

# DSG2-Directed CAR-T Cells Safely and Universally Eliminate Solid Tumors

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## Article

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# Abstract

CAR-T cell therapies are curative for advanced hematologic cancers, however that potential has yet to be realized in epithelia-derived solid tumors reflecting the limited portfolio of cancer-restricted, cell-surface targets. Desmoglein 2 (DSG2) is a desmosomal cadherin universally overexpressed on the surface of transformed epithelial cells, with normal protein expression believed to be junctionally-restricted between adjacent cells, creating a “window of opportunity” to eliminate solid tumors without toxicity. Here, we generated DSG2-directed CAR-T cells (αDSG2) that universally recognize and lyse assorted solid tumor cell lines in vitro and eliminated patient-derived and cell-derived colon, pancreatic, lung, prostate, breast, and liver tumors in vivo. Transgenic mice expressing human DSG2 experienced no toxicity following αDSG2 CAR-T cell administration. These studies reveal safe and robust antitumor activity of αDSG2 CAR-T cells and introduce a new class of junctionally-restricted antigens that can be safely and effectively targeted across solid tumor types.

## Introduction

Efforts to prevent, detect, and treat solid tumor malignancies have made significant progress in recent years, yet these tumor types collectively remain the primary cause of cancer-related mortality<sup>1,2</sup>. Immunotherapies, specifically adoptive chimeric antigen receptor (CAR)-T cell therapies, have emerged as powerful tools to overcome obstacles where traditional surgical, radiological, and pharmacological interventions fail. Within this system, patient T cells are collected, engineered to express a synthetic CAR surface molecule, expanded *ex vivo*, and then re-administered back to the patient. This process creates a population of T cells capable of bypassing highly restricted peptide-MHC interactions to bind cancer cell surface antigens directly and activate potent cytolytic effector functions. CAR-T cell therapies have produced curative results in patients with liquid tumors, resulting in several FDA-approvals<sup>3–5</sup>, but have yet to be successfully applied to solid tumors due to patient, tumor, and immune factors<sup>6</sup>, as well as the need for suitable antigen targets<sup>7</sup>. Currently, most solid tumor antigen targets are restricted to a limited profile of tissue and cancer cell types, diminishing their universality, and instead relegating CAR-T cell therapies to very narrow indications<sup>8</sup>. Likewise, target antigens are often associated with expression in normal tissues, creating a risk for on-target, off-tumor toxicity in patients<sup>9–12</sup>. While B-cell aplasia can be partially managed in patients receiving FDA-approved CD19- or BCMA-directed CAR-T cell therapies that non-discriminately target both healthy and malignant B-cells<sup>13</sup>, normal tissue toxicity by solid tumor-directed therapies can prove lethal<sup>14</sup>. Thus, there exists a critical need for cell therapy targets that can be applied to a broad range of solid tumor types, while simultaneously limiting on-target, off-tumor toxicities in normal tissues.

Traditional screening methods for cell therapy targets often seek conditions of absent or low target expression among normal tissues to limit off-tumor interactions while simultaneously exploiting the overexpression in cancer tissues<sup>15,16</sup>. However, these screening paradigms overlook the inherent disorganization of cancer, in which cell adhesion, position, and polarity are often disrupted by malignant

cell transformation<sup>17</sup>. Thus, targets that are protected and sterically inaccessible in normal tissues, creating a potential “window” for safe and effective targeting in cancer, are typically ignored by such screens. One prospective group of such proteins includes the superfamily of cadherins, a class of transmembrane glycoprotein “anchors” mediating adjacent cell-cell contact, stability, and adhesion<sup>18,19</sup>. Monoclonal antibody-based and peptide-based therapies directed at pro-oncogenic candidates E-cadherin<sup>20</sup>, N-cadherin<sup>21,22</sup>, and VE-cadherin<sup>23</sup> have proven effective and well-tolerated in both preclinical and clinical models. Moreover, CAR-T cells directed at cadherin 17 (CDH17) safely eliminated gastrointestinal (GI) and neuroendocrine tumors in mice, without toxicity in normal GI tissues expressing the protein<sup>24</sup> and are currently being evaluated in a phase 1/2 clinical trial (NCT06055439).

While promising, these potential targets are similarly limited to subsets of solid tumors. Desmoglein 2 (DSG2), a Ca<sup>2+</sup>-dependent transmembrane glycoprotein, is a member of a previously unexplored group of desmosomal protein targets within the larger cadherin superfamily. Ubiquitously expressed in simple and stratified epithelia, DSG2 was originally described as a constituent within the larger desmosome complex, linking adjacent cells to one another via the intercellular space and providing structural rigidity necessary to resist mechanical stress in tissues<sup>25</sup>. However, mounting evidence suggests that DSG2 also supports tumorigenesis<sup>26–29</sup> by modulating canonical cell adhesion<sup>30,31</sup> and promoting migration<sup>32</sup>, proliferation<sup>33</sup>, invasion<sup>30</sup>, and angiogenesis<sup>34</sup>. Analysis of publicly available RNAseq data demonstrates significant DSG2 overexpression among 20 different solid tumor types compared to normal tissues, with higher DSG2 expression often correlating with worse survival outcomes<sup>35</sup>. While DSG2 dysregulation underlies certain heritable cardiac disorders<sup>36</sup> with implications for infectious disease<sup>37</sup>, direct targeting of DSG2 in cancer remains unexplored.

In that context, we hypothesize that the overexpression of DSG2 and tissue disorganization in cancer can be exploited to create a universal solid tumor CAR-T cell therapy without collateral toxicity in normal tissues. Herein, we developed a third-generation CAR molecule that confers DSG2-specificity with potent cytolytic effector functions. We demonstrate universal cytotoxicity of eighteen solid cancer cell lines, constituting the six most lethal solid tumor types, representing >50% of all solid cancer deaths. We further establish that DSG2-directed CAR-T cells exhibit robust *in vivo* anti-tumor efficacy in eight primary and metastatic cell-derived and patient-derived solid tumor models. Importantly, we observe an absence of toxicity among normal tissues in a human DSG2 transgenic mouse model following CAR-T cell treatment. Together, these results demonstrate a cellular therapy targeting a new class of pro-tumorigenic junctional proteins that may be applied to large patient populations, addressing the current limited landscape of effective solid tumor immunotherapies.

## Results

### DSG2 is a broadly applicable solid tumor antigen

We evaluated DSG2 transcript expression in various human tumor types via The Cancer Genome Atlas (TCGA). DSG2 is expressed abundantly in epithelia-derived solid tumor types, but not tumors associated with brain, hematological, and soft tissue-derived cancers (**Fig. 1A**), mirroring DSG2 distribution among normal tissues. Similarly, ranked DSG2 protein expression via Human Protein Atlas (HPA) immunohistochemistry (IHC) scoring indicates abundant and universal staining across epithelial solid tumor types, but not within non-epithelial cancers (**Fig. 1B; Supplementary Fig. S1A**). The National Cancer Institute's Surveillance, Epidemiology, and End Results Program (SEER) cancer population statistics demonstrate that lung, colorectal, pancreatic, breast, prostate, and liver malignancies constitute >50% of all cancer-related deaths (**Fig. 1C**). Moreover, IHC staining of DSG2 via HPA confirms the abundance of membranous DSG2 staining in these six solid tumor types (**Fig. 1D**). Therefore, we believe these six tumor types are ideal representatives of solid cancers to investigate DSG2 as a universal solid tumor immunotherapy target. Based on these paradigms, we selected cell lines that stratify our six chosen solid tumor types based on aggregate transcriptional and proteomic DSG2 expression data retrieved from the Broad Institute's Cancer Cell Line Encyclopedia (CCLE) (**Supplementary Fig. S2A**). To validate CCLE expression data, we measured total DSG2 protein by immunoblot in 18 solid tumor cell lines, as well as DSG2-deficient (via CRISPR/Cas9) DLD-1 colorectal cancer (CRC) cells (**Supplementary Fig. S3A-S3C**), thereby serving as our DSG2 negative control cell model (**Fig. 1E**). Total DSG2 content demonstrates a stratification of DSG2 expression both within and across cancer types (**Fig. 1E**). However, because CAR-T cells can bind only surface antigen, we evaluated DSG2 surface expression by flow cytometry (**Fig. 1F**). Here, we quantified the average number of DSG2 molecules per cell and found variable levels of surface expression and quantity across cell lines, with the CRC cell line SW480 having the fewest DSG2 molecules/cell (740), and the prostate cancer cell line C4-2B having the greatest quantity (54,336). Together, these data demonstrate a heterogeneous, yet prevalent conservation of DSG2 expression across epithelia-derived solid tumors.

### **DSG2-directed CAR-T cells universally eliminate solid tumor cells *in vitro***

To generate a DSG2-directed CAR molecule, we sequenced and adapted an  $\alpha$ DSG2 monoclonal antibody (clone 6D8; **Supplementary Fig. S4A**) previously described to bind extracellular domains 3 and 4<sup>38,39</sup>. Antibody heavy and light chain variable regions were formatted into a single-chain variable fragment (scFv) and incorporated into a third-generation human CAR backbone containing 4-1BB, CD28, and CD3 $\zeta$  signaling domains and a T2A-linked GFP reporter (**Fig. 2A**). Lentivirus encoding  $\alpha$ DSG2 CAR was used to transduce primary human donor T cells in a 14-day manufacturing process, producing ~50% transduction efficiency (% GFP<sup>+</sup>) in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (**Fig. 2B; Supplementary Fig. S5A**).

