Kinetics of Tumor Destruction by Chimeric Antigen Receptor-modified T Cells

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The use of chimeric antigen receptor (CAR)–modified T cells as a therapy for hematologic malignancies and solid tumors is becoming more widespread. However, the infusion of a T-cell product targeting a single tumor-associated antigen may lead to target antigen modulation under this selective pressure, with subsequent tumor immune escape. With the purpose of preventing this phenomenon, we have studied the impact of simultaneously targeting two distinct antigens present on tumor cells: namely mucin 1 and prostate stem cell antigen, both of which are expressed in a variety of solid tumors, including pancreatic and prostate cancer. When used individually, CAR T cells directed against either tumor antigen were able to kill target-expressing cancer cells, but tumor heterogeneity led to immune escape. As a combination therapy, we demonstrate superior antitumor effects using both CARs simultaneously, but this was nevertheless insufficient to achieve a complete response. To understand the mechanism of escape, we studied the kinetics of T-cell killing and found that the magnitude of tumor destruction depended not only on the presence of target antigens but also on the intensity of expression—a feature that could be altered by administering epigenetic modulators that upregulated target expression and enhanced CAR T-cell potency.

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INTRODUCTION

T cells modified to express tumor-directed chimeric antigen receptors (CARs) have shown clinical efficacy in treating both hematological malignancies and solid tumors.[1–6] It is likely, however, that the most effective use of CAR-modified T cells will require additional engineering to enable them to overcome tumor immune escape mechanisms. One of the most important of these escape strategies is target antigen modulation under selective pressure.[7] This phenomenon has been reported as a cause of failure in both preclinical and clinical studies using adoptively transferred T cells with single antigen specificity to treat heterogeneous tumors,[7–9] and thus must be taken into consideration when designing CAR T-cell–based therapies.

To address this problem, we generated two CARs targeting the tumor-associated antigens (TAAs), mucin 1 (MUC1), and prostate stem cell antigen (PSCA), both of which are expressed on ~60% of human primary pancreatic tumor cells.[10–12] We then investigated, in a pancreatic tumor model, whether immune escape could be prevented by coadministering CAR T cells targeting two antigens present on the tumor cells.[13,14] As expected, when tested individually, selective pressure resulted in the emergence of a tumor subpopulation that lacked or had downregulated the target antigen, rendering the tumor insensitive to subsequent T-cell retreatment. However, we found that the coadministration of CAR T cells simultaneously targeting both TAAs was associated with a superior antitumor effect, which was nevertheless insufficient to produce complete tumor eradication.

To better understand this therapy failure, we developed an engineered tumor model where each tumor cell was modified to express a target antigen but at different levels of intensity, which could be tracked using a fluorescent marker. Using this platform, we discovered that (i) the magnitude of tumor cell killing mediated by CAR T cells was directly proportional to the frequency of target antigen-expressing tumor cells present, (ii) the kinetics of killing correlated with the intensity of antigen expression, and (iii) "low" antigen-expressing tumor cells were able to escape CAR-mediated killing, a phenomenon that could be ameliorated by combining CAR T cells with epigenetic modifiers.

RESULTS

T cells engineered to express CAR-PSCA can kill antigen-expressing targets

To target tumors expressing the TAA PSCA, we generated a retroviral vector encoding a humanized, codon-optimized CAR directed against PSCA. **Figure 1a** shows a graphical representation of the retroviral vector map. This transgenic molecule could be stably expressed on the surface of primary T cells with a mean of 89.9 ± 9% transduction efficiency as illustrated in **Figure 1b**. CAR-PSCA–modified T cells were phenotypically similar to nontransduced (NT) T cells (**Figure 1c**). Indeed, both populations (NT versus CAR-PSCA) were composed predominantly of $CD3^+$ T cells (95.2 ± 5.7 and 95.2 ± 3.5%), with a mixture of $CD4^+$ (19.2 ± 12.0 and 12.8 ± 6.3%) and $CD8^+$ (76.1 ± 15.5 and

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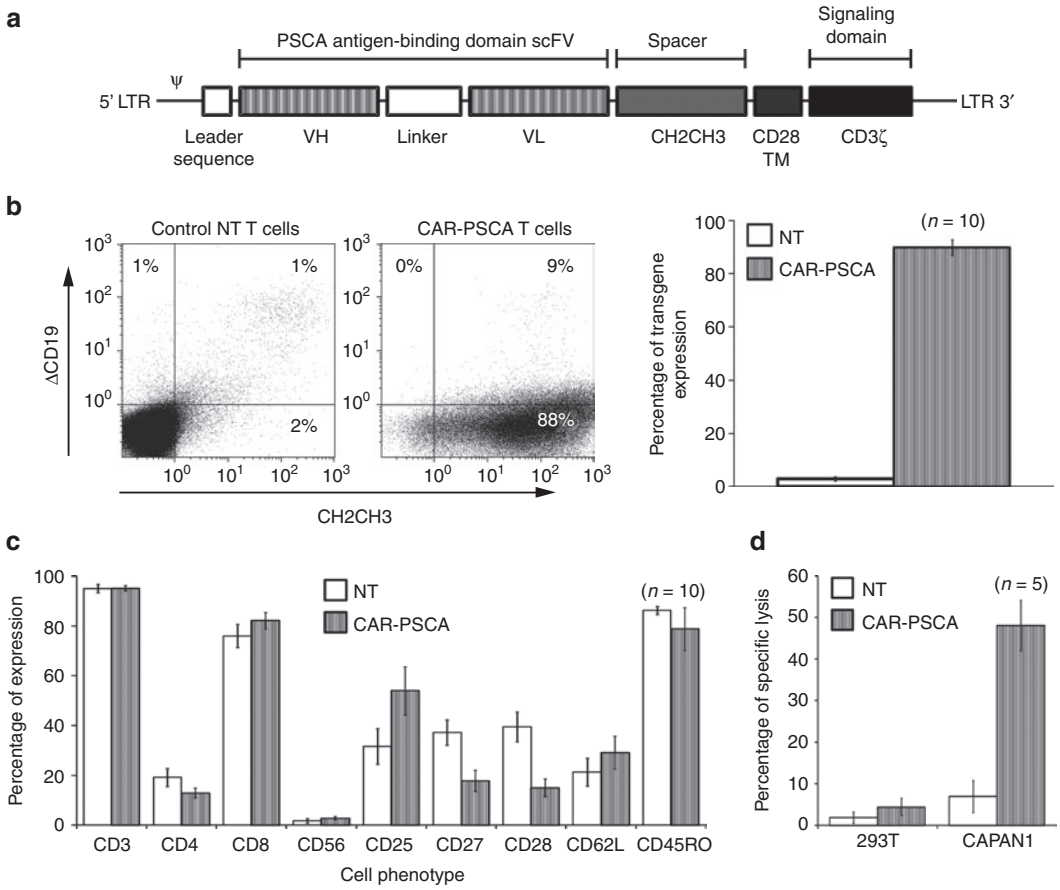

