Miltenyi Biotec), TCRγδ (Beckman Coulter or Miltenyi Biotec), TGFβRII (R&D Systems or Miltenyi Biotec), Vδ2, CD3 (Beckman Coulter), CD14, CD19, CD33, CD56, TCRαβ (Miltenyi Biotec), CD62L, CD95, CD45, CD45RO, CD45RA, CD70, NKG2D, DNAM1, CCR5, CCR7, CXCR3, CXCR4, 4-1BB, CD25, PD-1, TIM-3, LAG-3, and TIGIT with HuTruStain FcX (BioLegend), and Zombie Aqua viability dye (BioLegend) were used for flow cytometric staining (online supplemental table 1). Primary antibodies were purchased from BioLegend unless stated otherwise. CAR expression was measured using an intracellular FIX & PERM Cell Permeabilization Kit (ThermoFisher or Invitrogen/BioLegend) and anti-P2A antibody (Sigma Aldrich) followed by a PE-conjugated secondary antibody (Jackson ImmunoResearch). Strep-II tag expression was measured using streptavidin BV421 or PE (Biolegend) against a biotin-tagged anti-strep II antibody (Genscript). BD Quantibrite PE beads (BD Biosciences) were used to estimate the number of antibodies bound per cell. For phosphorylated SMAD2/3 (pSMAD2/3) detection, cells were serum starved for 2 hours in X-VIVO15 (without FBS) and then cultured with and without human recombinant TGFβ1 (20 ng/mL, R&D Systems) for 15 min. The cells were fixed (BD Cytofix Fixation Buffer), permeabilized (BD Phosflow Perm Buffer III), and stained with anti-pSMAD2/3 antibody conjugated to PE (BD Biosciences). Samples were acquired on the Novocyte (Agilent) flow cytometer and analyzed using FlowJo software (BD Biosciences).

Immunohistochemistry staining

Tumor Microarrays from multiple tumor types (Tissuearray.com) were stained using a validated protocol developed at HistoWiz laboratory with an anti-CD70 immunohistochemistry (IHC) mAb antibody (EPR26536-122, Abcam) on an automated Leica platform. Normal tissue sections from 33 types of human organs were derived from three different individuals (Tissuearray.com). The stained tumor and normal tissue sections were evaluated by a certified pathologist (HistoWiz) to identify the types of cells for which the signal was observed and provide scores for stain prevalence and intensity.

In vitro cytotoxicity assay

NucNIR-expressing adherent and suspension target cells were plated in tissue culture-treated (Sigma Aldrich) or poly-L-ornithine coated 96-well flat bottom plates (Corning). Effector cells ($Vδ1^+CAR^+$ viable cells) were cocultured with target cells at various E:T ratios in RPMI 1640 medium (Gibco) containing 10% FBS. Viable target cells expressing NucNIR were quantitated every 4 hours using the IncuCyte SX5 system (Sartorius). For the repeat stimulation cytotoxicity assay, effector cells were transferred 48 hours from the first stimulation and added to a new plate containing freshly plated target cells. Cultures conducted with TGFβ1 (20 ng/mL) had fresh reagent added during each stimulation. For the mixed tumor cytotoxicity assay, ADI-270 was cocultured with a 1:1 target cell mixture of A498-NucO and CD70 KO A498-NucNIR. Cytotoxicity Index (CI) was calculated by dividing the total NIR-object area ($μm^2$/well) of all time points by the value at time zero. Percent cytotoxicity was calculated by [(CI of target alone)−(CI of target+effector)/(CI of target alone)]×100.

Proliferation

Cell Trace Violet (CTV, ThermoFisher) working solution (2 µM) in 1X PBS (ThermoFisher) was used to label the cells following the manufacturer's instructions. CTV-labeled ADI-270 and anti-CD70 CAR Vδ1 T cells lacking dnTGFβRII armoring component were cocultured with A498 tumor cells (1:2 E:T ratio) for 7 days in the presence or absence of TGFβ1 (20 ng/mL). Cells were stained with propidium iodide (PI, BioLegend) and anti-Vδ1 antibody, then acquired on the Novocyte flow cytometer. Effector cells were evaluated for proliferation by determining the percentage of cell divisions with decreased CTV fluorescent intensity using FlowJo Proliferation Modeling software.

Targeted gene expression analysis

RNA was extracted from cell pellets of ADI-270 and unarmored anti-CD70 CAR Vδ1 T cells using the RNeasy Mini Kit (QIAGEN) after 24 hours of stimulation in non-tissue culture-treated plates precoated with human recombinant CD70 non-Fc tagged active trimer (5 µg/mL, AcroBio) and cultured with or without TGFβ1 (20 ng/mL). Gene expression levels were quantified using the nCounter CAR T Characterization Panel (NanoString) on the nCounter SPRINT Profiler (NanoString) according to the manufacturer's instructions. The nSolver Advanced Analysis software (NanoString) was used to normalize transcript expression levels and identify differentially expressed genes (DEGs, adjusted $p<0.05$ and $\log_2$-fold change >1 or <−1).

Multiplex cytokine quantification assay

ADI-270 was cocultured with target cells (1:1 E:T) for 24 hours in RPMI 1640+10% FBS. The MILLIPLEX Immunology Multiplex Assay Panel (Millipore) was used according to the manufacturer's protocol to measure cytokine and chemokine levels in supernatants on the Luminex FLEXMAP 3D Instrument (DiaSorin).

HvG susceptibility

Healthy donor PBMCs were activated with T Cell TransAct to upregulate CD70 expression. ADI-270 and donor-matched untransduced Vδ1 T cells were cocultured with activated PBMCs (targets) at E:T ratios of 1:1, 1:2, and 1:4 for 48 hours. Flow cytometry was used to quantify the frequency of $CD70^+$ PBMC targets in the cocultures.

Primed-alloreactive T cells against ADI-270 product/donors (≤1 match at HLA-A, HLA-B, and HLA-C alleles) were generated by coculturing enriched T cells (effectors) (EasySep Human T Cell Enrichment Kit, StemCell Technologies) from PBMCs with allogeneic monocyte-derived macrophages (targets) at a 10:1 E:T ratio in IL-2 (10 ng/mL) for 7 days. Macrophages were generated by isolating monocytes from PBMCs using human CD14 microbeads (Miltenyi) and cultured in RPMI 1640+10% FBS with 50 ng/mL of human recombinant macrophage colony-stimulating factor (M-CSF, R&D Systems) for 6 days. CTV-labeled ADI-270 and Vδ1 T cells expressing an irrelevant CAR (targets) were cocultured with the primed effector T cell allogeneic donors in a mixed lymphocyte reaction (MLR) assay at E:T ratios of 1:1, 1:2, and 1:4. After 24 hours, cells were analyzed using flow cytometry (Novocyte) to determine percent survival by quantitating absolute counts of $PI^{negative}$ (viable) ADI-270 and irrelevant CAR Vδ1 T cells, compared with the respective target alone controls. Percent survival was calculated using the equation (1−[(Vδ1 cell count alone control)−(Vδ1 cell count from coculture with allogeneic T cells)/(Vδ1 cell count alone control)])×100.

In vivo and ex vivo mouse studies

All mouse experiments were performed in accordance with the guidelines set by Adicet's Institutional Animal Care and Use Committee. In vivo efficacy studies were conducted using female NOD Cg-Prkdc^{scid} IL2rg^{tm1Wjl}/SzJ (NSG) mice (Jackson Lab), aged 6–9 weeks. Human tumor cell lines were subcutaneously (SC) implanted at a 1:1 ratio volume mixture with Matrigel (Corning). On reaching a predefined tumor volume between 100 and 500 mm^3, mice were randomized and subsequently injected intravenously with the appropriate number of CAR^+ Vδ1 T cells. Human IL-2 (13 000 IU; Proleukin) was administered intraperitoneally immediately prior to cell treatment and then thrice weekly for the duration of the study. Tumor volume was measured twice weekly using calipers. Mice were humanely euthanized when tumor volume reached 2000 mm^3 or when humane endpoints were reached. For ex vivo analysis of γδ T cells in tumor-bearing mice, CTV-labeled ADI-270 cells were intravenously injected into NSG mice bearing A498 tumors. On day 7 and day 14 post-treatment, blood, bone marrow, lung, spleen, and tumors were collected. Tissues were dissociated into single cells using the gentleMACS Dissociator (Miltenyi), and proliferation and immunophenotype were assessed using flow cytometry. To evaluate the kinetics, activity, and pharmacodynamics of ADI-270 in A498 tumor-bearing NSG mice, tumors and blood were collected on days 3, 7, and 14 post-treatment. Tumors were fixed in 10% neutral buffered formalin for 48 hours, preserved in 70% ethanol, and then embedded in paraffin. Tumor blocks were sectioned and processed for CD70 IHC staining and multiplex immunofluorescence (IF) for CD3/Ki67/Granzyme B (GzB) detection (Histowiz). Plasma was isolated from whole blood for human IFNγ quantitation using the FLEXMAP 3D Instrument.