We next sought to evaluate antigen recognition and effector cytokine potential of  $\alpha$ DSG2 CAR-T cells when co-cultured with adherent solid tumor cell lines. Following a 24-hour co-culture of  $\alpha$ DSG2 or control CAR-T cells with either wildtype DLD-1 or DSG2-deficient DLD-1 CRC cells, supernatants were collected, and the cytokine content was analyzed. Across three separate donors,  $\alpha$ DSG2 CAR-T cells generated a pro-inflammatory signature with increases in granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon- $\gamma$  (IFN $\gamma$ ), interleukin-2 (IL-2), and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) compared to control

CAR-T cells (**Fig. 2C**). Moreover, this cytokine signature was entirely abolished when DSG2 was removed from co-cultured DLD-1 cells via CRISPR/Cas9 knockout (**Fig. 2C**). Expectedly, elevated levels of innate and potentially anti-inflammatory cytokines were also observed in co-cultured supernatants of  $\alpha$ DSG2 CAR-T cells (**Supplementary Fig. S6A**). We next utilized the xCELLigence real-time cytotoxicity assay (RTCA) to evaluate  $\alpha$ DSG2 CAR-T cell cytotoxicity of DLD-1 target cells. RTCA demonstrates robust elimination of DLD-1 target cells by  $\alpha$ DSG2 CAR-T cells, with the majority of target cell cytotoxicity occurring in the first 10 hours of co-culture (**Fig. 2D, top**), while cytotoxicity is abolished by DSG2 deletion (**Fig. 2D, bottom**). To assess  $\alpha$ DSG2 CAR-T cell potency, we tested a broad range of effector-to-target (E:T) ratios by RTCA (**Fig. 2E**). All ratios above 0.5:1 achieved complete elimination of DLD-1 target cells within 36 hours, while lower 0.15:1 and 0.05:1 E:T ratios produced delayed cytotoxicity (**Fig. 2E**). This dose-response highlights the robust *in vitro* potency of  $\alpha$ DSG2 CAR-T cells compared to donor-matched control and mock untransduced T cells (**Fig. 2F**). Moreover, complete target cell cytotoxicity by  $\alpha$ DSG2 CAR-T cells is conserved across three independent donors compared to control CAR-T cells (**Fig. 2G**). To determine the universality of  $\alpha$ DSG2 CAR-T cells, we evaluated their cytotoxic potential against a panel of previously described (**Fig. 1E-F**) solid tumor cell line models. Like DLD-1 cells, we observed robust elimination of additional colorectal, pancreatic, lung, prostate, breast, and liver cancers in a 36-hour period compared to control CAR-T cells (**Fig. 2H; Supplementary Fig. S6B**). These data demonstrate that  $\alpha$ DSG2 CAR-T cells specifically recognize DSG2 antigen, produce potent effector functions, and universally eliminate solid tumor cell lines *in vitro*.

### **DSG2-directed CAR-T cells universally eliminate solid tumor cell xenografts *in vivo***

To evaluate the *in vivo* anti-tumor efficacy of  $\alpha$ DSG2 CAR-T cells, we established several cancer cell line-derived xenograft (CDX) models and tested the speed, potency, and universality of graft elimination post-treatment. As a first test, DLD-1 CRC cells were implanted subcutaneously in the flanks of NSG-MHC I/II DKO mice (**Fig. 3A**). This mouse strain was selected to minimize graft-versus-host disease (GvHD) from prolonged engraftment of adoptively transferred human CD4<sup>+</sup>/CD8<sup>+</sup> CAR-T cells and evaluate potential tumor recurrence post-treatment<sup>40,41</sup>. Once DLD-1 flank tumors achieved an average size of ~150 mm<sup>3</sup> (day 15), mice were randomized into two equal groups and injected intravenously (i.v.) with 5x10<sup>6</sup>  $\alpha$ DSG2 or control CAR-T cells. We observed complete elimination of all DLD-1 tumors by 28 days post-treatment and sustained remission in the  $\alpha$ DSG2 CAR-T cell-treated group for the duration of the experiment (**Fig. 3A**).

We further evaluated  $\alpha$ DSG2 CAR-T *in vivo* efficacy by selecting an appropriate clinical scenario of colorectal metastasis by challenging mice intraperitoneally (i.p.) with luciferase-expressing DLD-1 cells. Peritoneal metastasis is the second most common site of CRC metastasis, with peritoneal spread considered a terminal manifestation of the disease<sup>42–44</sup>. Moreover, this created an opportunity to evaluate the lowest effective dose of  $\alpha$ DSG2 CAR-T cells in tumor xenograft clearance, by which we titrated down from our previous conventional dose of 5x10<sup>6</sup> CAR-T cells to a significantly lower 1.5x10<sup>5</sup> CAR-T cell dose per mouse (**Fig. 3B-D**). As with the DLD-1 subcutaneous xenograft model, we observed

complete elimination of i.p. DLD-1 tumors and 100% survival of mice receiving a single  $5 \times 10^6$  dose of  $\alpha$ DSG2 CAR-T cells relative to donor-matched control CAR-T cells of the same quantity. Likewise, the next lowest dose of  $1.5 \times 10^6$   $\alpha$ DSG2 CAR-T cells eliminated DLD-1 tumors in four out of five mice, with sustained tumor control and survival observed in the fifth mouse for the duration of the experiment. Interestingly, although no significant tumor control was observed in the intermediate dose of  $5 \times 10^5$   $\alpha$ DSG2 CAR-T cells, one mouse receiving the lowest dose of  $1.5 \times 10^5$   $\alpha$ DSG2 CAR-T cells experienced tumor regression and sustained elimination for the duration of the experiment.

As with our previous *in vitro* studies and CRC xenograft i.p. model above, we chose to evaluate DSG2-directed CAR-T cell efficacy in a broad range of solid tumor types in the appropriate clinical setting. Pancreatic cancer, often identified in the advanced stages of the disease and associated with poor patient prognosis, suffers from a lack of effective intervention and therapies. Therefore, we chose to both model and treat pancreatic cancer in the context of peritoneal metastasis, the second most common site of dissemination<sup>45–47</sup>. Mice were therein challenged i.p. with luciferase-expressing BxPC-3 cells, a moderate DSG2 surface-expressing pancreatic cancer cell line (**Fig. 1F**), randomized into equivalent groups based on tumor burden via bioluminescence, and then treated with either  $\alpha$ DSG2 or control CAR-T cells. We observed complete elimination in 12 out of 14 mice treated with  $\alpha$ DSG2 CAR-T cells, with additional control, but eventual recurrence in two mice (**Fig. 3E**; **Supplementary Fig. S7A**).

We next evaluated DSG2-directed CAR-T cell efficacy in the context of both primary lung adenocarcinoma and lung-seeded prostate and breast cancer metastasis models (**Fig. 3F-H**). For our lung cancer model, we i.v. challenged mice with luciferase-expressing A549 cells, forming appreciable lung tumor foci measured via bioluminescent imaging. On day 18 post-implantation, tumor-bearing mice were equally randomized via tumor burden into  $\alpha$ DSG2 or control CAR-T cell treatment groups. We observed complete elimination of A549 lung tumor foci in all ten  $\alpha$ DSG2 CAR-T cell-treated mice seven days post-treatment relative to control CAR-T cells (**Fig. 3F**; **Supplementary Fig. S7B**). Although residual tumor was detected in the posterior region, abdomen, and long bone of three A549 i.v.-challenged mice (day 59; **Supplementary Fig. S7B**), nearly all animals experienced substantial survival benefit (**Fig. 3F**).

As with our lung cancer model, DU145 prostate cancer cells were seeded in the lung, forming appreciable lung tumor foci by day 39, and then treated with  $\alpha$ DSG2 or control CAR-T cells (**Fig. 3G**; **Supplementary Fig. S7C**). Again, we observed complete elimination of DU145 lung tumor foci in all  $\alpha$ DSG2 CAR-T cell-treated mice seven days post-treatment relative to control (**Fig. 3G**). Additionally, all bone and abdominal foci were eliminated 13 days following  $\alpha$ DSG2 CAR-T cell treatment (**Supplementary Fig. S7C**). Mice i.v.-challenged with luciferase-expressing MDA-MB-231 breast cancer cells also experienced complete elimination of lung tumor foci ten days after treatment with  $\alpha$ DSG2 CAR-T cells relative to control, with substantial survival benefit conferred in the two mice harboring residual metastasis in the bone and lower abdomen (**Fig. 3H**; **Supplementary Fig. S7D**).

Lastly, luciferase-expressing HepG2 liver cancer cells were implanted via i.p. challenge in mice, rapidly producing peritoneal liver metastasis (**Fig. 3I**; **Supplementary Fig. S7E**). Mice were randomized into

equivalent groups by bioluminescent tumor burden and treated on day 11 with either αDSG2 or control CAR-T cells. Peritoneal tumors were eliminated 38 days after αDSG2 CAR-T cell treatment relative to control with sustained clearance in three out of five animals for the duration of the experiment (**Fig. 3I; Supplementary Fig. S7E**). Taken together, these data demonstrate that DSG2-directed CAR-T cells broadly and rapidly eliminate solid tumor malignancies in the primary and metastatic settings, with sustained remission of disease following a single dose of CAR-T cells.

### **DSG2-directed CAR-T cells eliminate orthotopic and patient-derived pancreatic tumor xenografts *in vivo***

Although the above CDX models demonstrate the broad applicability of DSG2-directed CAR-T cell efficacy, we wished to further pursue therapeutic models that better reflect the human disease state. While useful, traditional heterotopic CDX models often fail to recapitulate the systemic tumor-organ microenvironment, including stromal and vascular barriers that may limit CAR-T cell trafficking and infiltration<sup>48,49</sup>. Therefore, we employed an orthotopic pancreatic cancer model to stress test αDSG2 CAR-T cell efficacy in the presence of extensive pancreatic desmoplastic stroma often associated with immunotherapeutic failure and poor patient survival outcomes<sup>50,51</sup>. Luciferase-expressing AsPC-1 pancreatic cancer cells were surgically implanted into the pancreatic tail of NSG mice, allowed to engraft for 7 days, and subsequently treated with αDSG2 or control CAR-T cells. While AsPC-1 cells possess the highest quantity of surface DSG2 among the three pancreatic cancer cell lines (**Fig. 1F**), *in vitro* cytotoxicity by αDSG2 CAR-T cells was among the slowest of all surveyed cell lines (**Fig. 2H**). Remarkably, all αDSG2 CAR-T cell-treated AsPC-1 orthotopic tumors experienced complete and sustained elimination 19 days post-treatment, with all six control mice reaching terminal endpoints 44 days post-treatment, highlighting the aggressiveness of this model (**Fig. 4A-C; Supplementary Fig. S7F**). Detection of circulating serum carcinoembryonic antigen (CEA), a common clinical biomarker of pancreatic cancer and abundantly expressed in AsPC-1 cells<sup>52,53</sup>, peaked at day 13 in αDSG2 CAR-T cell-treated animals but rapidly declined thereafter compared to control animals (**Fig. 4B**).