Figure 1 T cells can be engineered to recognize and kill pancreatic cancer cells expressing PSCA. (**a**) Retroviral vector map of the first-generation humanized, codon-optimized CAR-PSCA. (**b**) Shows the transduction efficiency of CAR-PSCA on primary T cells by detecting the CH2CH3 domain. (**c**) Shows the phenotype of NT and CAR-PSCA T cells, $n = 10$. (**d**) Shows the cytolytic ability of NT and CAR-PSCA T cells in a 6-hour chromium release assay (at a E:T of 10:1) using as a target the PSCA$^+$ tumor cell line CAPAN1 ($n = 5$). CAR, chimeric antigen receptor; LTR, long terminal repeat; scFv, single-chain variable fragment; VH, variable heavy; VL, variable light.

82.2 ± 10.5%) populations, and the same proportion of CD3$^+$ T cells in both NT and transduced populations expressed the central memory markers CD62L, CD27, and CD45RO. CAR-modified T cells were able to kill PSCA$^+$ pancreatic cancer cells CAPAN1 (48 ± 6% specific lysis at 10:1 E:T ratio), but not PSCA-negative 293T targets, and NT T cells produced only background levels of lysis (7 ± 4 and 4 ± 1% specific lysis of CAPAN1 and 293T cells, respectively) (**Figure 1d**).

Targeting a heterogeneous tumor using a monospecific CAR T-cell product selects immune escape variants

To determine whether CAR-PSCA T-cell treatment could produce tumor elimination *in vivo*, we engrafted severe combined immunodeficiency mice with 1×10^6 CAPAN1, which naturally express PSCA and were modified to express enhanced green fluorescence protein (eGFP)-Firefly luciferase (CAPAN1-eGFP-FFLuc) to allow for *in vivo* tumor detection. Once the tumor was established, mice received a single infusion of either NT or CAR-PSCA T cells (30×10^6 cells). As shown in **Figure 2a**, CAR-PSCA T-cell treatment resulted in an initial antitumor response (day 28 posttreatment), which was followed by rapid tumor progression such that by days 42 and 56 posttreatment, the tumor signal was similar in magnitude between the two groups (**Figure 2a**, right panel).

We modeled this phenomenon of immune escape *in vitro* by coculturing CAPAN1-eGFP-FFLuc cells with NT or CAR-PSCA T cells at a 1:5 ratio. After 72 hours, residual tumor cells were quantified by flow cytometry, gating on GFP$^+$ cells, whereas T cells were excluded by costaining with a CD3-directed antibody. Similar to our *in vivo* findings, we observed that while coculture of NT T cells with the pancreatic cancer cells CAPAN1 had no impact on tumor cell growth, CAR-PSCA T-cell treatment resulted in an initial antitumor response, reflected by a 82 ± 9% reduction in tumor cell numbers (**Figure 2b**). However, the tumor population that survived initial CAR T-cell exposure was resistant to subsequent retreatment, as shown in **Figure 2c**. To determine the mechanism of resistance, we performed immunohistochemical analysis on tumor cells that had received a single treatment with either NT or CAR-PSCA T cells. As shown in **Figure 2d**, while CAR-PSCA T cells eliminated CAPAN1 cells expressing high levels of PSCA antigen, a residual PSCA low/negative subpopulation remained, which subsequently outgrew. These residual tumor cells did, however, continue to express a second untargeted TAA, MUC1 (**Figure 2d**).