Statistics

Statistical analyses were performed using Prism (V.10, GraphPad). The tests selected to determine statistical significance and defined p values are stated in the figure legends.

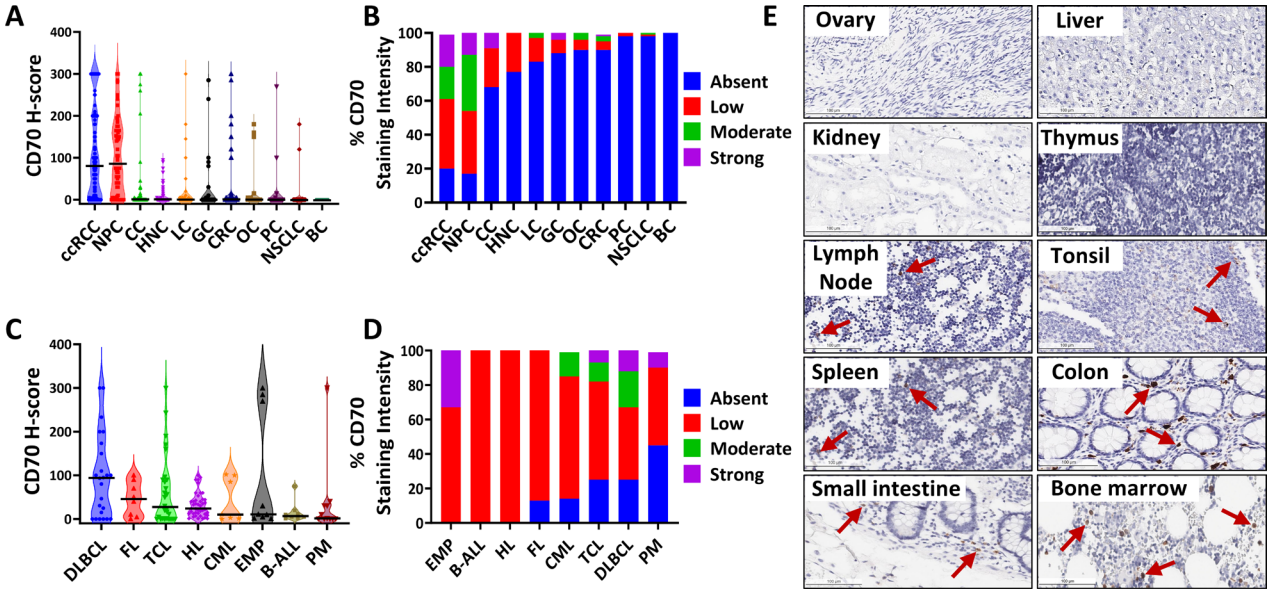

Figure 1 CD70 is expressed on multiple solid and hematologic tumors but has minimal expression on normal tissues. CD70 expression by IHC was assessed across 11 solid tumor indications (A, B) and eight hematologic malignancies (C, D). The H-score was calculated as (1×percentage of low staining)+(2×percentage of moderate staining)+(3×percentage of strong staining) within the target region, ranging from 0 to 300. The horizontal black bar represents the median H-score value. H-score staining intensity metric: 0=absent, 0<low≤100, 100<moderate≤200, 200<strong≤300. The number of tumor samples represented for each indication: clear cell renal cell carcinoma (ccRCC=192), nasopharyngeal cancer (NPC=60), cervical cancer (CC=71), head and neck cancer (HNC=70), laryngeal cancer (LC=80), gastric cancer (GC=72), colorectal cancer (CRC=208), ovarian cancer (OC=80), prostate cancer (PC=192), non-small cell lung cancer (NSCLC=192), breast cancer (BC=104), diffuse large B cell lymphoma (DLBCL=24), follicular lymphoma (FL=8), T cell lymphoma (TCL=28), Hodgkin's lymphoma (HL=43), chronic myelogenous leukemia (CML=7), extramedullary plasmacytoma (EMP=9), B cell acute lymphoblastic leukemia (B-ALL=8), and plasma cell myeloma (PM=11) (E) Representative IHC staining images of CD70 expression on normal tissues (×10, magnification). Arrows indicate positive CD70 cell staining. The scale bar (black line) represents 100 µm. IHC, immunohistochemistry.

RESULTS

CD70 is broadly expressed across multiple cancer indications

The aberrant expression of CD70 has been described in multiple solid and hematologic malignancies.[21 23] To confirm CD70 as an appropriate tumor-associated antigen, IHC staining on primary tumor cores and normal tissue sections was performed. Of the profiled 11 solid tumor types, overall H-scores and percentage of CD70-positive samples were highest in clear cell renal cell carcinoma (ccRCC) and nasopharyngeal carcinoma with lower but meaningful frequencies in other indications (figure 1A,B, online supplemental figure 1). CD70 was also expressed in multiple hematologic malignancies with high H-scores in diffuse large B cell lymphoma, follicular lymphoma, T cell lymphoma, and Hodgkin's lymphoma (HL) (figure 1C,D). Acute myeloid leukemia (AML) samples were not included in the assessment; however, a high percentage of AML blasts and stem/progenitor cells positive for CD70 expression has been reported.[25 28] In contrast, analysis of IHC staining of normal tissues found no CD70 expression across various epithelial cell types. As would be predicted, rare and faint staining of immune cells (identified as lymphocytes, macrophages, or mast cells), particularly in the gastrointestinal tract and in lymphocyte-predominant organs such as tonsil, lymph node, spleen, bone marrow, small intestine, and colon was observed (figure 1E). Together, these results support the rationale for targeting CD70, which is expressed in a broad range of tumor types with minimal expression in normal tissues.

ADI-270 is highly enriched for Vδ1 anti-CD70 CAR T cells that possess a favorable phenotype

To activate and specifically expand the Vδ1 T cell subset, which represents a small fraction of PBMCs (0.2%–1.0%), a similar CAR-T manufacturing process as previously described was used[8] (figure 2A), including transduction with a γ-RV encoding the CAR construct depicted in figure 2B. CD27 is endogenously expressed on Vδ1 T cells.[8 29] To accurately determine CAR expression in ADI-270, an anti-P2A antibody recognizing the portion of the self-cleaving peptide retained on the C-terminal, cytoplasmic end of the CAR moiety after separation from dnTGFβRII was used (figure 2C). The correlation of intracellular P2A expression with CAR surface expression was assessed using a version of the anti-CD70 CAR with an extracellular epitope tag. These studies found a correlation between the $P2A^+$ population and surface CAR expression (online supplemental figure 2A). Furthermore, CD27 surface level percentage and geometric mean fluorescence intensity were increased on ADI-270 compared with untransduced donor-matched