Due to long-term culture and propagation, cancer cell lines often lose their original tumor architecture and genetic heterogeneity, thereby limiting the predictive response to therapy in the clinic. Therefore, like our orthotopic model above, we employed the use of a pancreatic PDX model in mice to further recapitulate the human disease state, highlighted by intrinsic tumor cell and antigen heterogeneity<sup>54–56</sup>. Tumor fragments from PDX039, a treatment-naïve surgically resected stage IV human pancreatic adenocarcinoma sourced from the NCI's Patient-Derived Models Repository (PDMR), were passaged once *in vivo*, and then implanted subcutaneously in NSG mice and allowed to achieve an average size of ~150 mm<sup>3</sup> before treatment with either αDSG2 or control CAR-T cells. Four out of five αDSG2 CAR-T cell-treated mice experienced rapid tumor decline 25 days post-treatment, with sustained elimination for the duration of the experiment compared to control mice (**Fig. 4D-F**). Circulating serum CEA peaked 7 days post-treatment in PDX039-challenged mice, before then declining to near pre-treatment levels one week thereafter in αDSG2 CAR-T cell-treated mice relative to controls (**Fig. 4E**). Together, these results demonstrate DSG2-directed CAR-T cell efficacy is preserved in preclinical models that simulate the

complex architecture and heterogeneity of human tumors. Moreover, these data further complement an extensive list of solid tumor settings in which DSG2-directed CAR-T cells may be deployed when other therapeutic options are limited.

### **DSG2-directed CAR-T cells demonstrate no toxicity in human DSG2 transgenic mice**

Although invaluable for determining *in vivo* CAR-T cell efficacy, mice (including the immunodeficient NSG mouse model used above) have only moderate species homology in the DSG2 extracellular domains with human<sup>57</sup>, eliminating mouse Dsg2 recognition by αDSG2 CAR-T cells (**Supplementary Fig. S8B-C**). To address these xenogeneic differences, we instead used an immunocompetent human DSG2 transgenic (hDSG2<sup>Tg</sup>) mouse model to evaluate potential DSG2-related toxicities among normal tissues expressing the transgene. Originally generated to study species B human adenovirus host receptor binding<sup>58</sup>, the hDSG2<sup>Tg</sup> mouse model has since been extensively validated to express human DSG2 with similar levels and functionality to that of humans<sup>59–64</sup>. With this, we devised a syngeneic adoptive transfer model, whereby CD8<sup>+</sup> T cells were isolated from hDSG2<sup>Tg</sup> donor mice, transduced with the DSG2-directed CAR construct possessing murine, instead of human, 4-1BB, CD28, and CD3ζ signaling domains (**Supplementary Fig. S8A**), and administered to hDSG2<sup>Tg</sup> recipient mice for signs of normal tissue toxicity. Importantly, murine T cells possessing this construct produced a robust *in vitro* effector response with durable antitumor efficacy in human cancer xenograft models (**Supplementary Fig. S8B-S8D**). Due to the immune competency of the model, hDSG2<sup>Tg</sup> recipient mice were first lymphodepleted with cyclophosphamide (CTX) pre-conditioning on days -3 and -1 prior to treatment. Mice then received a single i.v. dose of 5x10<sup>6</sup> αDSG2 or control mouse CAR-T cells (**Fig. 5A**). Following treatment, no overt signs of toxicity or significant changes in body weight were observed relative to control CAR-T cells (**Fig. 5B**). Mouse serum cytokines analyzed on day 3 post-treatment indicated no atypical signs of cytokine release syndrome (CRS)-related toxicities compared to either baseline pre-treatment collection (day 0) or when compared to control-treated mice (**Fig. 5C**). Moreover, no significant differences were observed between αDSG2 or control CAR-T cell-treated hDSG2<sup>Tg</sup> mice for organ damage-related serum biomarkers in liver (**Fig. 5D**), heart (**Fig. 5E**), kidney (**Fig. 5F**), or their associated ions (**Supplementary Fig. S9A**). Necroscopy and scored histopathology evaluated by a blinded pathologist indicated no abnormal tissue infiltration or toxicity by αDSG2 CAR-T cells in tissues/organs collected on day 14 (**Fig. 5G**). Thus, no CAR-T cell toxicities were detected in any normal tissues or organs of mice engineered to express a human DSG2 transgene, indicating that DSG2-directed CAR-T cells may be effective for all solid cancers without toxicity in patients.

## **Discussion**

Immunotherapy has demonstrated tremendous potential in the treatment of solid tumor malignancies over the past few decades. Where traditional interventions have often failed, immune checkpoint blockade (ICB) has succeeded, culminating in thousands of ongoing clinical trials and >100 FDA approvals for various solid tumors and disease states<sup>65,66</sup>. However, ICB is generally limited to tumors

with high neoantigen densities <sup>67</sup> and is often associated with significant toxicities <sup>68</sup>. Adoptive CAR-T cell therapies offer a more targeted approach with potentially fewer systemic side effects. ICB dependence on functional neoantigen processing and presentation is completely bypassed in CAR-T cell therapies, eliminating a prominent tumor cell-intrinsic escape mechanism <sup>69,70</sup>. Given the continuous successes of CD19- and BCMA-directed CAR-T cell therapies across various hematological malignancies, our data justifies a parallel, if not exceedingly effective broad-spectrum CAR-T cell-directed therapy targeting universally expressed DSG2 in solid tumors.

Although this study specifically investigates the effectiveness of DSG2-directed CAR-T cells in the six most lethal forms of solid cancer (lung, colon, pancreatic, prostate, breast, and liver), there remains extensive applicability beyond those evaluated. TCGA reveals increased DSG2 expression among a variety of solid tumors in addition to those six (**Fig. 1A**) and other groups have independently explored elevated DSG2 expression and dysregulation among cervical <sup>71</sup>, endometrial <sup>72</sup>, ovarian <sup>73</sup>, gastric <sup>74,75</sup>, esophageal <sup>76</sup>, and various squamous cell carcinomas <sup>77-79</sup>. Beyond empirical evaluation of every known solid tumor type, our justification for selecting the six most lethal tumor types seems appropriate as a first step in addressing the feasibility of universal application. Within these six tumor types, cancer cell line selection was based on 1) usage by the field (body of evidence associated with that cell line), 2) median expression profile (discounting outliers), and 3) consistency with clinical landscape (such as triple-negative breast cancer). By utilizing three cancer cell lines per tumor type, and adhering to the stringency of these criteria, we justify a representative profile of DSG2-directed CAR-T therapy in solid tumors. Interestingly, while the exact tumor-intrinsic factors determining candidacy for αDSG2 CAR-T cell efficacy remain incompletely explored, variable levels of DSG2 surface expression do not appear to directly influence cytotoxicity kinetics (**Fig. 1F** and **Supplementary Fig. S6B**). Notably, SW480 colorectal cancer cells, with only 740 DSG2 molecules/cell, have approximately the same  $T_{50}$  cytotoxicity as C4-2B prostate cancer cells (3.3 vs. 3.6hr), possessing 54,336 DSG2 molecules/cell. While higher antigen expression does not necessarily equate to increased cytotoxicity, in the absence of a correlation, this result is difficult to interpret. Moreover, direct tuning of DSG2 expression within individual cell lines, ignoring cell-intrinsic differences, has never been directly modulated to test this <sup>80</sup>.

Like *in vitro* cancer cell selection, extending efficacy to the *in vivo* setting requires careful consideration of how and when αDSG2 CAR-T cells would likely be deployed clinically. In hematological settings, CAR-T cells are traditionally used as third-line and, more recently, second-line therapies, both targeting relapsed or refractory disease <sup>81,82</sup>. For solid tumors, this typically substantiates as metastasis following initial treatment. Rather than evaluate all tumor types subcutaneously, an imperfect model system for most metastases, xenografts were modeled in either the peritoneal or pulmonary compartments depending on tumor type and metastatic niche. Although highly effective in all xenograft models tested, some αDSG2 CAR-T cell-treated animals experienced initial tumor control, followed by eventual recurrence. In all cases, survival was extended considerably relative to control-treated animals (BxPC-3, A549, and MDA-MB-231), but residual tumor persistence occurred in some animals until terminal endpoints were achieved. Interestingly, in the two i.p. BxPC-3-challenged mice with recurrence, tumors manifested as a

single subcutaneous-like mass affixed to the inner peritoneum, rather than diffuse carcinomatosis. In our i.v.-challenged A549 (lung) and MDA-MB-231 (breast) CDX models, additional tumors manifested in what appears to be the femur-tibial junction by intravital imaging, indicating metastasis beyond initial colonization of the lung. These instances of incomplete tumor eradication, albeit rare, may indicate potential spatiotemporal settings or compartments that restrict CAR-T cell trafficking following initial treatment with  $\alpha$ DSG2 CAR-T cells. In future studies, it may be prudent to evaluate the persistence of these tumors upon direct administration of CAR-T cells into the local tumor environment (i.p. CAR-T cells following i.p. challenge) or upon a subsequent dose.