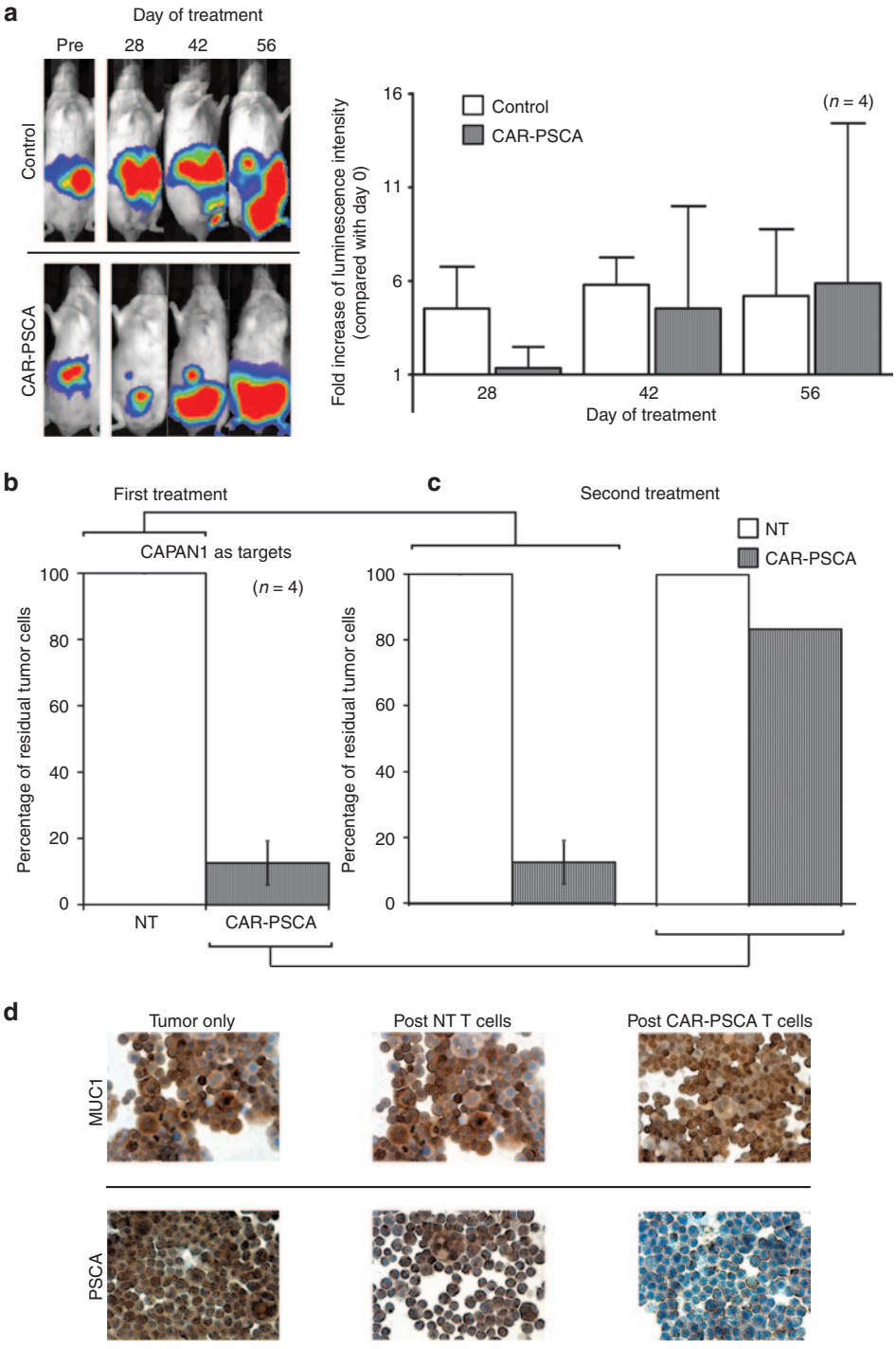

Figure 2 Targeting a heterogeneous tumor with monospecific CAR T cells leads to tumor immune escape. (**a**) Shows the bioluminescence signal from severe combined immunodeficiency mice engrafted with CAPAN1-eGFP-FFLuc at different time points after treatment with CAR-PSCA or NT T cells (data are plotted as fold change of luminescence intensity compared with day 0). (**b**) Shows the percentage of residual tumor cells after a 72-hour coculture with a 5:1 E:T ratio, using NT T cells or CAR-PSCA T cells and CAPAN1 tumor cells ($n = 4$). (**c**) Shows the antitumor effect of NT and CAR-PSCA T cells on tumor cells that were resistant to an initial T-cell treatment ($n = 4$). (**d**) Shows PSCA and MUC1 antigen expression in untreated CAPAN1 tumor cells and in tumor cells following treatment with NT or CAR-PSCA T cells. FFLuc, Firefly luciferase; eGFP, enhanced green fluorescence protein; NT, nontransduced; PSCA, prostate stem cell antigen.

Tumor immune escape occurs even using T cells modified to express a second- or third- generation CAR

It is possible that more rapid or complete killing of cells expressing low levels of target antigen might prevent emergence of tumor immune escape variants. Recent reports have shown that T cells modified to express CARs containing costimulatory endodomains (second- and third-generation CARs) have increased proliferation, cytokine production, and prolonged *in vivo* persistence.[15,16]

To discover whether tumor immune escape could also be prevented using a later generation CAR construct, we made a CAR targeting PSCA that incorporated CD28 (second generation) or CD28 and 41BB (third generation) costimulatory endodomains. **Supplementary Figure 1a** shows the first-, second-, and third-generation CAR-PSCA retroviral vector maps, and **Supplementary Figure 1b** shows the expression of each in transduced primary T cells. T cells expressing both second- and third-generation CAR constructs proliferated more than the first-generation construct when cultured with K562 cells modified to express PSCA antigen (**Supplementary Figure 1c**), but all had equivalent cytolytic activity against pancreatic cancer cells CAPAN1 in both short-term (6-hour) ^{51}Cr release (**Supplementary Figure 1d**) and long-term (72-hour) coculture assays (**Supplementary Figure 1e**), leaving behind the same residual resistant tumor subpopulation.

T cells modified with a CAR targeting MUC1 specifically kill antigen-expressing targets, but tumor heterogeneity leads to tumor immune escape

Since heterogeneity of target antigen expression is an evident cause of tumor immune resistance and escape, we next determined whether concomitant targeting of a second TAA, MUC1, would overcome this problem. We generated a retroviral vector encoding a CAR directed against MUC1, with a truncated CD19 molecule (ΔCD19) as a marker.[17] **Figure 3a** shows a representation of the retroviral vector map. Transduction efficiency of CAR-MUC1 on T cells was determined by detection of the CH2CH3 domain (CAR-MUC1) and ΔCD19, which demonstrated a mean efficacy of 83.1% (±11.5%) **Figure 3b**. The T-cell phenotype was unaffected by the transduction with CAR-MUC1 (**Figure 3c**) and were composed predominantly of $CD3^+$ T cells (95.2 ± 5.7%), with $CD4^+$ (19.2 ± 12.0%) and $CD8^+$ (76.1 ± 15.5%) subpopulations, a subset of which expressed the central memory markers CD62L, CD27, and CD45RO. Transgenic CAR-Muc1 T cells were able to specifically kill CAPAN1 cells, which naturally express MUC1 antigen (35 ± 5% specific lysis at 10:1 E:T ratio), and had no activity against 293T cells, which are MUC1-negative targets (**Figure 3d**).