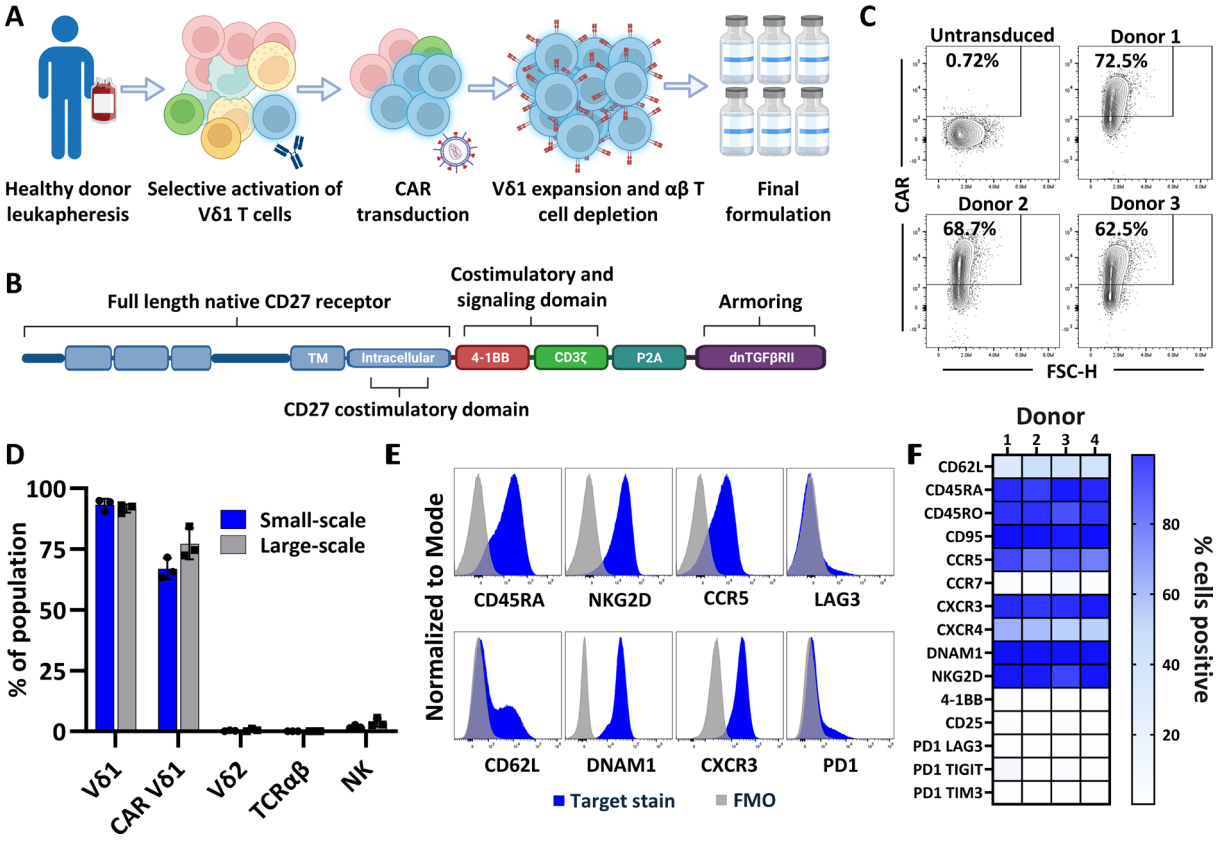

Figure 2 ADI-270 drug product is highly pure for Vδ1 CAR$^+$ T cells with a favorable phenotype. (A) Illustration of key manufacturing steps to generate ADI-270 and (B) schematic of the anti-CD70 CAR construct including dnTGFβRII. Created using BioRender (https://biorender.com). (C) P2A intracellular staining of ADI-270 from three different donors. Untransduced Vδ1 T cells were used as the P2A negative control gate. (D) Percent cell composition (mean±SD) at the end of the ADI-270 manufacturing process post-thaw from small and large-scale productions using three different donors. (E, F) Representative flow cytometric histogram plots (gray=FMO, blue=target stain) and heatmap of cell surface marker percentages (gated on Vδ1$^+$ cells) associated with T cell memory, chemotactic migration, innate receptors, activation, and exhaustion expressed on ADI-270 from four different donors. FMO, fluorescence minus one.

controls, suggesting transgene expression on ADI-270 cells (online supplemental figure 2B). Flow cytometry analysis of ADI-270 final product averaged across both small and large-scale productions found a high purity of Vδ1 T cells (92.5%±2.1%), of which 72.0%±7.5% were CAR$^+$ (figure 2D). Cell impurities consisting of Vδ2, αβ T, and NK cells were 0.39%±0.42%, 0.05%±0.04%, and 2.77%±1.53%, respectively (figure 2D). The T cell memory phenotype of human γδ T cells has been defined based on the coexpression of CD27 and CD45RA.[8 29] The extracellular domains of the anti-CD70 CAR and endogenous CD27 are identical, so the memory phenotype of ADI-270 cannot be identified using CD27$^+$ populations. Analysis of other T cell memory phenotypic markers on ADI-270 determined that a portion of the cells expressed CD62L with high levels of CD45RA, CD45RO, and CD95 (figure 2E,F). These combinations of markers are similarly expressed by ADI-001, which uses a comparable manufacturing process as ADI-270 to promote a less differentiated CD27$^+$CD45RA$^+$ γδ T cell memory phenotype.[8] In addition, ADI-270 displayed minimal coexpression of exhaustion-associated markers PD-1, LAG-3, TIGIT, and TIM-3 (figure 2E,F). In summary, the ADI-270 manufacturing process results in cells with a favorable, less-differentiated, non-exhausted phenotype that has been associated with improved CAR-T cell functionality.[30]

Effective tumor homing and infiltration of CAR T cells is dependent on the expression of a diverse repertoire of chemokine receptors.[31] ADI-270 expressed the chemokine receptors CXCR3, CXCR4, and CCR5 (figure 2E,F). The ligands for these receptors are enriched in tumors and can chemotactically attract T cells to the tumor site.[31–33] Vδ1 T cells are known to express NK cell receptors (NKRs) such as NKG2D and DNAM1,[34] and high expression of these receptors on ADI-270 was observed (figure 2E,F). The combination of chemokine receptors and NKRs supports the potential enhanced tumor trafficking and diverse antitumor activity of ADI-270.

dnTGFβRII protects ADI-270 from the immunosuppressive effects of TGFβ

To overcome the immunosuppressive effects of TGFβ, ADI-270 is armored with dnTGFβRII (figure 3A). Compared with anti-CD70 Vδ1 T cells lacking the armoring component (online supplemental figure 3A), surface TGFβRII was detectable on unstimulated ADI-270, and TGFβRII