Due to the absence of cross-species recognition by our DSG2-directed scFv, we evaluated the safety of our CAR in the context of a syngeneic, but humanized DSG2 transgenic mouse (hDSG2<sup>Tg</sup>) model<sup>58–64</sup>. Mimicking human DSG2 expression, functionality, and location within normal cell-cell junctions was critical for elucidating any potential  $\alpha$ DSG2 CAR-T cell-related toxicities in normal tissues. Herein, an absence of toxicity post-treatment was observed, indicated by no significant changes in body weight, serum cytokines, organ damage-related serum biomarkers, or histopathology compared to control CAR-T cells (**Fig. 5**). Absence of toxicity in this model may suggest that DSG2 is expressed within an immunologically restricted space between normal cells, thereby sparing it from recognition by CAR-T cells. Interestingly, circulating DSG2-specific autoantibodies were identified in patients with pemphigus, an autoimmune disease canonically associated with pathogenic anti-DSG1 and anti-DSG3 autoantibodies triggering blistering of the skin and mucous membranes<sup>83</sup>. In agreement with the safety of  $\alpha$ DSG2 CAR-T cells in hDSG2<sup>Tg</sup> mice, DSG2-specific antibodies produce no pathology in patients, aligning with a model of DSG2 compartmentalization within desmosomes. Our previous animal model studies investigating GUCY2C-directed CAR-T cells in colorectal cancer similarly demonstrate anticancer efficacy without normal intestinal toxicity, reflecting anatomical segregation of GUCY2C to the luminal surface of intestinal epithelia<sup>84,85</sup>, supported by GUCY2C CAR-T cell safety in ongoing clinical trials (ChiCTR2100053828, ChiCTR2100044831, NCT06197178, NCT05319314, NCT05287165). Similarly, CDH17-directed CAR-T cells are believed to functionally ignore CDH17 antigen expressed between normal intestinal epithelial cells due to lateral junction occlusion abutted by tight junctions<sup>24</sup>. Moreover, claudin 18.2, a tight-junction molecule predominantly found in normal gastric epithelium, becomes accessible on the cell surface during gastric tumorigenesis, thereby permitting effective and safe CAR-T cell therapy in mouse models<sup>86</sup> and patients<sup>87</sup>. We propose a similar model of DSG2 safety, in which DSG2 between adjacent normal cells is functionally ignored by CAR-T cells but, through malignant transformation, loses polarity in tumor cells and is therefore targetable by CAR-T cell therapy. However, unlike CDH17, GUCY2C, and claudin 18.2, which are expressed by a limited number of solid cancers, DSG2 is universally expressed by epithelial-derived solid cancers. Thus, DSG2 may serve as a first-in-class universal CAR-T cell target in solid cancers, necessitating further clinical investigation for safety and efficacy in patients and the development of mass-manufacturable CAR-T production processes that could produce  $\alpha$ DSG2 CAR-T cells for the ~10M patients dying from solid cancers annually around the world.

# Methods

## Public Expression Data

The Cancer Genome Atlas (TCGA) transcriptional expression data were generated using Bioconductor v3.18, “RTCGA.rnaseq” RStudio package, RSEM normalization, accessed 11-01-2015. Human Protein Atlas (HPA) patient tumor protein expression and categorization were determined by internal HPA IHC staining criteria: i) staining intensity, ii) fraction of stained cells, and iii) subcellular localization. Graph percentages are based on number of medium/high IHC-scored tissues divided by total number of available patient specimens per tumor type for that dataset.

## Cell lines

Cell lines, ATCC designations, and their associated culture conditions are outlined in **Supplementary Table 1**. Cells were maintained at a low passage number in a 37°C humidified incubator at 5% CO<sub>2</sub> and regularly tested for mycoplasma (ATCC, #30-1012K). Cell lines were STR profiled (ATCC, #135-XV) to confirm reference cell identity with American Type Culture Collection (ATCC).

DLD-1 (DSG2-CRISPR-KO) cell lines were generated by seeding  $0.4 \times 10^5$  wildtype DLD-1 cells in a 24-well plate and then co-transfecting (ThermoFisher Scientific, #CMAX00008) with a synthetic gRNA pool targeting exon 3 of the DSG2 locus (sgRNA1; UCUGAUCUUGCAGAAGAAAG, sgRNA2; AAGACAAAUAUACCAAAAGG, sgRNA3; AGAAGAAACACCAUUUUUUC) and SpCas9 2NLS nuclease per the manufacturer’s protocol (CRISPR Gene Knockout Kit v2, SYNTHEGO Corporation). 72 hours post-transfection, cells were passaged for both monoclonal colony establishment and genomic DNA isolation (ThermoFisher Scientific, #K182001) in parallel. Target region deletion was detected via PCR (Promega, #M712) of genomic DNA (Forward Primer: GTCATGTCATCTCTGGCCTGT, Reverse Primer: TCCTCTTGCATCCAAAGCGT) and the amplicon was subsequently extracted/purified (ThermoFisher Scientific, #K210025) from an agarose gel and analyzed via Sanger sequencing. DSG2 knockout efficiency was determined via comparison of DLD-1 sgRNA(+) and sgRNA(-) genomic DNA sequencing analyzed via SYNTHEGO’s Inference of CRISPR Edits (ICE) tool. Monoclonal DLD-1 cell colonies were established via limiting dilution (0.8 cells/100µL per well), cultured ~3 weeks, and screened via genomic Sanger sequencing, genomic DNA PCR, flow cytometry, and immunoblot for the absence of DSG2.

## Immunoblots

Total protein was extracted from cultured cell lines and mechanically dissociated PDX tissues via cold M-PER extraction reagent (ThermoFisher Scientific, #78501) supplemented with protease and phosphatase inhibitors. Lysates were sonicated and protein content was quantified via BCA biuret assay (ThermoFisher Scientific, #23227). Lysates were prepared in 4X LDS sample buffer (ThermoFisher Scientific, #NP0007), reduced with 2.75mM β-Mercaptoethanol (EMD Millipore, #444203), and then boiled at 100°C for 5 min. 15µg of protein was loaded per lane in a 4-12% Bis-Tris gel (ThermoFisher Scientific, #NP0336) and transferred to nitrocellulose membrane via an iBlot 2 semi-dry transfer

(ThermoFisher Scientific, #IB21001). Membranes were blocked for 2 hours in phosphate-buffered saline (PBS) + 0.1% Tween-20 and 10% milk at room temperature, followed by 4°C overnight incubation with primary antibodies: (αDSG2; Abcam, #ab150372) and (αGAPDH; Cell Signaling Technology, #2118). Anti-rabbit horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Labs, #111-035-144) was then incubated with membranes for 1 hour, washed, developed with Dura chemiluminescent substrate (ThermoFisher Scientific, #34075), and imaged using a Bio-Rad ChemiDoc MP Imaging Station.

## **Flow Cytometry and Cell Surface Quantification**

DSG2 protein cell surface quantification was enumerated via flow cytometry with BD QuantiBRITE-PE beads (BD Biosciences, #340495) for reference. Cell lines were thawed, cultured in appropriate media (**Supplementary Table 1**) until ~90% confluency was achieved, at which point cells were trypsinized and then quenched with ice-cold media. Cell suspensions were transferred to polystyrene FACS tubes, washed twice with cold FACS buffer, and incubated for 15 min with FC Block (BD Bioscience, #564219) on ice to minimize non-specific staining. Cells were then washed, incubated with DSG2-PE antibody (Clone: CSTEM28; ThermoFisher Scientific, #12-9159-42) for 45 min at 4°C in the dark, washed again, and finally spiked with SYTOX-Red LIVE/DEAD (ThermoFisher Scientific, #S34859) viability stain just prior to analysis. FACS (BD FACSCelesta™) gating strategy via FCS/SSC, single cell, and viability was determined for each cell line due to variable size and complexity. DSG2<sup>+</sup> gating was determined by comparison to identical cell lines stained with IgG2bκ-PE isotype control (ThermoFisher, #12-4732-81). Quantitative comparisons were made with BD QuantiBRITE-PE beads ran in unison as a pre-calibrated standard with known quantities of PE-fluorophore-conjugated beads. Assuming a 1:1 fluorophore to antibody ratio per the manufacturer, DSG2 surface molecule number was extrapolated by comparing analyzed DSG2-PE fluorescent intensity to a BD QuantiBRITE-PE calculated standard curve. Approximate DSG2 molecules/cell were calculated by subtracting from isotype control antibody background signal. Results were analyzed using FlowJo v10.9.

## **Real-Time Quantitative PCR**

RNA was extracted from cultured cell lines via TRIzol™ reagent (ThermoFisher Scientific, #15596026), separated via chloroform phase extraction, precipitated by isopropyl alcohol, washed via 75% ethanol, and air-dried. RNA was reconstituted in DEPC-treated water and purity was measured with a Nanodrop 1000 (ThermoFisher Scientific). Approximately 200 ng of RNA was reverse transcribed to complementary DNA (cDNA) using the TaqMan Reverse Transcription kit according to kit directions (Thermo Fisher Scientific, #N8080234). Transcripts were quantified by qRT-PCR using TaqMan primer probes (ThermoFisher Scientific, DSG2: #Hs00170071\_m1; GAPDH: #Hs99999905\_m1) on a QuantStudio-5 (ThermoFisher Scientific), with TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, #4318157), per kit instructions. Relative transcript abundance was calculated by the  $2^{-\Delta\Delta C_t}$  method and normalized to that of GAPDH.

## **Primary Human T Cell Isolation and Culture**

Healthy human CD4<sup>+</sup> and CD8<sup>+</sup> T cells were isolated from fresh human peripheral blood leukopaks (STEMCELL Technologies, #200-0470) via negative pan T cell selection (Miltenyi Biotec, #130-096-535). T cells were cryopreserved in animal component-free CryoStor10 (STEMCELL Technologies, #07930) for short and long-term storage. As needed, human T cells were thawed drop-wise into human T cell culture medium consisting of: RPMI-1640 (Corning, #10-041-CV), 10% heat-inactivated fetal bovine serum (Gibco, #A38400-01), 250mM *N*-Acetyl-L-cysteine (Sigma-Aldrich, #A9165), 1% Insulin-Transferrin-Selenium (Gibco, #41400-045), 1% GlutaMAX (Gibco, #35050-061), 1% *D*-glucose solution (Gibco, #A24940-01), 1% sodium pyruvate (Gibco, #11360-070), 1% MEM non-essential amino acids (Gibco, #11140-050), 1% HEPES (Gibco, #15630-080), 1% penicillin-streptomycin (Gibco, #15140-122), and 55µM 2-mercaptoethanol (Gibco, #21985-023), supplemented with 10 ng/mL recombinant human IL-7 and IL-15 (NCI BRB Preclinical Repository). Cultures were maintained at 37°C and 5% CO<sub>2</sub>.