To determine whether CAR-MUC1 T-cell monotherapy would also lead to tumor immune escape, we engrafted severe combined immunodeficiency mice with the CAPAN1-eGFP-FFLuc cells and treated them with either NT or CAR-MUC1 T cells. Treatment with CAR-MUC1 T cells produced an initial antitumor response, measurable by a decrease in the tumor signal at day 28. However, this was followed by rapid tumor progression (**Figure 4a**). To confirm that this escape was due to tumor

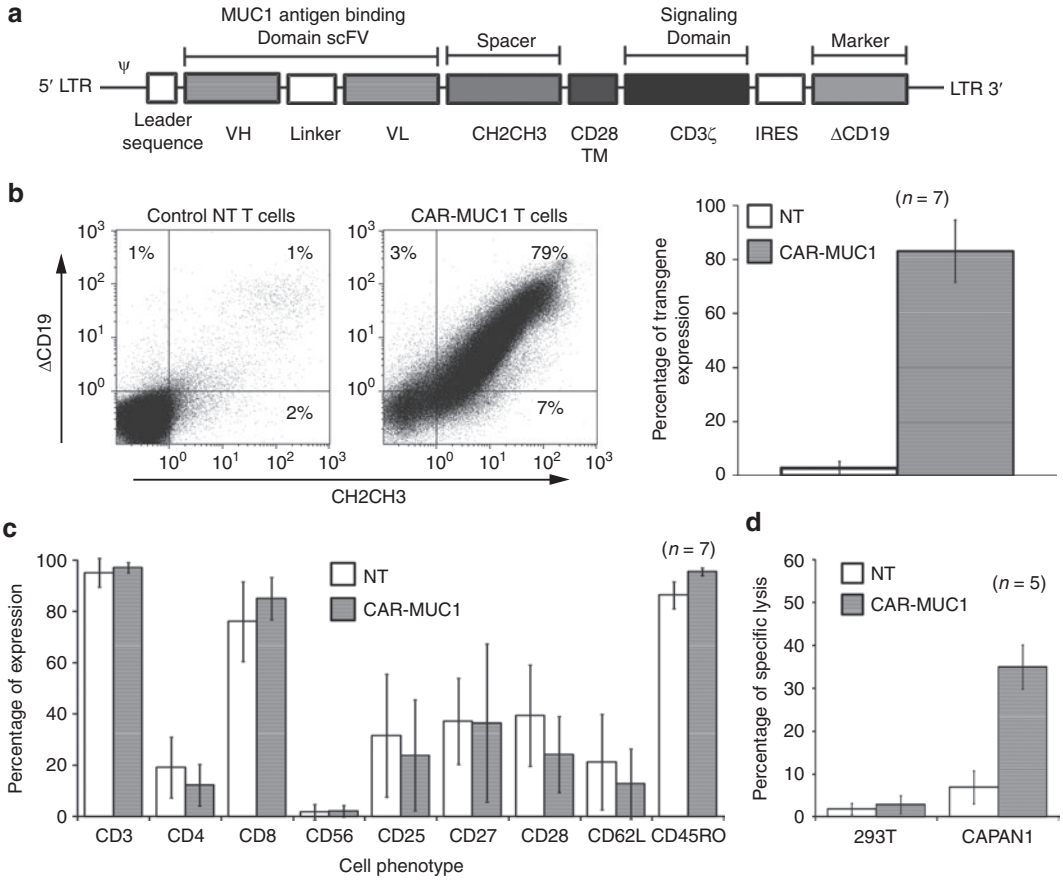

Figure 3 CAR T cells modified to recognize MUC1 kill $MUC1^+$ tumor cells. (**a**) Shows a retroviral vector map of a first-generation MUC1-specific CAR coexpressing a truncated form of CD19 (ΔCD19). (**b**) Shows the transduction efficiency of CAR-MUC1 on primary T cells—double-positive CH2CH3 and ΔCD19 cells (left panel) and summary data for seven donors (right panel). (**c**) Shows the T-cell phenotype of NT and CAR-MUC1 T cells ($n = 7$). (**d**) Shows the cytolytic activity of NT and CAR-MUC1 T cells in a 6-hour chromium release assay (at a E:T of 10:1) using as targets the $MUC1^+$ tumor cell line CAPAN1.

antigen modulation, we cocultured NT or CAR-MUC1 T cells with CAPAN1-eGFP-FFLuc cells for 72 hours, and while CAR-MUC1 T-cell treatment resulted in an initial reduction of tumor cells (66 ± 21%) (**Figure 4b**), residual tumor cells remained insensitive to retreatment (**Figure 4c**), likely due to decreased antigen expression, as confirmed by immunohistochemistry (**Figure 4d**). These residual tumor cells did, however, continue to express the untargeted PSCA antigen.