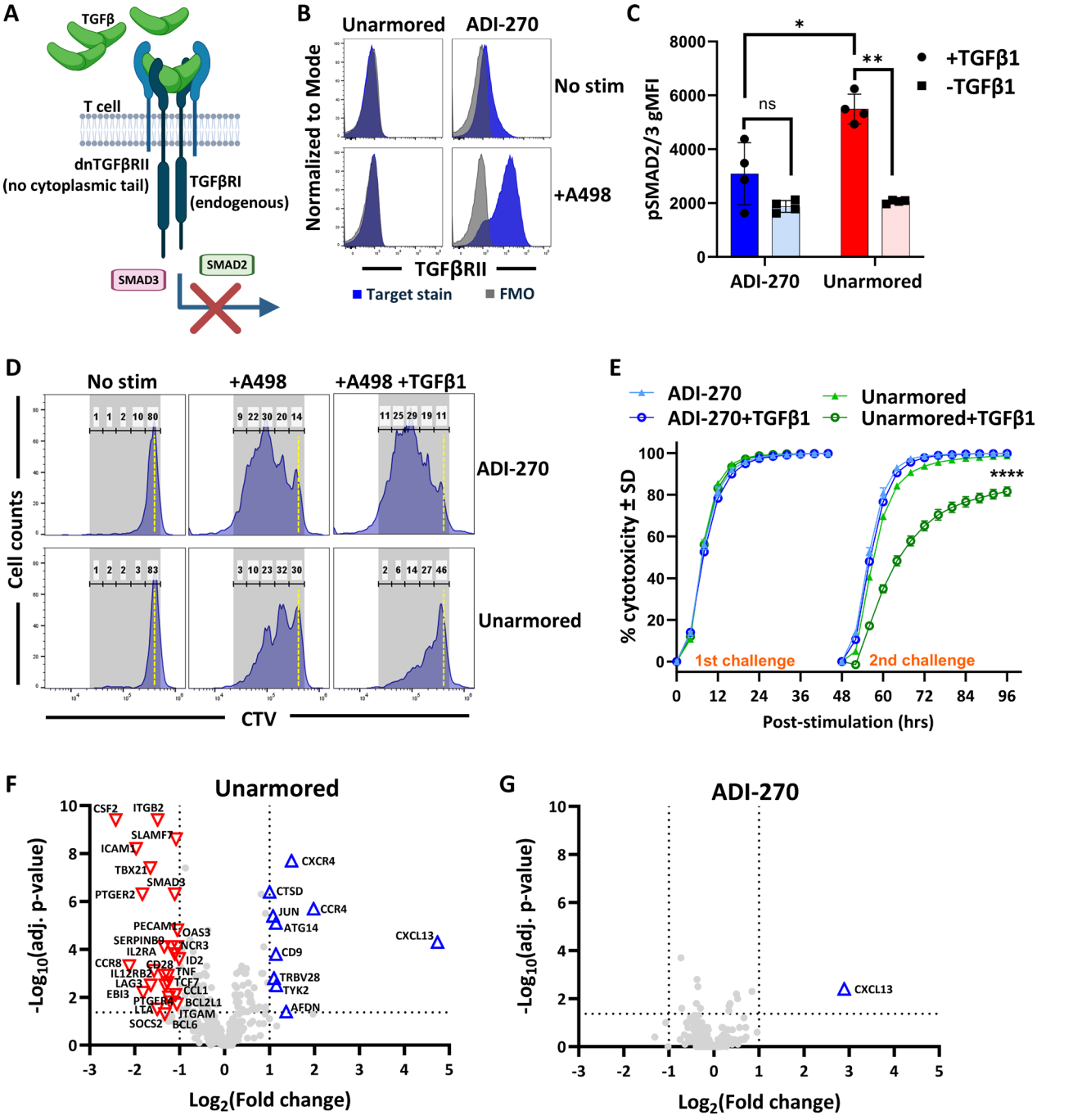

Figure 3 dnTGFβRII armoring protects ADI-270 from the immunosuppressive effects of TGFβ. (A) Illustration of TGFβ-signaling inhibition by dnTGFβRII. Created using BioRender (https://biorender.com). (B) Histogram plots of cell surface TGFβRII expressed on ADI-270 and unarmored anti-CD70 Vδ1 T cells (lacking dnTGFβRII) stimulated with and without A498 tumor cells for 24 hours (gray=FMO, blue=target stain). Data are representative of three independent experiments. (C) Intracellular pSMAD2/3 expression in ADI-270 and unarmored anti-CD70 Vδ1 T cells stimulated with (circle) and without (square) TGFβ1. The bar graphs represent the average gMFI of pSMAD2/3 from four different donors (symbols). Paired t-test, *p<0.05, **p<0.005. (D) Histogram plots of the cell division percentages from CTV-labeled ADI-270 and unarmored anti-CD70 Vδ1 T cells stimulated with and without A498 tumor cells for 7 days in the presence or absence of TGFβ1. The yellow dotted lines represent 'division zero'. Data are representative of four independent experiments. (E) Cytotoxicity of ADI-270 (blue) and unarmored anti-CD70 Vδ1 T cells (green) against A498 tumor cells (two challenges) in the presence and absence of TGFβ1. Percent cytotoxicity (mean±SD) is derived from three biological replicates. Data are representative of three independent experiments. Two-way ANOVA, ****p<0.0001. (F) Volcano plots showing DEGs from Nanostring bulk transcriptional profiles of unarmored anti-CD70 Vδ1 T cells and (G) ADI-270 stimulated with recombinant CD70 protein in the presence and absence of TGFβ1. Red and blue triangles represent DEGs downregulated and upregulated, respectively, in TGFβ1 treated versus untreated conditions. Data are derived from three different donors. The nSolver software was used to identify DEGs (ie, $-\log_{10}$(adj.p-value)>1.3 and $\log_2$-fold change >1 or <−1). ANOVA, analysis of variance; CTV, Cell Trace Violet; DEGs, differentially expressed genes; FMO, fluorescence minus one.

expression was further upregulated on CAR-mediated activation (figure 3B). To assess the biological impact of dnTGFβRII expression in ADI-270, phosphorylated SMAD2 and 3 (pSMAD2/3) levels, the direct downstream mediators of TGFβ signaling, were measured following stimulation with TGFβ1. Significantly reduced levels of pSMAD2/3 were observed for ADI-270 compared with unarmored anti-CD70 Vδ1 T cells after stimulation with TGFβ1 (figure 3C). These results suggest that the levels of dnTGFβRII in the absence of CAR activation are sufficient to reduce TGFβ1-mediated signaling in ADI-270.

In vitro proliferation and cytolytic activity of ADI-270 on CAR-mediated activation in the presence of TGFβ1 were also evaluated. The number of cell divisions (figure 3D, online supplemental figure 3A) and target cell killing of $CD70^+$ A498 tumor cells (figure 3E) for the unarmored anti-CD70 Vδ1 T cells was reduced in the presence of TGFβ1. In contrast, the proliferation and cytotoxicity profile of ADI-270 remained unchanged, further supporting the functionality of dnTGFβRII in protecting against the immunosuppressive effects of TGFβ1.

To gain additional insight into the protective effect of dnTGFβRII against TGFβ1, T cell-related gene expression profiling of ADI-270 and unarmored anti-CD70 CAR Vδ1 T cells stimulated with recombinant CD70 was performed, either in the presence or absence of TGFβ1. A total of 37 significant DEGs were identified from unarmored anti-CD70 CAR Vδ1 T cells when exposed to TGFβ1 (figure 3F, online supplemental table 2). Gene ontology and pathway analysis of the downregulated DEGs showed an association with T cell activation and cytokine-mediated responses (online supplemental figure 3B). In contrast, minimal transcriptional changes were observed for ADI-270 in the presence of TGFβ1 (figure 3G). These results demonstrate that armoring with dnTGFβRII protects ADI-270 against TGFβ1-mediated changes in T cell effector activity.

ADI-270 has robust antitumor activity against CD70-expressing tumor cells in vitro

Heterogeneous expression or loss of target antigen within the TME can impact the effectiveness of CAR T cell therapies.[35] To investigate the antitumor potential of ADI-270, in vitro cytotoxicity studies were performed against multiple solid and hematologic tumor cell lines that were quantified for CD70 expression (figure 4A). Robust cytotoxicity of ADI-270 was observed against the entire panel of tumor cells evaluated, including the CD70 low-expressing cell lines MM1.S and H1975 (figure 4B). The cytotoxicity of ADI-270 is primarily driven by CAR activation, as unengineered Vδ1 T cells exhibited lower activity against various tumor cell lines, which appear to be cell line-dependent (online supplemental figure 4A). To further assess the antitumor activity of ADI-270, in vitro cytotoxicity was compared with allogeneic αβ T cells that were engineered to express clinically tested reference scFv-based anti-CD70 CARs. ADI-270 and the αβ reference CAR T cells functioned similarly against CD70-high A498 tumor cells. However, ADI-270 retained a high degree of cytotoxicity against CD70-low ACHN tumor cells compared with the anti-CD70 CAR αβ T cell references (figure 4C). Notably, this improved activity was not dependent on dnTGFβRII armoring (online supplemental figure 4B).

Although the mechanism of tumor killing by ADI-270 is predominately CAR-mediated, CAR-independent activity (innate and/or adaptive immunity driven by NKRs and the γδ TCR) may contribute to and complement the targeting of $CD70^+$ cells. To assess the CAR-independent killing potential using a tumor model in which heterogeneous antigen expression is present, CD70 KO A498 tumor cells were cultured with ADI-270 in the presence or absence of $CD70^+$ A498 tumor cells. Moderate activity was observed against CD70 KO A498 tumor cells alone, but cytotoxicity was significantly higher when $CD70^+$ cells were included in the tumor mixture (figure 4D). Importantly, cytolytic activity was absent against a panel of CD70-negative normal cells (online supplemental figure 4C), suggesting preferential tumor-specific targeting by ADI-270 that CAR activation may potentiate. These data provide insights into the mechanism of action of ADI-270 to target and kill a range of CD70-expressing and CD70-negative tumor cells, potentially overcoming the limitations associated with heterogeneous tumor antigen expression.

ADI-270 is associated with a favorable cytokine and chemokine profile

CAR T cells elicit their antitumor activity in multiple ways, including the release of soluble mediators. Assessment of supernatants from cocultures of ADI-270 with A498 tumor cells showed increased levels of proinflammatory cytokines GM-CSF, IFNγ, IL-5, IL-6, TNFα, and IL-13 when compared with unstimulated cells (figure 4E). The chemotactic ligand MCP-1 (CCL2) was highly upregulated, and IL-8 (CXCL8), MIP1α (CCL3), MIP1β (CCL4), and RANTES (CCL5) were moderately increased. Furthermore, cytokine and chemokine profiles for ADI-270 and the unarmored control were similar (online supplemental figure 5A), suggesting dnTGFβRII armoring does not significantly alter the cytokine and chemokine release profile.