### Lentiviral Production and Functional Titer

Low-passage HEK293T/17 cells (ATCC, CRL-11268) were co-transfected with third-generation pCDH-EF1α-CAR-T2A-GFP transfer plasmid, pRSV-Rev (Addgene, #12253) and pMDLg/pRRE (Addgene, #12251) packaging plasmids, and pMD2.G (Addgene, #12259) envelope plasmid, using Lipofectamine 3000 (ThermoFisher Scientific, #L3000001). Supernatants were collected at 24 and 48 hours post-transfection, combined, and concentrated overnight at 4°C using PEG-8000 (Sigma-Aldrich, # 25322-68-3) with gentle rotation. Supernatants were pelleted at 1600xg for 1 hour and resuspended 1:200 their original volume in lentiviral storage buffer (10mM Tris pH 7.5, 10% lactose, 25mM proline) and stored at -80°C.

The functional titer of lentivirus was evaluated by transducing 5x10<sup>5</sup> HEK293T/17 cells with concentrated lentivirus at 10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup> dilutions in triplicate with 0.8 µg/mL polybrene (Sigma-Aldrich, #TR-1003-G) for 72 hours. Cells were trypsinized, stained for LIVE/DEAD (ThermoFisher Scientific, #S34859), and endogenous GFP-expression was measured via flow cytometry (BD FACSCelesta) and analyzed using FlowJo v10.9. Titers were calculated using the following formula:

$$\text{Transforming Units/mL} = \frac{(\text{Cell \# at Inoculation} * \% \text{ GFP})}{(\text{Inoculum Volume} * \text{Viral Dilution Factor})}$$

### T Cell Activation and Lentiviral Transduction

Human T cells were thawed and immediately activated via anti-CD2/CD3/CD28 antibody-conjugated magnetic beads (Miltenyi Biotec, #130-091-441) in culture media supplemented with cytokines. After 24 hours, CAR-encoding lentivirus was added at MOI of 5 with 0.8 µg/mL polybrene, incubated for an additional 48 hours before magnetically removing activation beads (Miltenyi Biotec, #130-092-168), and then transferring to a G-Rex®6M culture system (Wilson Wolf, #80660M) for 10 additional days.

## xCELLigence Cytotoxicity Assays

All cytotoxicity assays were conducted using an xCELLigence Real-Time Cell Analysis (RTCA) SP instrument (Agilent Technologies, #380601030). E-plates (Agilent Technologies, #5232368001) were equilibrated with 100  $\mu$ L target cell media, followed by addition of  $1.0\text{--}3.0 \times 10^4$  target cells/well in 50  $\mu$ L. Continuous 15 min cell impedance sweeps were performed until a cell-index of  $\geq 1.0$  was achieved, followed by addition of effector cells in 50  $\mu$ L serum-free media at 5:1 (Effector:Target) ratio unless otherwise specified. All assays were performed in triplicate wells per condition with 15 min continuous sweeps for  $\geq 36$  hours after normalizing to the time of effector cell addition using the xCELLigence “Immunotherapy” module. For analysis, all cytotoxicity curves were normalized between untreated target cells (0% cytotoxicity) and target cells treated with 0.25% Triton X-100 (100% cytotoxicity).

## Murine Xenograft Experiments

*In vivo* studies were conducted in compliance with Thomas Jefferson University Institutional Animal Care and Use Committee (IACUC)–approved protocol #01529. Eight to 12-week-old NOD.Cg-*Prkdc*<sup>scid</sup>*H2-K1<sup>b</sup>-tm1Bpe**H2-Ab1<sup>g7-em1Mvw</sup>**H2-D1<sup>b</sup>-tm1Bpe**Il2rg<sup>tm1Wjl</sup>*/SzJ (NSG-MHC I/II DKO) mice (The Jackson Laboratory; Strain #025216) were used for all *in vivo* experiments. For subcutaneous DLD1 and A431 tumor models, mouse flanks were implanted with  $1 \times 10^6$  cancer cells and measured biweekly via caliper. When tumors achieved an average size of  $\sim 150 \text{ mm}^3$ , mice received  $5 \times 10^6$  CAR<sup>+</sup> human donor-matched  $\alpha$ DSG2 or control  $\alpha$ CD19-directed CAR-T cells ( $\sim 1 \times 10^7$  total T cells). For intraperitoneal tumor models, mice were implanted i.p. with  $1 \times 10^6$  luciferase-expressing (AddGene, Plasmid #105621) DLD-1, BxPC-3, or HepG2 cells and measured weekly via bioluminescence imaging (PerkinElmer, IVIS Lumina LT Series III) and analyzed using Aura v2.3.1 software (Spectral Instruments Imaging). When the average bioluminescent signal achieved  $\sim 1 \times 10^7$  photons/sec/cm<sup>2</sup>/sr, mice were randomized into groups and received  $5 \times 10^6$  human donor-matched CAR-T cells (unless otherwise specified, i.e. dose response). For tumor lung models, mice were injected i.v. with  $1 \times 10^6$  luciferase-expressing A549, DU145, or MDA-MB-231 cells via tail vein and measured weekly via bioluminescence imaging. When tumor lung foci reached appreciable size, mice were randomized into groups and received  $5 \times 10^6$  human donor-matched CAR-T cells. AsPC-1 pancreatic orthotopic tumors were established by surgically implanting  $2.5 \times 10^5$  luciferase-expressing AsPC-1 cells via 28G syringe into the mouse pancreas tail. The surgical wound site was closed, and analgesic and antibiotic were applied. When the average bioluminescence signal achieved  $\sim 1 \times 10^7$  photons/sec/cm<sup>2</sup>/sr, mice were randomized into equal groups and received  $5 \times 10^6$  human donor-matched CAR-T cells. Pancreatic patient-derived xenografts (PDXs) were established by surgically implanting second passage (P=2) tumor fragments suspended in 50% Matrigel (Corning, #354230) solution via trocar needle into mouse scruff. The surgical wound site was closed, and analgesic and antibiotic were applied. Tumors were then measured biweekly via calipers. When tumors achieved an average size of  $\sim 150 \text{ mm}^3$ , mice received  $5 \times 10^6$  human donor-matched CAR-T cells. The PDX model 193399\_133\_R [Lot# JW0KK4] used in this study was developed by NCI PDMMR. Serum was collected

weekly via capillary eye bleeds for circulating CEA protein quantification. All CAR-T cells were administered i.v. via tail vein injection.

### **Murine CAR-T Safety Evaluation**

DSG2-directed CAR-T cell safety was evaluated in an immunocompetent human DSG2 transgenic (hDSG2<sup>Tg</sup>) murine system<sup>58</sup>. Donor hDSG2<sup>Tg</sup> T cells were isolated from dissociated murine splenocytes, activated (Miltenyi Biotec, #130-093-627), and transduced with a gammaretrovirus containing a murine version of the “6D8” third-generation CAR molecule (i.e., murine CD28, 4-1BB, and CD3ζ domains)<sup>84,85</sup>, with CAR transduction efficiency evaluated by bicistronic GFP expression via flow cytometry. Recipient hDSG2<sup>Tg</sup> mice (aged 6 to 8 weeks) were twice lymphodepleted with cyclophosphamide (100mg/kg/dose) and adoptively transferred with 5x10<sup>6</sup> mouse CAR-T cells directed to human HER2 (clone 4D5; control) or human DSG2 (clone 6D8). Mouse body weight and condition score were measured biweekly. Peripheral blood was collected via retro-orbital eye bleed on days 0 (pre-treatment, baseline) and 3 for cytokine determination, and day 14 for blood chemistry and organ toxicity profiling (IDEXX BioAnalytics) post-treatment. On day 14, all mice were euthanized, and key organs were taken, fixed in 10% formalin, paraffin-embedded, sectioned, and stained for H&E for pathology evaluation by a treatment-blinded pathologist.

### **ELISA**

Effector cytokine release was determined via co-culture of CAR-T cells with pre-plated DLD-1 and DLD-1 (CRISPR-DSG2<sup>-/-</sup>) colorectal cancer cells at a 5:1 E:T ratio in triplicate. Following 24-hour co-culture, medias were collected, cells and debris were removed via centrifugation, and supernatants were stored at -80°C until assayed. Cytokine levels were determined via a human cytokine multiplexed proinflammatory array (Eve Technologies Corporation, #HDF15) with the exception of IFNγ (ThermoFisher Scientific, #EHIFNG) for three separate human donors. Mouse serum cytokine ELISA quantification was conducted by Eve Technologies via Mouse Cytokine Proinflammatory Focused 10-Plex Discovery Assay® Array (MDF10).

Circulating serum levels of carcinoembryonic antigen (CEA) were analyzed following CAR-T cell treatment of AsPC-1 pancreatic orthotopically challenged NSG mice. Blood was collected weekly via retro-orbital eye bleeds and serum was separated from whole blood by 2000xg centrifugation for 10 min at 4°C. Serum was then diluted 10-fold and incubated overnight in a 4°C shaking incubator within pre-coated CEA ELISA wells (ThermoFisher Scientific, #EHCEA). Similarly, pancreatic PDX039-challenged NSG mice were analyzed following an identical serum collection and dilution procedure, but analyzed via a higher-sensitivity CEA ELISA (Abcam, #ab264604).