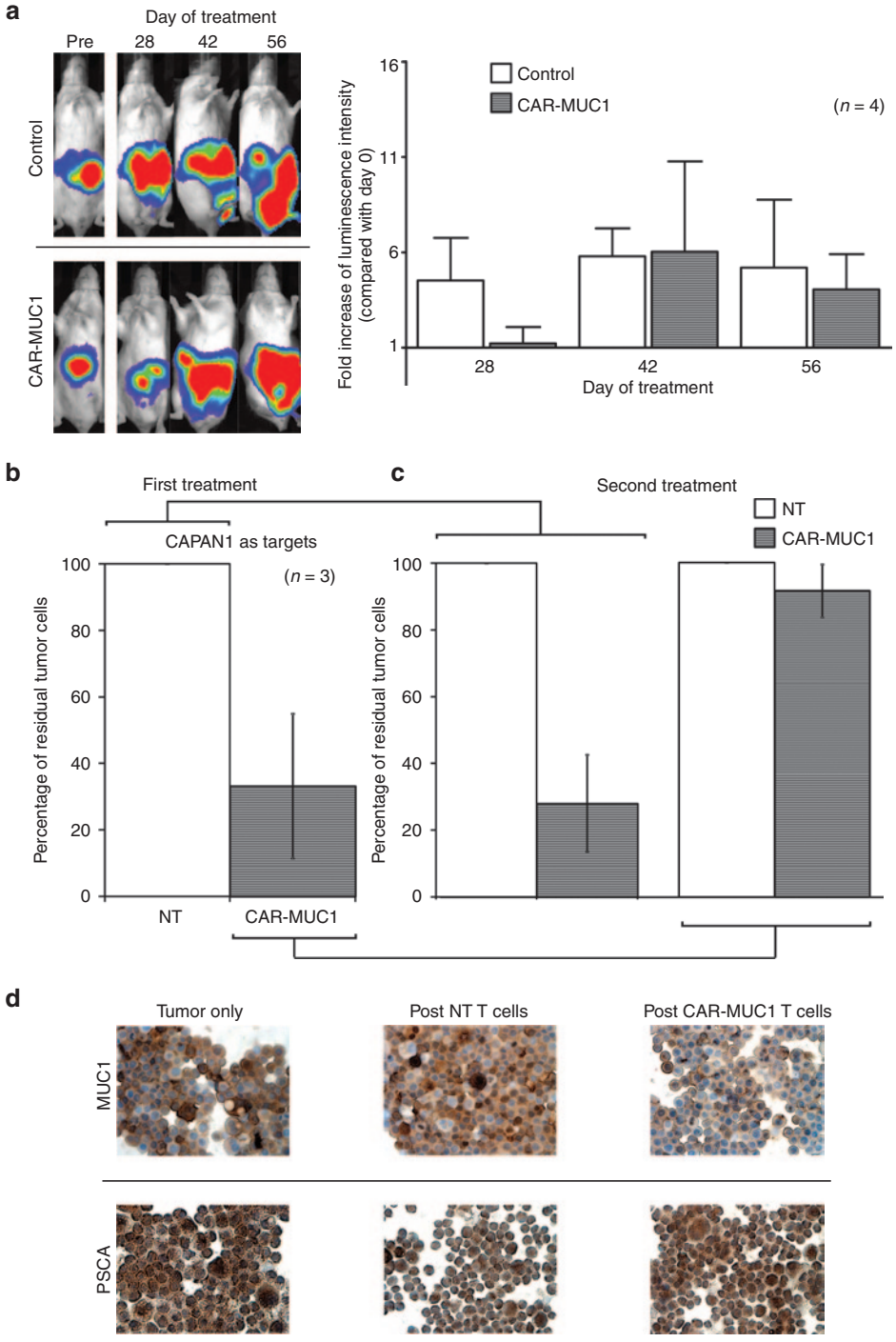

Figure 4 Targeting a heterogeneous tumor with MUC1–specific CAR T cells also leads to tumor immune escape. (**a**) (left) shows the bioluminescence signal from severe combined immunodeficiency mice engrafted with CAPAN1-eGFP-FFLuc at different time points after treatment with CAR-MUC1 or NT T cells (data are plotted as fold change of luminescence intensity compared with day 0 (right). (**b**) Shows the percentage of residual CAPAN1 tumor cells after a 72-hour coculture experiment at a 5:1 E:T ratio with NT or CAR-MUC1 T cells ($n = 4$). (**c**) Shows the antitumor effect of NT and CAR-MUC1 T cells on tumor cells that were resistant to an initial T-cell treatment ($n = 4$). (**d**) Shows PSCA and MUC1 antigen expression on untreated CAPAN1 tumor cells and in tumor cells following treatment with NT or CAR-MUC1 T cells. FFLuc, Firefly luciferase; eGFP, enhanced green fluorescence protein.

Combination of CAR T cells targeting two TAAs produces superior antitumor activity

To determine whether dual-targeted CAR therapy would produce superior antitumor effects, we cultured the pancreatic cancer cell line CAPAN1, which naturally expresses both PSCA and MUC1, with both CAR-PSCA and CAR-MUC1 T cells simultaneously. In a short-term (6-hour) cytotoxicity assay, combination therapy produced superior tumor cell killing (75 ± 8% specific lysis, E:T 10:1) compared with single antigen-specific T cells (35 ± 5% and 48 ± 6% specific lysis, CAR-MUC1 and CAR-PSCA, respectively) (**Figure 5a**). Similar results were obtained after a 3-day coculture, in which treatment with CAR-MUC1 T cells alone reduced tumor cells by 65 ± 13%, treatment with CAR-PSCA T cells alone eliminated 82.1 ± 9% of tumor cells, whereas dual-targeted therapy was superior, resulting in a 96.6 ± 1% reduction (**Figure 5b**).

To address whether dual CAR-targeted therapy could result in tumor elimination, we engrafted severe combined immunodeficiency mice with CAPAN1-eGFP-FFluc cells. As shown in **Figure 5c**,**d**, treatment with a combination of CAR-MUC1 and CAR-PSCA T cells produced superior antitumor effects compared with either tested individually. However, this effect was not sustained, and by day 63, the tumor recurred in all animals. Thus, dual-targeted therapy is also insufficient to eliminate all cancer cells.