Excessive levels of proinflammatory soluble mediators can lead to severe systemic inflammatory responses in patients treated with CAR αβ T cell therapies.[36] The secreted cytokines and chemokines levels were compared between ADI-270 and anti-CD70 CAR αβ T cell references in the presence of A498 tumor cells (online supplemental figure 5B). Levels of GM-CSF, IL-2, and IL-3, known mediators of inflammation, were significantly higher in cultures containing CD70 CAR αβ T cell references. When macrophages were added to the culture, a significant upregulation of IFNγ, TNFα, and MIG was observed in cultures with CAR αβ T cell references compared with ADI-270 (online supplemental figure 5B). These results suggest a potential for lower incidence of severe cytokine release syndrome (CRS) and macrophage activation syndrome associated with ADI-270 compared with CAR

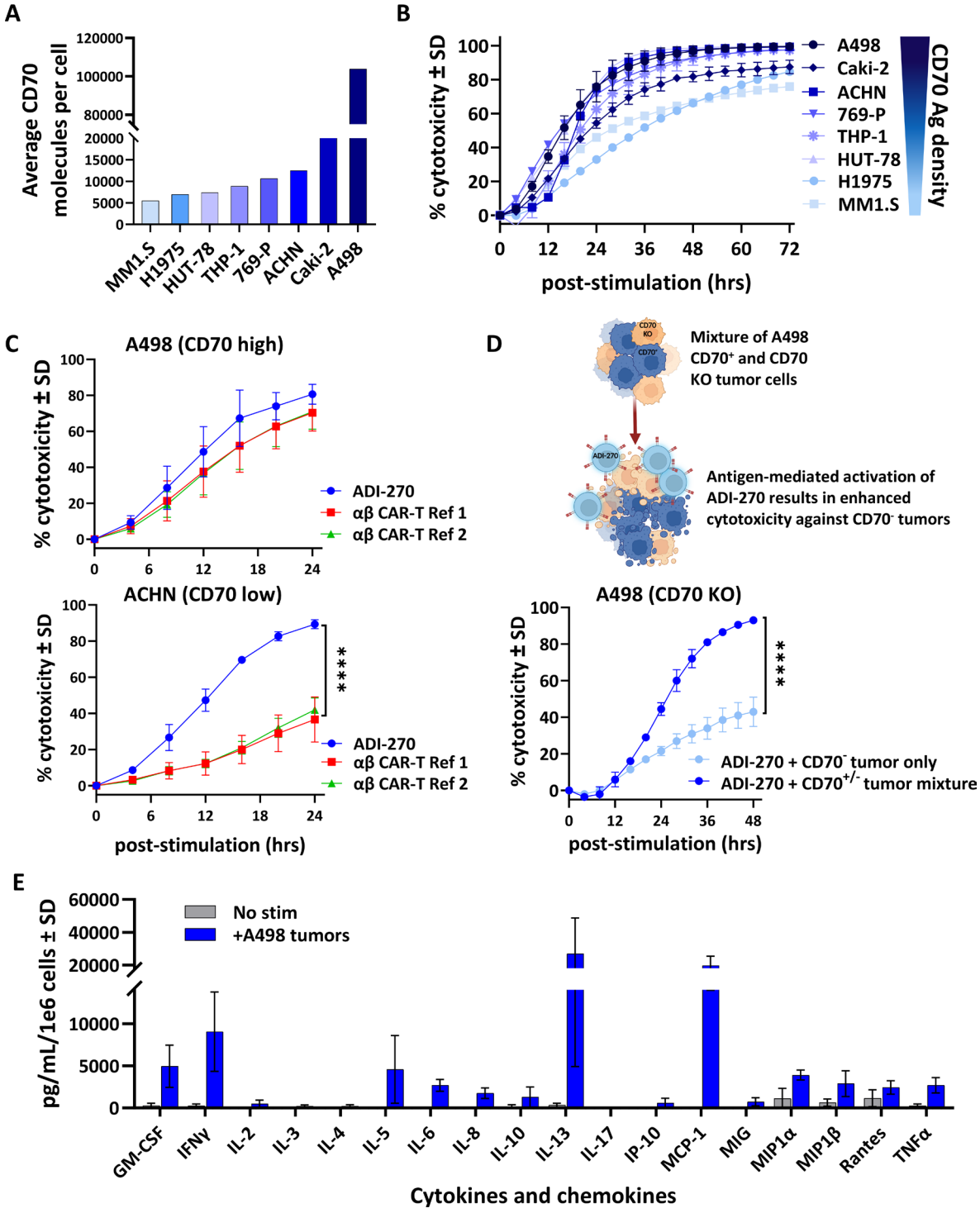

Figure 4 ADI-270 in vitro antitumor activity extends to CD70-low and CD70-negative tumor cell lines. (A) CD70 expression in different tumor cell lines using BD Quantibrite beads. The y-axis represents the average number of CD70 molecules per cell. (B) The cytotoxic potential of ADI-270 was measured against a panel of cells derived from hematologic and solid tumors with varying levels of CD70 expression at a 1:1 E:T ratio for 72 hours. Percent cytotoxicity (mean±SD) is derived from three biological replicates. Data are representative of three different independent experiments. (C) Comparison of cytotoxicity between ADI-270 and anti-CD70 CAR αβ T cell references against A498 and ACHN tumor cells at a 1:1 E:T ratio for 24 hours. Percent cytotoxicity values (mean±SD) are from three different donors. Two-way ANOVA, ****p<0.0001. (D) Illustration of the $CD70^{+/-}$ tumor mixture assay. Created using BioRender (https://biorender.com). The cytotoxicity of ADI-270 against CD70 KO A498 cells alone (light blue) and in a mixture with $CD70^{+}$ A498 cells ($CD70^{+/-}$, dark blue) at a 1:1 E:T ratio for 48 hours. Percent cytotoxicity values (mean±SD) are from two different donors. Two-way ANOVA, ****p<0.0001. (E) Cytokine and chemokine detection of supernatants from ADI-270 after a 24 hours coculture with A498 tumor cells. Mean concentration values (pg/mL/1e6 cells±SD) are from three different donors. ANOVA, analysis of variance; E:T, effector-to-target.