### **Intracellular Cytokine Staining (ICS)**

48-well polystyrene plates were pre-coated 24 hours in advance with 10 µg/mL of recombinant DSG2 protein (R&D Systems; #947-DM-100, #7699-DM-050) or positive control αHIS antibody (ThermoFisher

Scientific, MA1-21315) at 4°C.  $1 \times 10^6$  αDSG2 CAR-T cells were then added to wells along with protein transport inhibitor (ThermoFisher Scientific, #00-4980-03) and incubated for 6 hours alongside PMA/Ionomycin-treated (ThermoFisher Scientific, #00-4970-03) positive control T cells. T cells alone without antigen served as a negative control. Cells were then collected, washed, surface stained (BioLegend, #100708), permeabilized (BD Biosciences, #554723), stained intracellularly with anti-IFNγ (BioLegend, #505810) and anti-TNFα (BioLegend, #506324) antibodies, and fixed (BD Biosciences, #554714) prior to analysis. FACS was conducted on a BD LSR II flow cytometer and results analyzed via FlowJo v10.9.

## **Statistical Analysis**

Data and statistical analysis were performed as indicated in figure legends using GraphPad Prism v10 software. A Student's *t* test was used for all comparisons between two groups with Welch's correction to account for unequal variances. One-way analysis of variance (ANOVA) was used for comparisons of more than two groups. All results are presented as the means ± standard deviation (SD) unless otherwise specified.

## **Declarations**

### ***Supplemental Information***

Supplementary Figures S1-S9 and Supplementary Table S1: including cell line origin and conditions.

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**Author Contributions**

R.D.C. conceptualized the study, planned and executed the experiments, analyzed the data, prepared the figures, and wrote the manuscript. L.W., T.R.B., O.K., M.C., R.E.S., P.J., and M.M.C. contributed to executing or analyzing both *in vitro* and *in vivo* experiments. A.A.E., A.C., J.S.K., A.K.L., J.R.B., T.J.M.K., V.V.G., and E.H. contributed to executing or analyzing *in vivo* experiments. J.K.W. contributed to therapy design and study guidance. A.L. generated model systems. M.G.M. and S.A.W. provided guidance, validation, investigation, and editing. A.E.S. provided study supervision, validation, investigation, project administration, writing, and editing.

**Declaration of Interests**

R.D.C. and M.G.M report pending patents related to the submitted work. S.A.W. is a member of the Board of Directors for Targeted Diagnostics & Therapeutics, Inc. which provided research funding outside this work and has a license to commercialize inventions unrelated to this work. A.E.S. reports personal fees from Targeted Diagnostics and Therapeutics (TDT), Inc. unrelated to this work and Vittoria Biotherapeutics Inc. related to the submitted work; pending patents related to the submitted work; and CAR-T cell-related patents licensed to Vittoria Biotherapeutics.

**Resource Availability**

**Lead Contact**

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Adam E. Snook (Adam.Snook@jefferson.edu).

**Materials Availability**

Plasmids generated in this study will be made available upon request and completion of a Material Transfer Agreement.

**Data and Code Availability**

This study did not generate any novel datasets. Publicly available transcriptional and proteomic data was accessed and retrieved from The Cancer Genome Atlas (TCGA), Human Protein Atlas (HPA), and the Broad Cancer Dependency Map (DepMap) Project databases.

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Figures

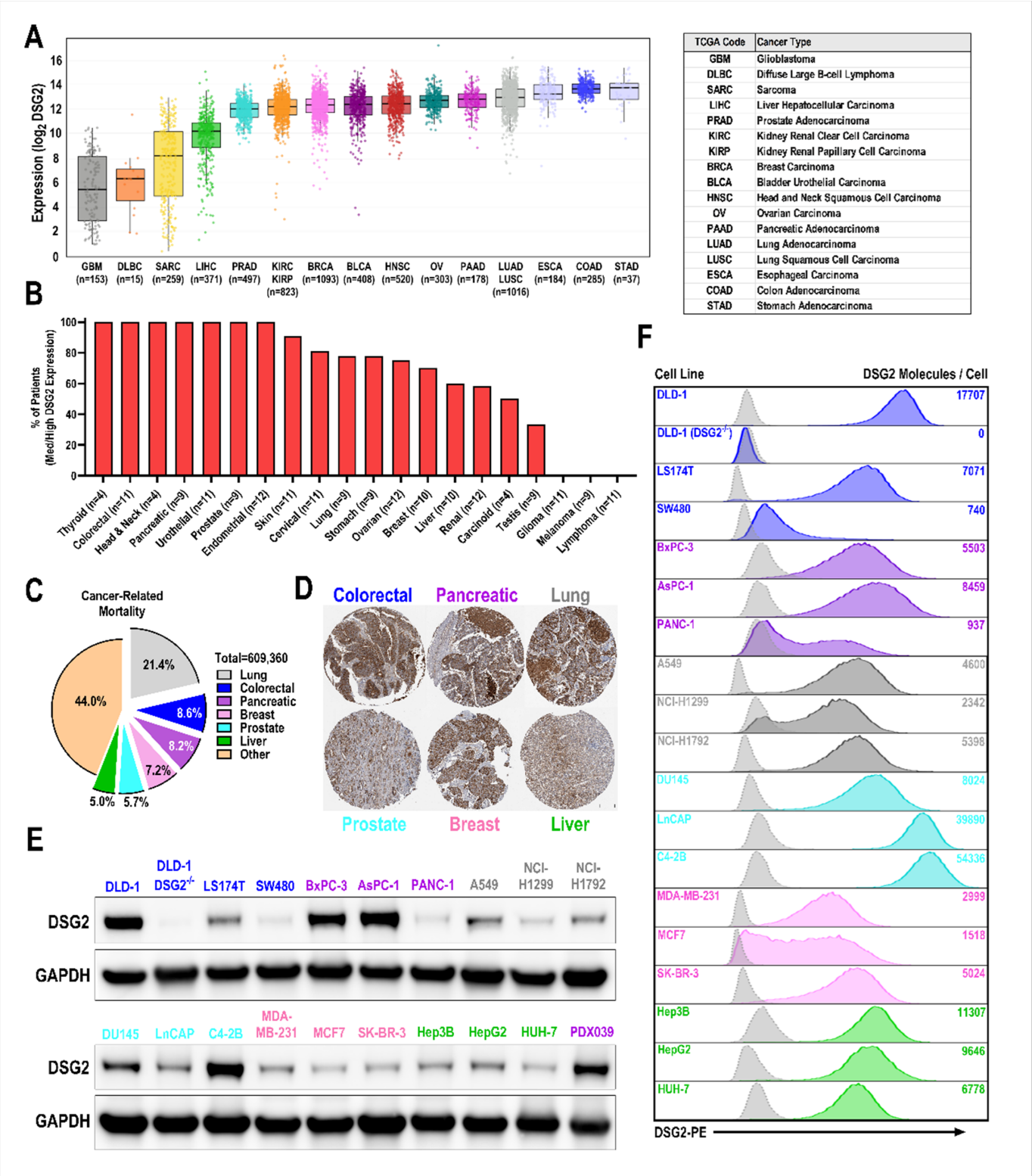


Figure 1

**DSG2 is abundantly and universally expressed across epithelia-derived solid tumors.** (A) *DSG2* mRNA expression in TCGA-cataloged tumors. (B) Percentage of patient tumors scoring medium/high for DSG2 protein via Human Protein Atlas (HPA) IHC. Percentage based on number of medium/high IHC-scored

tissues divided by total number of available patient specimens per tumor type. CAB025122 dataset. (C) 2023 indexed Surveillance, Epidemiology, and End Results (SEER) cancer statistics highlighting the six tumor types producing the most U.S. cancer-related deaths. (D) Representative DSG2 protein IHC in HPA-cataloged solid tumors representing the six largest cancer mortality contributors. CAB025122 dataset. Scale bar, 200  $\mu$ m. (E) Total DSG2 protein in various cancer cell line lysates measured by immunoblot and cataloged by tumor type: colorectal (dark blue), pancreatic (purple), lung (grey), prostate (cyan), breast (pink), liver (green), and pancreatic patient-derived xenograft tumor tissue (purple). (F) DSG2 cell-surface expression measured by flow cytometry relative to isotype-matched controls (left; dotted light grey). Inset values (right) indicate average DSG2 molecules per cell line. n=2 replicates/cell line. See also Supplementary Fig. S1-S3.