Generation of an artificial system to model tumor immune escape

To better understand the mechanism behind this therapy failure, we developed an engineered tumor model by transgenically expressing either MUC1 or PSCA TAAs in 293T cells. Thus, we generated two retroviral vectors: the first encoding the MUC1 antigen and coexpressing the fluorescent tag mOrange and the second encoding the PSCA antigen and coexpressing GFP. The intensity of the fluorescent tag correlated with the intensity of antigen expression, as shown in **Supplementary Figure 2** for MUC1, thus allowing us to monitor, in real time, the antitumor activity of our CAR T cells. **Figure 6a** shows the retroviral vector maps, and **Figure 6b** shows the expression of MUC1/mOrange and PSCA/GFP in 293T cells. To ensure that each tumor cell expressed a target antigen, we subsequently sorted these cells to achieve pure populations that were either 100% MUC1 expressing (mOrange$^+$) or PSCA expressing (GFP$^+$).

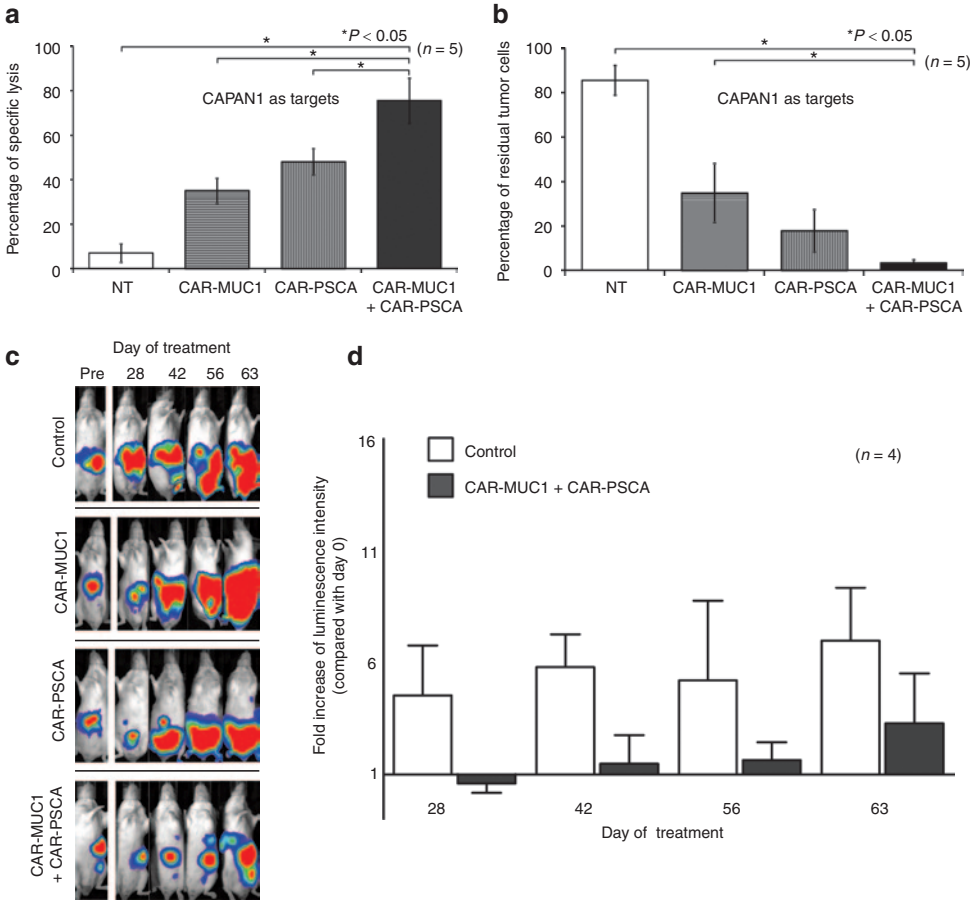

Figure 5 Targeting tumors using a dual CAR approach produces superior antitumor activity. (**a**) Shows the cytolytic effect of NT, CAR-MUC1, CAR-PSCA, or the combination in a 6-hour Cr^{51} release assay using CAPAN1 as a target (10:1 E:T ratio; $n = 5$). *indicates a P value <0.05 using Student's t-test. **(b)** Shows the number of residual CAPAN1 tumor cells after a 72-hour coculture experiment using the same panel of effectors and targets (E:T of 5:1; $n = 5$). **(c)** Shows bioluminescence images of representative mice engrafted with CAPAN-eGFP-FFluc and treated with NT, CAR-MUC1, CAR-PSCA, or the combination, whereas (**d**) shows summary results for the NT and dual-targeted groups ($n = 4$) at pre or 28, 42, 56, or 63 days posttreatment. Data are plotted as fold change of luminescence intensity compared with day 0. FFLuc, Firefly luciferase; eGFP, enhanced green fluorescence protein; MUC1, mucin 1; PSCA, prostate stem cell antigen.