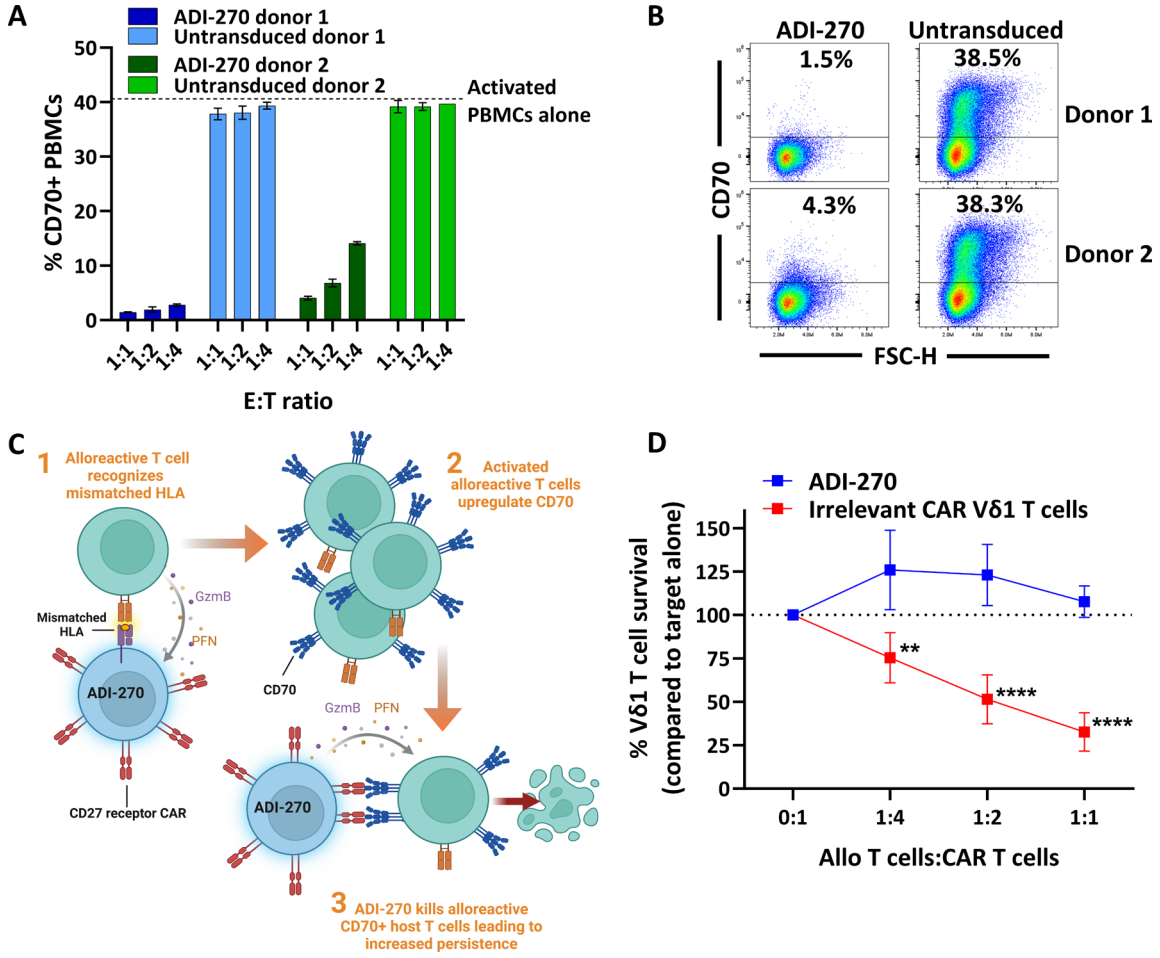

Figure 5 ADI-270 is associated with reduced in vitro killing by alloreactive T cells. (A) Percentage of activated CD70$^+$ PBMCs was determined using flow cytometry after a 48 hours coculture with ADI-270 or untransduced Vδ1 T cells derived from two different donors. Bar graphs represent the percent CD70 (mean±SD) of activated PBMCs from duplicate biological replicates. The dashed horizontal line represents the percent CD70$^+$ from activated PBMCs alone control wells. (B) Representative flow cytometric pseudocolor plots displaying the gated populations of CD70$^+$ activated PBMCs when cocultured with ADI-270 or untransduced Vδ1 T cells at a 1:1 E:T ratio. Values within the gate represent the percentage of CD70$^+$ cells. (C) Illustration of a proposed mechanism of ADI-270 HvG armoring for increased persistence. Created using BioRender (https://biorender.com). (D) Percent survival of ADI-270 and irrelevant CAR Vδ1 T cells (matched donor) in the presence of primed allogeneic T cells (three different donors) at E:T ratios of 1:4, 1:2, and 1:1 after a 24 hours coculture. The irrelevant CAR consists of a human scFv sequence, a CD8α hinge and transmembrane domain, and 4-1BB and CD3ζ signaling domains. Two-way ANOVA, **p<0.01, ****p<0.0001. ANOVA, analysis of variance; PBMCs, peripheral blood mononuclear cells; E:T, effector-to-target.

αβ T cells, based on a favorable in vitro inflammatory cytokine profile.

ADI-270 has increased in vitro survival against alloreactive T cells

The anti-CD70 CAR-mediated activity of ADI-270 is anticipated to target subsets of activated lymphocytes expressing CD70. The on-target off-tumor killing potential of ADI-270 was assessed against PBMCs expressing CD70. Cocultures of activated PBMCs with ADI-270 substantially reduced the frequency of CD70$^+$ target cells in an E:T ratio-dependent manner compared with untransduced Vδ1 T cell controls (figure 5A,B). With the confirmed targeting against CD70$^+$ lymphocytes by ADI-270, a proposed mechanism of action of HvG armoring for ADI-270 is illustrated in figure 5C. Using an MLR assay, ADI-270 showed significantly improved survival against primed alloreactive T cells compared with the irrelevant CAR control across multiple E:T ratios (figure 5D). These results suggest ADI-270 has the potential to reduce HvG rejection by eliminating CD70$^+$ alloreactive T cells.

ADI-270 demonstrates robust in vivo potency in multiple solid and hematologic tumor xenografts

The in vivo efficacy of ADI-270 was investigated in multiple tumor xenograft models in NSG mice using SC-implantation. The level of CD70 expression in the human tumor xenografts was verified using IHC (online supplemental figure 6). A single intravenous administration of ADI-270 resulted in robust tumor control against A498 (ccRCC, CD70-high) and H1975 (non-small cell lung cancer, CD70-low) solid tumor models (figure 6A). Additionally, ADI-270 displayed significant activity against hematologic-derived tumors with low levels of CD70 expression,

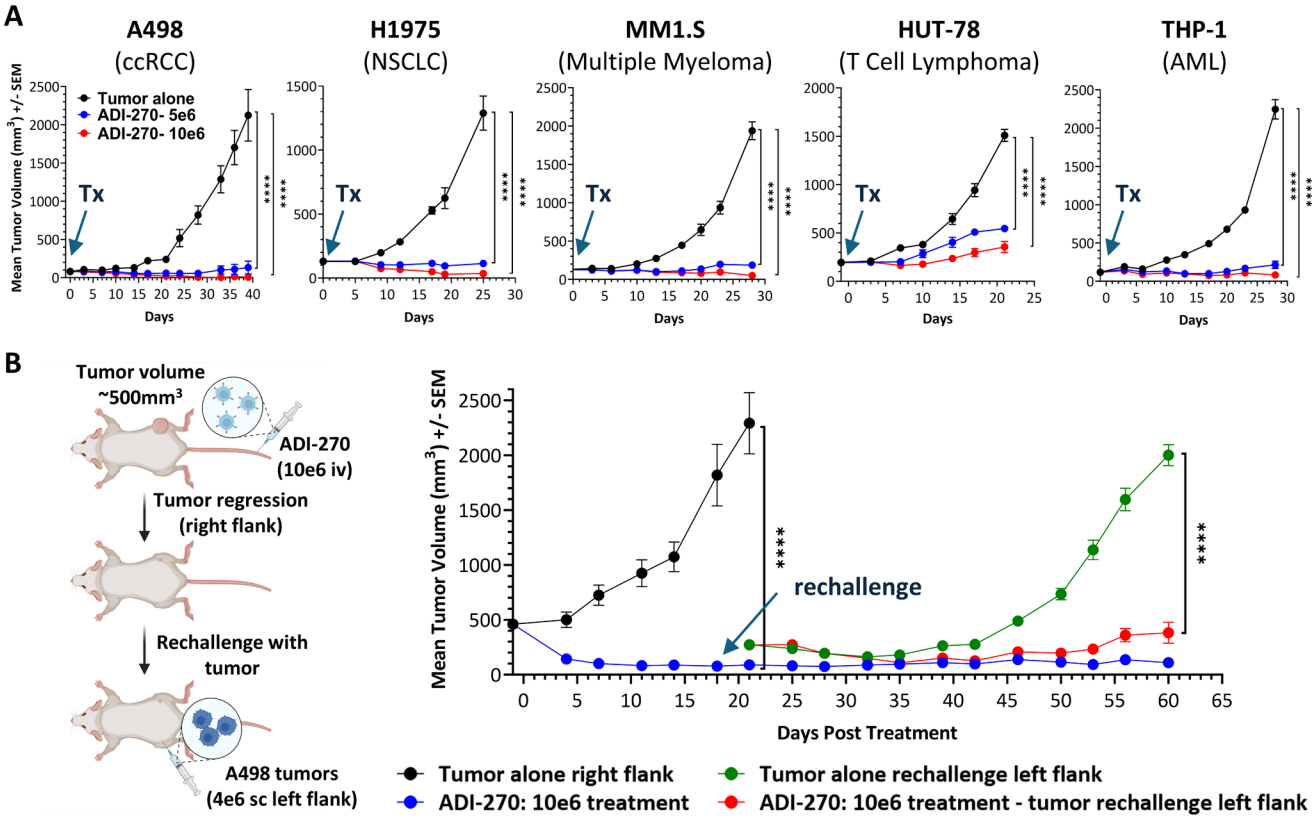

Figure 6 ADI-270 shows strong potency and systemic persistence in vivo. (A) In vivo tumor growth control of ADI-270 against SC-implanted A498 (4e6 cells), H1975 (2e6 cells), MM1.S (1e6 cells), HUT-78 (2e6 cells), and THP-1 (2e6 cells) tumors in NSG mice. Arrows represent the day of ADI-270 treatment (Tx), which occurred 5–7 days post-tumor implant. Data shown are the mean tumor volume±SEM from five mice per group and are representative of two independent experiments. Statistical comparison between tumor alone and ADI-270 treated mice was determined using two-way ANOVA, ****p<0.0001. (B) Study schematics of the in vivo tumor rechallenge model in NSG mice. Created using BioRender (https://biorender.com). Tumor growth control of ADI-270 against primary and secondary SC-implanted A498 tumors. The data shown are the mean tumor volume±SEM from five mice per group and are representative of two independent experiments. The arrow indicates the day of the second tumor challenge. Two-way ANOVA, ****p<0.0001. ANOVA, analysis of variance; ccRCC, clear cell renal cell carcinoma; NSCLC, non-small cell lung cancer; AML, acute myeloid leukemia; SC, subcutaneously.

including MM1.S, HUT-78, and THP-1. In comparison to unarmored anti-CD70 CAR Vδ1 T cells, ADI-270 was associated with increased tumor control against A498 tumor cells that secrete TGFβ1 (online supplemental figure 7A,B) and prolonged the survival of mice in a patient-derived xenograft AML high tumor-burden model (online supplemental figure 7C), further supporting dnTGFβRII functionality. Lastly, a single treatment of ADI-270 in mice bearing large, established A498 tumors (500 mm^3 on average) resulted in tumor regression and prolonged tumor growth inhibition. When the same mice were rechallenged with a second dose of tumor cells on the opposite flank (18 days postinitial treatment), significant tumor growth inhibition was observed relative to ADI-270-naïve controls (figure 6B). These observations highlight the ability of ADI-270 to both persist in the mouse following tumor eradication and mount a secondary response at a distant location.