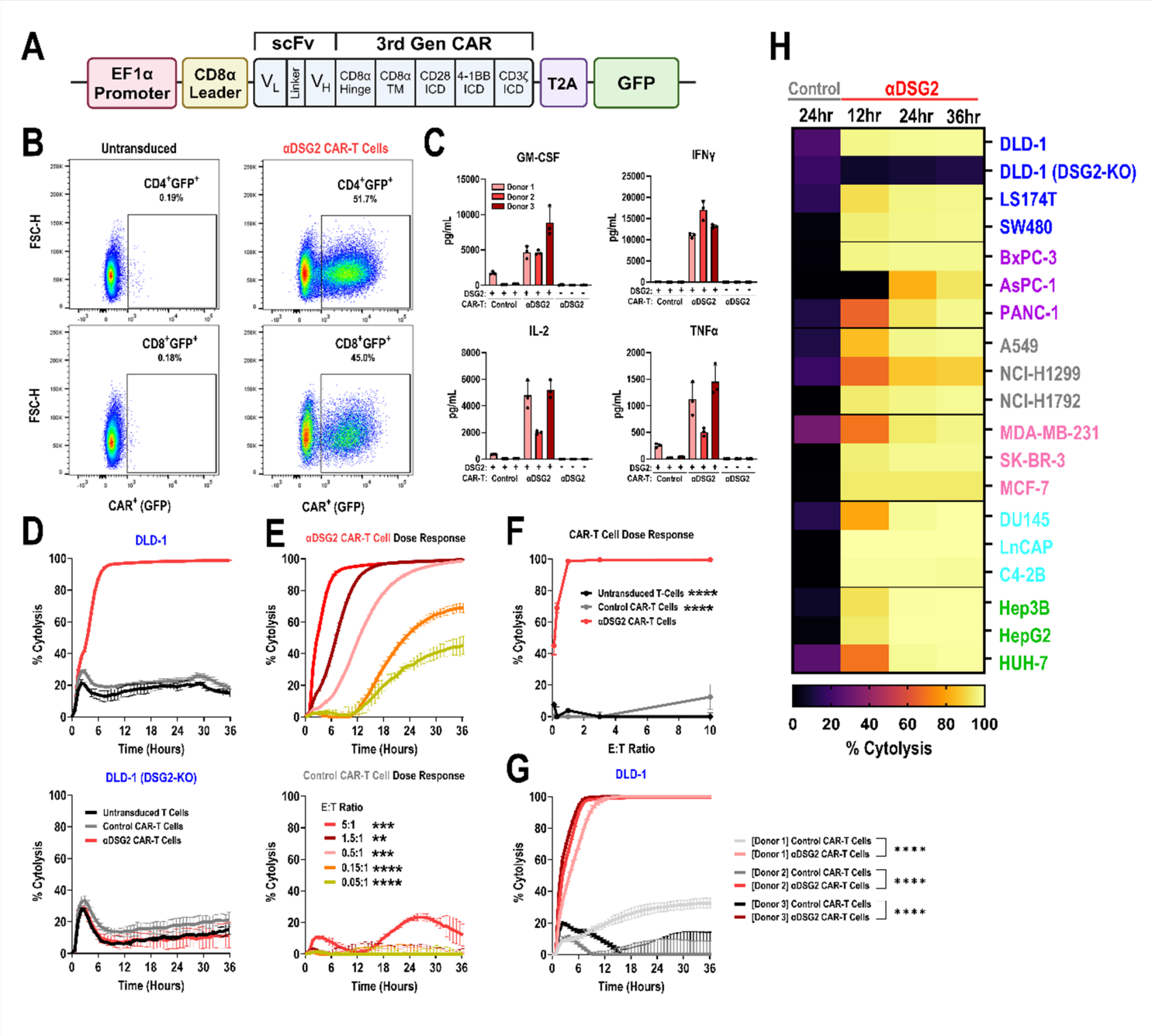
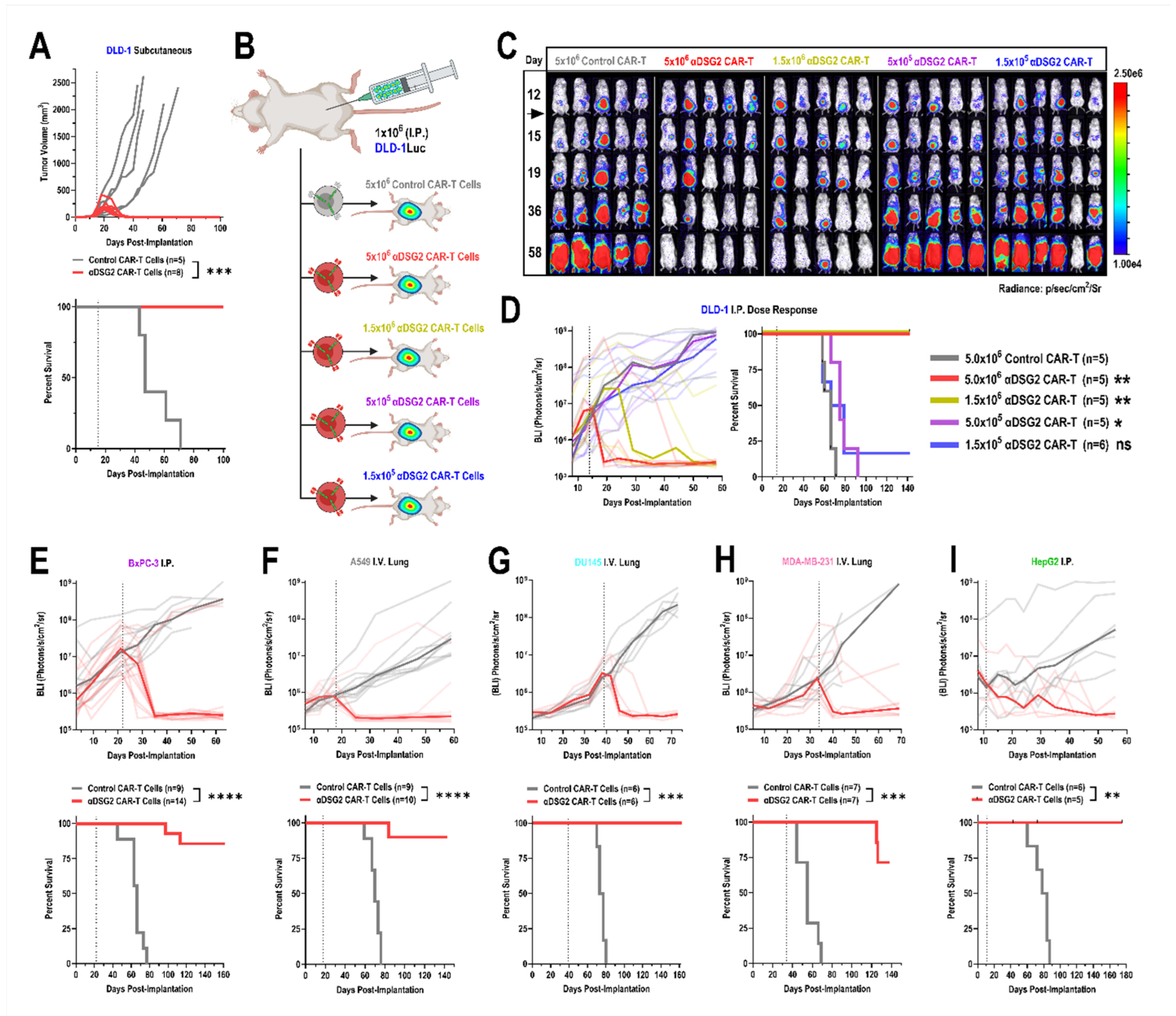


Figure 2

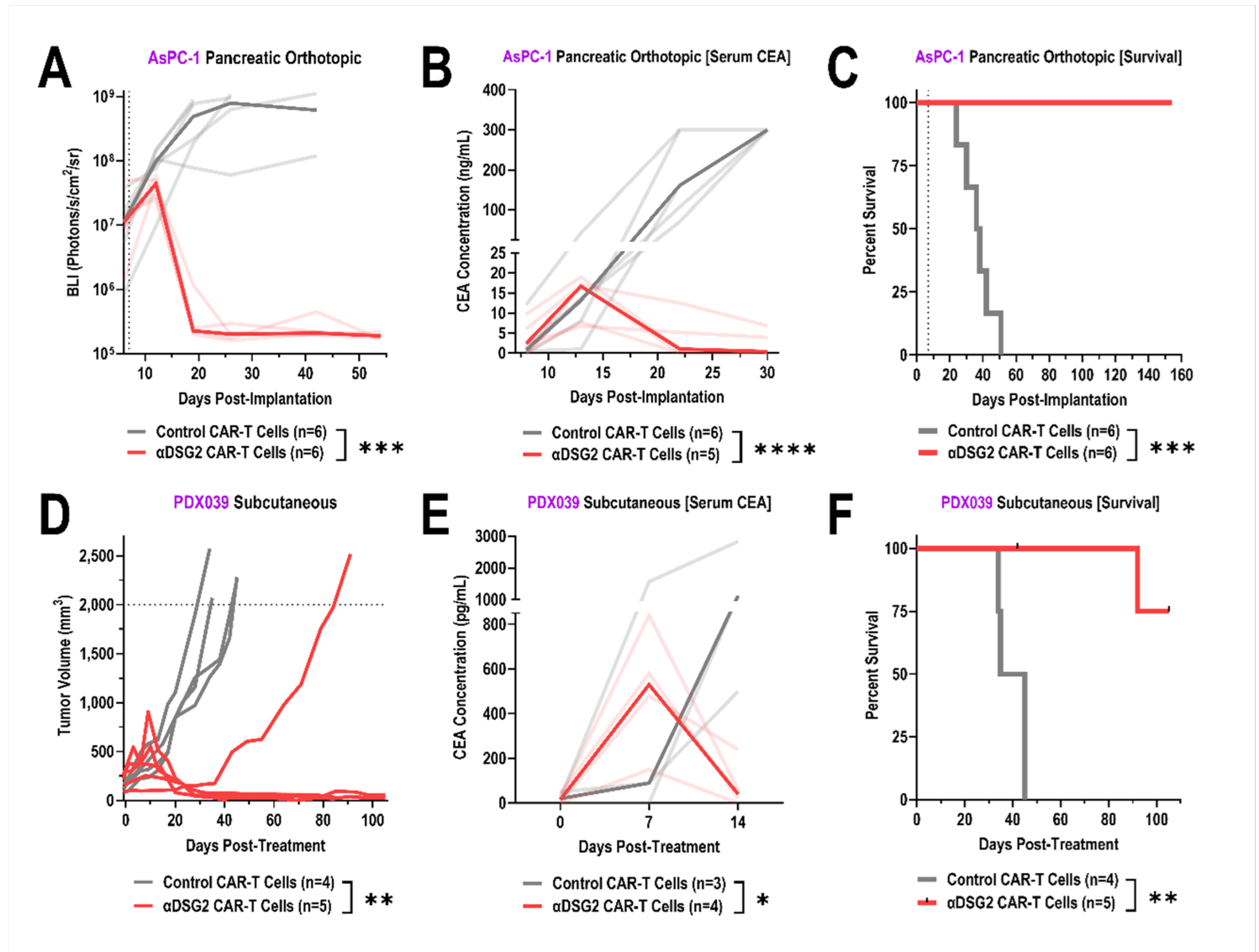
**DSG2-directed CAR-T cells recognize and eliminate DSG2-expressing cancer cells *in vitro*.** (A) Schematic diagram of the third-generation  $\alpha$ DSG2 CAR, T2A, GFP reporter construct. (B) Representative  $\alpha$ DSG2 CAR lentiviral transduction efficiency (GFP fluorescence) in human T cells. (C) Effector cytokine secretion in supernatants of  $\alpha$ CD19 (control) or  $\alpha$ DSG2 CAR-T cells co-cultured with wildtype (DSG2+) or DSG2-deficient (DSG2-) DLD-1 colorectal cancer cells in three donors after 24 hours. Data represented as mean  $\pm$  SD; n=3 technical replicates/donor. (D) Cytolysis of wildtype (top) and DSG2-deficient (bottom) DLD-1 cells by  $\alpha$ DSG2 CAR-T cells at 5:1 E:T (Effector:Target) ratio compared to donor-matched untransduced and control CAR-T cells. (E) Comparison of DLD-1 target cell cytolysis between  $\alpha$ DSG2 (top) and control CAR-T cells (bottom) at a decreasing E:T ratio. (F) Composite cytolysis of DLD-1 target cells at decreasing E:T ratios of untransduced, control, or  $\alpha$ DSG2CAR-T cells from **E** at 36 hours. (G) Cytolysis of DLD-1 target cells by  $\alpha$ DSG2 CAR-T cells compared to control CAR-T cells in three separate donors (5:1 E:T). (H) Composite heat map of  $\alpha$ DSG2CAR-T cell cytolysis across various solid tumor cell lines [colorectal (dark blue), pancreatic (purple), lung (grey), prostate (cyan), breast (pink), and liver (green)] relative to control CAR-T cells. Individual cytolysis curves for **H** are shown in **Supplementary Figure S6B**. All cytolysis graphs (**D-H**) depict group mean cytolysis  $\pm$  SD; n $\geq$ 3 technical replicates/CAR or T cell type. Cytolysis curve significance determined by area under curve (AUC) calculation and compared using an unpaired *t* test with Welch's correction. \*\*, *P* < 0.01; \*\*\*, *P* < 0.001; \*\*\*\*, *P* < 0.0001. See also Supplementary Fig. S3-S6.