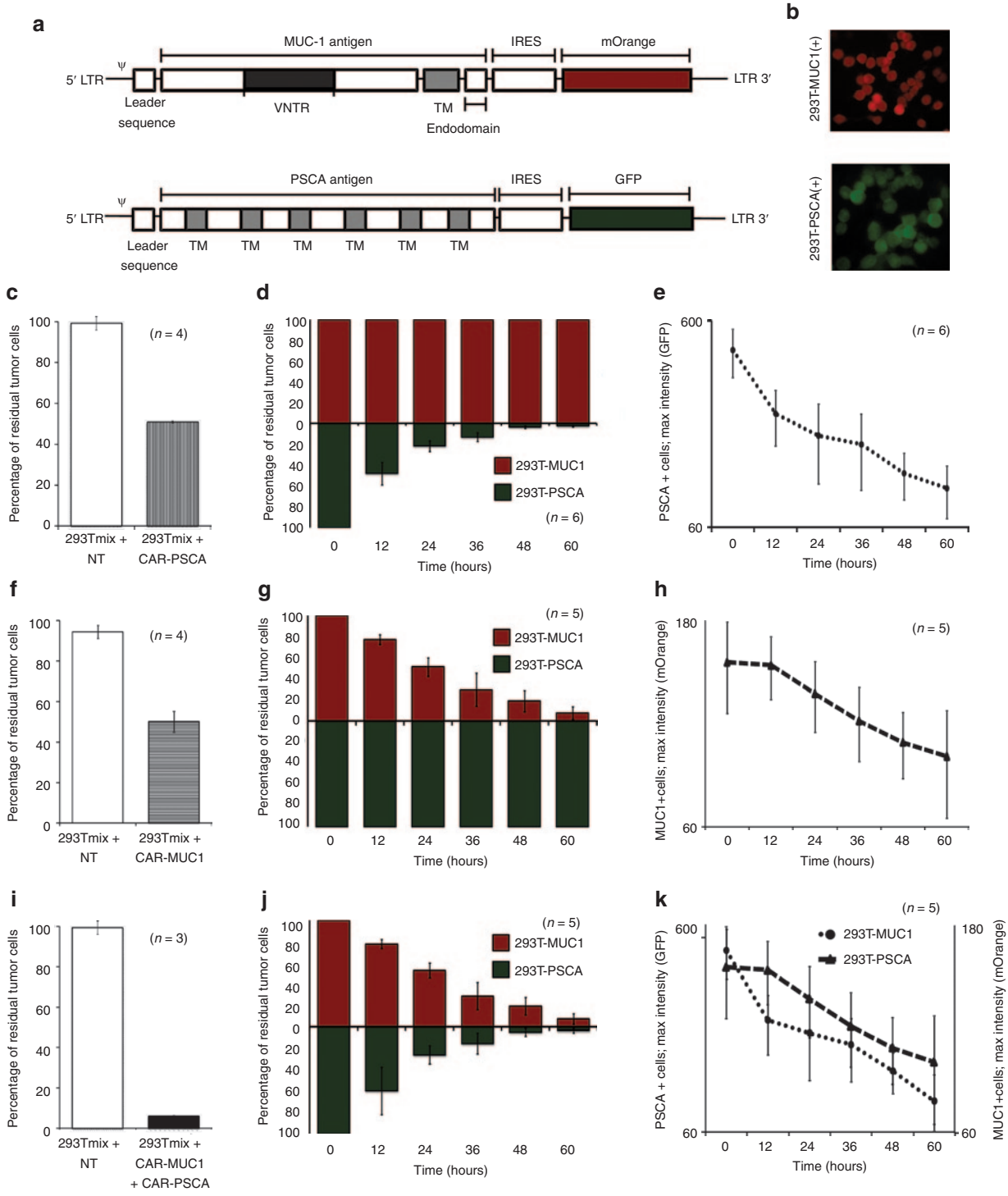

Figure 6 Characterizing the tumor immune escape phenomenon using an artificial tumor model. (**a**) Shows two retroviral vector maps, the first encoding the TAA MUC1, which has a variable number of proline-rich segments that are tandemly repeated (variable number tandem repeat (VNTR))[37] and coexpressing mOrange. The second encodes the TAA PSCA, which contains six transmembrane portions,[38] and coexpressing GFP. **(b)** Shows the mOrange and GFP expression on selected transduced 293T cells. **(c)** Shows residual 293T cells (1:1 mixture of MUC1- and PSCA-expressing cells) 72 hours after treatment with NT or CAR-PSCA T cells at an E:T 10:1 (n = 6). **(d)** Shows the proportion of residual GFP^+ and $mOrange^+$ tumor cells after CAR-PSCA T-cell treatment. **(e)** Shows the intensity of GFP, which serves as a surrogate for PSCA antigen expression, (**f**) shows residual 293T cells (1:1 mixture of MUC1- and PSCA-expressing cells) 72 hours after treatment with NT or CAR-MUC1 T cells at a E:T 10:1 (n = 5). **(g)** Shows the proportion of residual GFP^+ and $mOrange^+$ tumor cells after treatment with CAR-Muc1, (**h**) shows the intensity of mOrange as a surrogate for MUC1 antigen expression, (**i**) shows residual 293T cells (1:1 mixture of MUC1- and PSCA-expressing cells) 72 hours after treatment with NT or or the combination of CAR-MUC1 and CAR-PSCA T cells (n = 5), (**j**) shows the proportion of residual GFP^+ and $mOrange^+$ tumor cells after treatment with the combination of CAR-MUC1 and CAR-PSCA T cells, and (**k**) shows the intensity of GFP and mOrange as surrogates for PSCA and MUC1 antigen expression (n = 5). CAR, chimeric antigen receptor; IRES, internal ribosome entry site; LTR, long terminal repeat; MUC1, mucin 1; TM, transmembrane.