ADI-270 rapidly infiltrates and kills tumors in vivo

To better understand the kinetics of ADI-270 tumor infiltration and killing, tissues collected from A498 tumor-bearing NSG mice were analyzed by IHC, IF, and flow cytometry. Inhibition of tumor growth by ADI-270 (figure 7A) was associated with a substantial reduction in $CD70^+$ tumor cells as early as day 3, followed by complete elimination by day 7 post-treatment (figure 7B). IF staining revealed rapid tumor infiltration of ADI-270 cells (human $CD3^+$) at day 3 post-treatment that were double positive for the proliferative and cytolytic markers, Ki67 and GzB, respectively (figure 7C). The percentage of ADI-270 cells within the tumor decreased on day 7 and day 14, which coincided with the reduction in $CD70^+$ cells. In addition, the level of the proinflammatory cytokine IFNγ in plasma was highest on day 3 post-treatment (figure 7D). Flow cytometry analysis of γδ T cells isolated from xenograft tumors and several mouse organs further demonstrated infiltration, proliferation, and activation of ADI-270 preferentially within the tumor, with minimal expansion in non-tumor tissues (figure 7E–G).

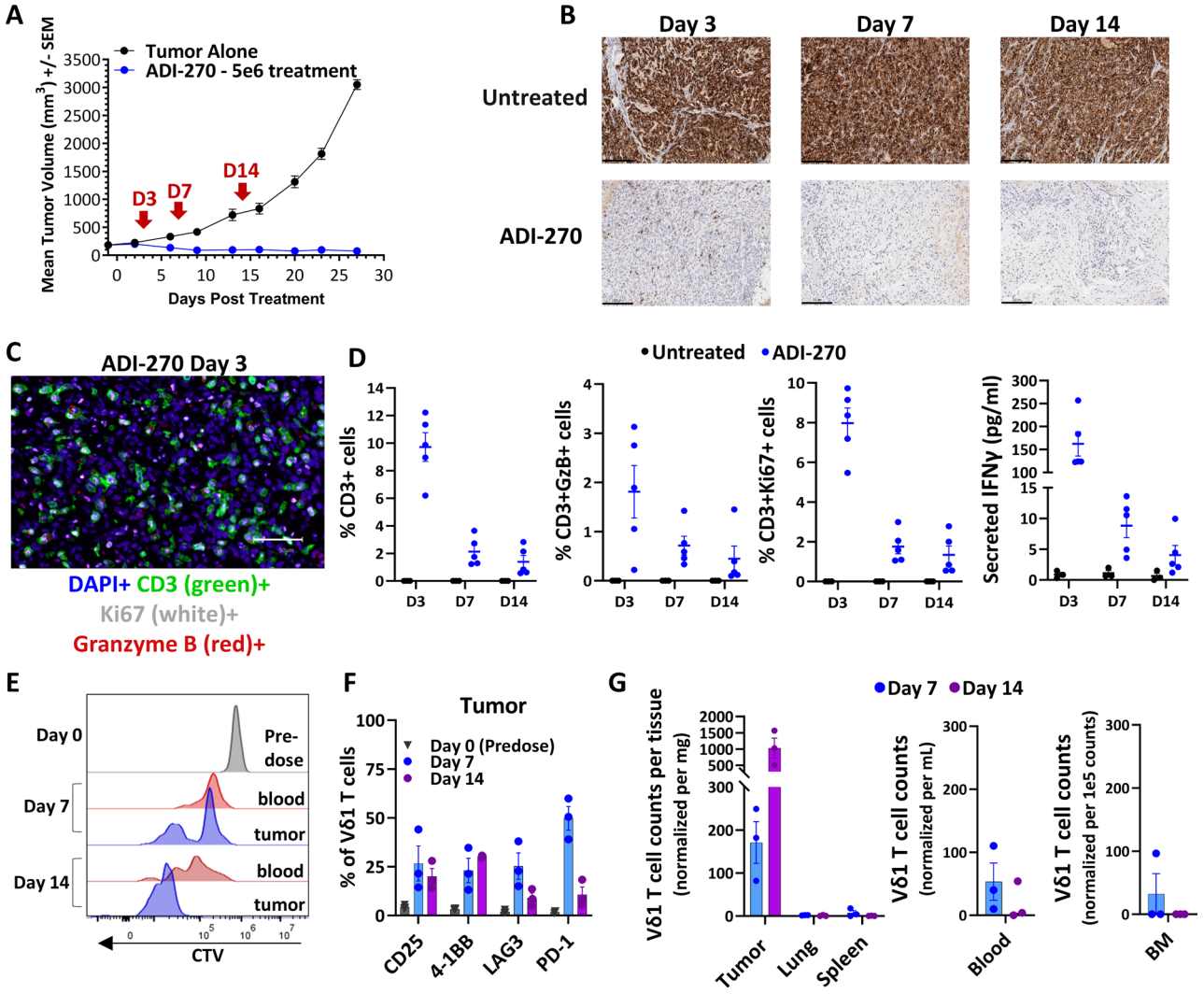

Figure 7 ADI-270 shows rapid tumor-specific infiltration, activation, proliferation, and potent killing of CD70⁺ A498 xenografts. (A) Tumors and plasma were harvested from NSG mice at the indicated time points (red arrows) post-treatment with ADI-270. (B) Representative IHC staining of CD70 on FFPE tumor tissues from untreated and ADI-270 treated NSG mice at different time points. The scale bar (black line) represents 200 µm. (C) Representative multiplex IF staining (×60, magnification) for CD3/Ki67/GzB/DAPI on tumors harvested from NSG mice at day 3 post-treatment with ADI-270. The scale bar (white line) represents 50 µm. (D) The percentage of CD3⁺ cells expressing activation markers (Ki67 and GzB) was quantified using digital image analysis (Histowiz) based on multiplex IF staining. Secreted IFNγ levels from plasma were measured using multiplex Luminex technology. Data values (mean±SEM) for ADI-270 treated and untreated conditions are from 5 and 3 mice per group (symbols), respectively. (E) Tissues from A498-bearing NSG mice receiving a single IV dose of CTV-labeled ADI-270 were harvested on days 7 and 14. Representative proliferation histogram plots of CTV-labeled ADI-270 from tumors and blood as indicated by the dilution of the CTV dye compared with the day 0 control. (F) Staining for activation-associated markers on ADI-270 and (G) the absolute counts of ADI-270 were measured from tumors, lung, spleen, blood, and bone marrow (BM) on days 7 and 14. The Vδ1 counts were normalized per mg of tissue (tumor, lung, spleen), per mL of blood, or per 1e5 BM cell counts. Data (mean±SEM) are from 3 mice per time point (symbols). CTV, Cell Trace Violet; IF, immunofluorescence; IHC, immunohistochemistry; FFPE, formalin-fixed paraffin-embedded.

DISCUSSION

The TME of solid tumors poses significant challenges that limit the efficacy of CAR T cell therapies. ADI-270, an allogeneic γδ CAR T cell approach consisting of Vδ1 T cells engineered to express an anti-CD70 CAR in cis with dnTGFβRII armoring, was developed to address these challenges. ADI-270 demonstrated robust killing against multiple CD70-expressing tumor cell lines and sustained activity towards heterogeneous CD70-expressing tumors, highlighting the intrinsic antitumor properties associated with Vδ1 T cells.[7] Armoring with dnTGFβRII significantly enhanced the ability of ADI-270 to maintain effector function in the presence of the immunosuppressive factor TGFβ1. In vivo, ADI-270 showed rapid infiltration and elimination of CD70⁺ tumor cells in NSG mice, with prolonged persistence necessary to inhibit the growth of a second tumor xenograft challenge. Lastly, as an allogeneic approach, ADI-270 was associated with increased in vitro survival against alloreactive T cells. Together, these

preclinical proof-of-concept data provide support for the clinical development of ADI-270 in CD70$^+$ cancers.