**Figure 3**

**DSG2-directed CAR-T cells eliminate DSG2-expressing solid tumor cell xenografts *in vivo*.** (A) Tumor growth curves (top) and mouse overall survival (bottom) of subcutaneous DLD-1 colorectal tumors after control (CD19) or DSG2-directed CAR-T cell treatment (vertical dotted line denotes treatment on day 15). (B-D) αDSG2 CAR-T cell dose response in mice bearing peritoneal DLD-1 metastases. (B) Schematic of luciferase-expressing DLD-1 i.p. implantation and treatment with control or decreasing quantities of αDSG2 CAR-T cells. (C) Bioluminescence images of mice with i.p. luciferase-expressing DLD-1 tumors; arrow denotes treatment on day 14. (D) Tumor burden (BLI) over time (left) and associated overall survival (right). Individual mouse BLI are shown with a bolded overlay indicating the median BLI of all mice per group. Vertical dotted line denotes treatment on day 14. (E-I) Metastatic tumor models were established in NSG mice. Tumor burden (via BLI) and overall survival were monitored after control or

$\alpha$ DSG2 CAR-T cell treatment. Vertical dotted lines denote treatment day. (E) BxPC-3 metastatic pancreatic tumor model (i.p.). Treatment on day 22. (F) A549 lung adenocarcinoma model (i.v.). Treatment on day 18. (G) DU145 metastatic prostate cancer model (i.v.). Treatment on day 39. (H) MDA-MB-231 metastatic breast cancer model (i.v.). Treatment on day 34. (I) HepG2 metastatic liver cancer model (i.p.). Treatment on day 11. All survival was analyzed using the log-rank (Mantel-Cox) test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ ; ns, not significant. All panels employed  $5 \times 10^6$  CAR+ T cells, except B-D as indicated. See also Supplementary Fig. S7.



**Figure 4**

**DSG2-directed CAR-T cells eliminate orthotopic and patient-derived pancreatic tumors.** (A-C) Tumor burden quantified over time by BLI (A), circulating serum CEA levels (B), and overall survival (C) in mice surgically implanted with luciferase-expressing AsPC-1 pancreatic cancer cells in the pancreas following control or  $\alpha$ DSG2 CAR-T cell treatment; vertical dotted line denotes treatment on day 7. (D-F) Tumor burden quantified over time via caliper measurement (D), circulating serum CEA levels (E), and overall survival (F) in mice challenged subcutaneously with patient-derived pancreatic cancer xenograft tissue.

Mice received either control or αDSG2 CAR-T cells when tumors reached ~150 mm<sup>3</sup>. Longitudinal dotted line indicates euthanasia criterion. Individual mouse BLI (A) and serum CEA concentrations (B,E) are shown with a bolded overlay indicating the median of all mice per group. Tumor growth and serum CEA were analyzed by two-way ANOVA. All survival was analyzed using the log-rank (Mantel-Cox) test. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001; \*\*\*\*, P < 0.0001.

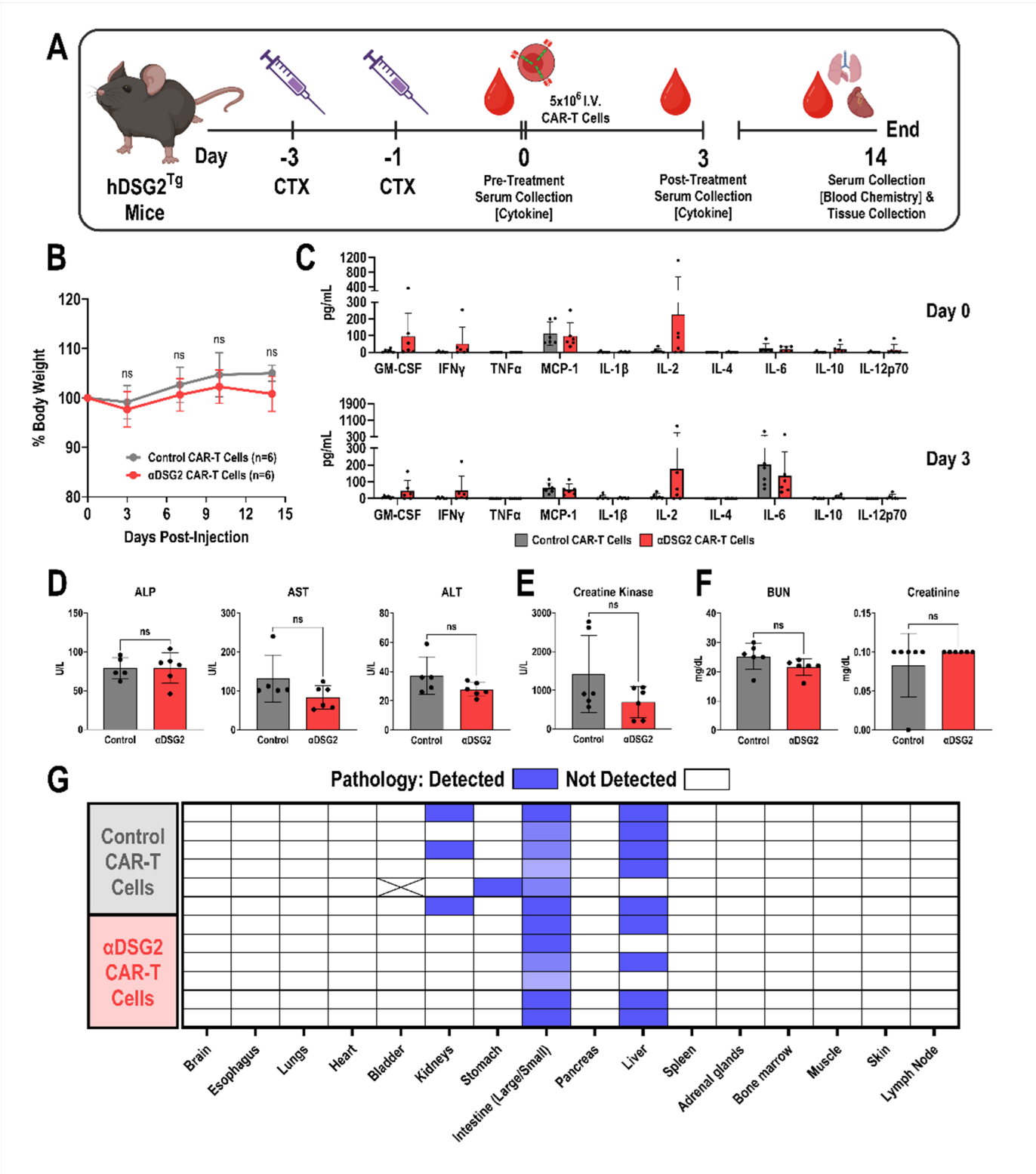


Figure 5

**DSG2-directed CAR-T cells are safe in a human DSG2 transgenic mouse model.** (A) Safety study design. Mice were randomized (n=6/group; 3 male, 3 female) and twice pre-conditioned with cyclophosphamide (CTX; 100mg/kg i.p.). Blood was collected prior to control or αDSG2 CAR-T cell treatment (day 0) for baseline circulating cytokine quantification and comparison to peak cytokine levels 3 days after CAR-T cell administration. At two weeks post-treatment, blood and tissues were collected for organ-toxicity biomarker and histopathology evaluation, respectively. (B) Percent body weight change normalized to day 0 starting body weight prior to CAR-T cell treatment. (C) Serum cytokine levels in peripheral blood pre- (day 0) and post- (day 3) CAR-T cell treatment. (D-F) Serum biomarkers associated with liver (D), heart (E), and kidney (F) pathology in peripheral blood at study end (day 14). ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urine nitrogen. (G) Treatment-blinded histopathology evaluation of organs collected at study end (day 14). Color gradient denotes inflammation score: 1-4. Rows indicate individual animals. All data in **B-F** are presented as means ± SD. Body weight significance (B) determined by two-way ANOVA. Organ-damage biomarker analysis (D-F) was performed by unpaired *t* test with Welch's correction. *P* > 0.05 was considered not statistically significant, ns. See also Supplementary Fig. S8-S9.

## Supplementary Files

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