The expression level of targeted antigens on tumors plays a significant role in the functional potency of CAR T cells.[37 38] The prevalence of CD70 positivity across multiple cancers was confirmed through IHC staining, but the intensity of expression substantially varied between and within tumor samples. The full-length natural receptor CD27 was selected as the antigen targeting moiety of the CAR based on the high affinity and specificity for CD70.[39] The characterized role for natural CD27:CD70 receptor interactions is important for providing critical survival and proliferative signals for optimal activation of γδ T cells.[40] Thus the full-length CD27 receptor (including its signaling domain) in the CAR construct may provide further benefit to ADI-270. Strategies targeting CD70 by engineering its natural receptor (CD27) as the antigen-recognition moiety of a CAR have demonstrated superior preclinical antitumor activity compared with scFv-based approaches.[25–27] Observations of increased cytotoxicity of ADI-270, when compared with scFv-based anti-CD70 CAR αβ T cell references, against lower-expressing CD70$^+$ tumor cells support the potential for improved efficacy (figure 4C), particularly given the heterogeneity of CD70 expression across primary patient tumors (figure 1). The contribution of CAR-independent activity associated with ADI-270 may extend tumor killing (figure 4D), emphasizing the potential advantage for broader heterogeneous tumor targeting by Vδ1 T cells that has been previously reported.[5 41] Several CD27 receptor CAR therapies, which include NK cell-based therapies that also possess innate-associated antitumor pathways, are actively being explored in the phase 1 setting for both hematologic and solid cancers (NCT05092451, NCT05703854, NCT02830724).

The manufacturability of Vδ1 T cells may represent a barrier for development, as Vδ1 T cells represent only a small percentage (0.2%–1.0%) within PBMCs. A successful manufacturing process was established for ADI-270 that achieves clinically relevant Vδ1 T cell yields and avoids significant fratricidal events observed with other anti-CD70 CAR approaches, the latter outcome due to the upregulation of CD70 on activated T and NK cells.[42 43] Genetic modifications such as CRISPR-editing to knock out endogenous CD70 expression or the screening of scFvs to identify anti-CD70 CARs that mask CD70 in cis are strategies intended to minimize fratricide.[42–44] γδ T cells express CD70 post-activation,[40] yet the incorporation of the CD27 receptor-based CAR did not negatively impact expansion cell yields (online supplemental figure 8). The CD27 receptor-based CAR may lead to cis binding, preventing CD70 from being accessible for targeting, as observed for anti-CD70 CAR masking.[43] Another possibility is the expansion of CD70-negative γδ T cells resistant to fratricide, which could represent findings from studies targeting CD7 that show the self-selection of fratricide-resistant CD7-negative CAR T cells.[45] In addition, ADI-270 demonstrated robust cytotoxicity and sustained persistence leading to the inhibition of secondary tumor challenges in mouse models in vivo, which suggests CAR-mediated activation does not induce fratricide that could potentially interfere with potency. These results are also consistent with other groups that have reported using CD27 receptor-based CAR approaches to modify αβ T cells and NK cells.[25 27 46 47]

Activated TGFβ can be elevated in primary tumor tissues[48 49] and is associated with anti-inflammatory and immunosuppressive effects.[15] TGFβ has been shown to impair the antitumor activity of γδ T cells, specifically the Vγ9Vδ2 T cell subset,[50] but the effects on Vδ1 T cells are less understood. Data from these studies provide insight into the transcriptional and functional changes induced by TGFβ1 on activated CAR Vδ1 T cells. Transcripts related to pathways involved in T cell activation, cytokine signaling, and differentiation that include CSF2, IL2RA, LAG3, TBX21, TCF7, TNF, BCL2L1, BCL6, and EBI3 were downregulated in antigen-stimulated unarmored CAR Vδ1 T cells when exposed to TGFβ1 (figure 3F), which coincided with reduced in vitro cytotoxicity and proliferation (figure 3D,E). Surface expression of TGFβRII was highly upregulated following CAR-mediated activation in ADI-270, indicating an increased presence of dnTGFβRII compared with the unarmored control (figure 3B), allowing ADI-270 to remain refractory to the inhibitory effects of TGFβ1 (figure 3D,E). While TGFβ receptors can be sequestered within intracellular pools, certain signaling events facilitate their trafficking towards the plasma membrane.[51] Several clinical studies evaluating dnTGFβRII armored CAR T cells for metastatic castration-resistant prostate cancer, relapsed HL, and advanced hepatocellular carcinoma have reported acceptable safety profiles and promising clinical responses,[52–54] further supporting the rationale for armoring to counteract the immunosuppressive effects of TGFβ.

Approaches to evade host rejection and create hypoimmune allogeneic CAR T cells are currently being evaluated to increase persistence and efficacy. Gene editing to disrupt MHC, overexpression of inhibitory receptors such as HLA-E and CD47, and partial HLA matching have been adapted to allogeneic CAR T cells to evade host-mediated allorejection, but clinical results demonstrating improved persistence using these methods remain in progress.[55–57] Alternative strategies have aimed to directly target and kill alloreactive T cells to reduce HvG rejection.[58–60] The upregulation of CD70 on activated lymphocytes has been associated with alloreactive T cells in postallogeneic stem cell transplant settings.[61] Increased CD70$^+$CD4$^+$ T cells were detected in blood, and CD70$^+$CD8$^+$ T cells located in the skin were defined as alloreactive memory T cells and associated with acute GvHD.[61] Preclinical models indicate that ADI-270 has the capacity to target and kill CD70$^+$ T cells, and in the presence of primed alloreactive T cells, ADI-270 showed increased survival compared with non-CD70 targeting CAR controls in short-term killing assays. Strategies to target alloreactive CD70$^+$ T cells have been explored by other groups using allogeneic anti-CD70 CAR αβ T cells, such as ALLO-316.[62] Extended and improved cellular kinetics

(CK) of ALLO-316 was observed as host CD70$^+$ T cells were reduced in patients' blood post-treatment in a phase I clinical study.[62] The killing of host-alloreactive T cells may create a larger window of treatment efficacy to eliminate tumors as patients begin to reconstitute their immune cells postlymphodepletion.

Preclinical in vivo studies have demonstrated robust potency of ADI-270 across multiple human tumor xenograft models expressing different levels of CD70 (online supplemental figure 6) with no signs of xenogeneic GvHD or acute toxicities (data not shown). The translation of these results to successful clinical development will be informed by insights gained from current clinical trials using CAR-modified γδ T cell approaches. ADI-001, an allogeneic CAR Vδ1 T cell therapy targeting CD20, is being evaluated in subjects with relapsed/refractory (R/R) B cell malignancies (NCT04735471). From interim data, ADI-001 has achieved meaningful clinical activity with a 63% complete response rate across all dose levels with no severe incidences of CRS or ICANS,[63] toxicities often associated with CAR αβ T cell therapies. Data from in vitro studies of ADI-270 revealed significantly lower levels of cytokines associated with severe neurotoxicity,[64] including IL-2, IL-3, IFNγ, and GM-CSF compared with anti-CD70 CAR αβ T cell references. The favorable inflammatory profile associated with ADI-270 may prove advantageous in minimizing dose-limiting toxicities for improved clinical efficacy. Importantly, evaluation of patient samples post-treatment with ADI-001 revealed robust expansion in peripheral blood with CK and Cmax values comparable to what has been reported for autologous anti-CD19 CAR αβ T cell therapies.[65] Furthermore, ADI-001 was detected at high levels in lymph node samples post-treatment, indicating efficient tissue homing consistent with the predicted biology of γδ T cells.[65] The encouraging data obtained thus far with CAR Vδ1 T cells in the clinical setting, together with the HvG armoring provided by targeting CD70, suggest ADI-270 has the potential for enhanced tumor trafficking and extended persistence, which may contribute to clinical responses.

In summary, these preclinical proof-of-concept studies demonstrate the potential of ADI-270 to exhibit robust potency and persistence against multiple CD70-expressing cancers. The armoring of ADI-270 provides resilience to attenuation by TGFβ, and CD70-targeting can reduce alloreactive rejection by T cells. Supported by these data, ADI-270 has the potential to target multiple CD70$^+$ cancers, and initial clinical Phase 1/2 evaluation in patients with R/R ccRCC is ongoing (NCT06480565).

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Competing interests KPN, YC, AGT, YV, MA, MS-B, MS, PM, SRYG, RK, JZ, AA, JTSW, EBS, KMW, AD, JS, DB, PP, HB, CJR, SP, AJ, MH, AB, SG and BTA are employees of Adicet Therapeutics. GL is currently an employee of the FDA. AG-R is currently an employee of Kyverna Therapeutics.

Patient consent for publication Not applicable.

Ethics approval All mouse experiments were performed in accordance with the guidelines set by Adicet's Institutional Animal Care and Use Committee (IACUC), Protocol # 2024-01.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request. All data relevant to the study are included in the article or uploaded as supplementary information. Data are available upon reasonable request. All data relevant to the study are included in the article or uploaded as supplemental information.

ORCID iD

Kevin P Nishimoto http://orcid.org/0009-0008-9537-4414

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