To mimic a heterogeneous tumor population, the sorted cells were mixed at a 1:1 ratio and initially treated with either NT or CAR-PSCA T cells for 72 hours. As expected, NT T-cell treatment had no impact on residual tumor cell numbers, whereas treatment with CAR-PSCA T cells alone reduced the number of engineered tumor cells by 50.9 ± 1% (**Figure 6c**), reflecting a selective reduction in the GFP$^+$ (PSCA expressing) population (98.1 ± 1%). To examine the kinetics of CAR T-cell killing we quantified, by flow cytometry, tumor cell numbers over time (0, 12, 24, 36, 48, and 60 hours). As shown in **Figure 6d**, there was a progressive decrease in the number of GFP$^+$ tumor cells, which was most pronounced within the first 24 hours post-treatment (45.4 ± 11% reduction between 0 and 12 hours and 29.7 ± 4% reduction between 12 and 24 hours), and less marked thereafter (10.7 ± 4% from 24 to 36 hours, 10.2 ± 1% from 36 to 48 hours, and 1 ± 0.9% from 48 to 60 hours). Nevertheless, a residual PSCA-expressing subpopulation (1.9%) remained. To next investigate whether sensitivity to CAR-mediated killing was linked to the intensity of target antigen expression on tumor cells, we also measured the fluorescence intensity of GFP at the same time points. As shown in **Figure 6e**, tumor cells expressing the highest antigen levels were killed first, whereas those with lowest expression survived.

We repeated these studies but substituting CAR-MUC1 T cells as the effector population. After 72 hours of treatment with CAR-MUC1 T cells, the total number of tumor cells had decreased by 49.9% (±5%) (**Figure 6f**), representing a selective and gradual reduction in MUC1/mOrange$^+$ tumor cells over time (**Figure 6g**), until only MUC1 "low" tumor cells remained (**Figure 6h**), whereas the PSCA$^+$ population was unaffected.

Next, to determine whether dual-targeted CAR therapy would produce superior antitumor effects, we cocultured the heterogeneous tumor cell population (1:1 mix of MUC1/mOrange$^+$ and PSCA/GFP$^+$ 293T cells) with both CAR-MUC1 and CAR-PSCA T cells. As expected, the combination of these two effector T cells products resulted in superior antitumor effects. However, even this combination approach failed to eradicate all tumor cells, with 6.0 ± 3% targeted cells remaining (3.9 ± 2% residual MUC1/mOrange$^+$ and 1.9 ± 1% residual PSCA/GFP$^+$ cells) (**Figure 6i**). Again, these residual subpopulations reflected cells with the lowest intensity of target antigen expression, judged by mOrange and GFP fluorescence (**Figure 6j**,**k**). Thus, sensitivity to CAR T-cell treatment is related to both the proportion of cells expressing the targeted antigen and the intensity at which the antigen is expressed.

Finally, to determine whether CAR T-cell potency could be improved by combination with conventional epigenetic modulators, which can increase TAA expression by demethylating DNA, we cultured CAPAN1 cells that had been previously treated with CAR-MUC1 T cells and consequently expressed only low levels of the target antigen with 1 μmol/l of decitabine, a hypomethylating agent. As expected, decitabine exposure resulted in an increase in the intensity of MUC1 expression from 3.5 to 26.0 (relative mean fluorescence intensity) after 4 days of treatment (**Supplementary Figure 3a,b**), resensitizing previously resistant tumor cells to CAR-MUC1 T-cell killing (**Supplementary Figure 3c**).

DISCUSSION

CAR-targeted therapy is rapidly gathering momentum as a cancer treatment, but early clinical reports indicate that the use of monospecific T cells may be associated with the outgrowth of tumor cells that lack or have downregulated the targeted antigen.[7–9] Therefore, using pancreatic cancer as a model, we evaluated whether simultaneously targeting two TAAs frequently coexpressed by tumor cells could prevent such an occurrence. We found that while CAR T cells with single tumor specificity (MUC1 or PSCA) produced antitumor effects *in vitro* and *in vivo*, heterogeneous target antigen expression allowed the outgrowth of antigen loss variants, thus mirroring the clinical scenario. To our surprise, however, even simultaneous treatment with both CAR-MUC1 and CAR-PSCA T cells was insufficient to produce tumor elimination. To uncover the mechanism behind this tumor resistance, we developed an artificial tumor model, which allowed us to determine that tumor sensitivity to CAR therapy is associated not only with the proportion of cells expressing the targeted antigen(s) but also the intensity at which these antigen(s) are expressed.

A number of clinical studies have now demonstrated that CAR-modified T cells directed to TAAs can effectively traffic to distant tumor sites, expand and persist *in vivo*, penetrate even bulky disease, and treat disseminated tumors. For example, T cells modified with a GD2-specific CAR given to children with advanced neuroblastoma produced 5 responses in 11 children with active relapsed disease, including 3 complete remissions.[3,18] Similarly, T cells engrafted with a CAR targeting the CD19 molecule on B-cell lymphomas have produced rapid and sustained responses in three of nine patients with chronic lymphocytic leukemia and in one of two patients with acute lymphoblastic leukemia.[1,9,19,20] Thus, while CAR technology has clearly been associated with clinical efficacy, tumor heterogeneity may play a role in those who fail to eliminate their tumor following the administration of a monospecific product or who subsequently relapse. This phenomenon has been previously reported in studies using T cells whose native receptors are tumor specific. For example, Riker *et al.*[8] found that administration of T cells specific for the melanoma-associated peptide gp100 was followed by the outgrowth of tumors lacking the target antigen, and similar effects have been observed when EBV-associated malignancies are treated with cytotoxic T lymphocytes of restricted specificity[21] (Bollard *et al.*, personal communication). Similarly, Grupp *et al.* recently reported a clinical relapse of a CD19-negative tumor in an individual initially infused with CD19-directed CAR T cells for treatment of acute lymphoblastic leukemia.[9] To prevent such an eventuality, investigators have therefore explored whether simultaneously targeting multiple TAAs expressed by hematological malignancies may be superior. In preclinical models Mihara *et al.*[13] showed that additive cytotoxic effects could be achieved *in vitro* by combining CAR T cells targeting both CD19 and CD38. Our current study extends this approach to a solid (pancreatic cancer) tumor model and uses MUC1- and PSCA-directed T cells to model the phenomenon of tumor immune escape due to antigen heterogeneity.

As expected, the combination of CAR products produced superior antitumor effects compared with either alone. However, we consistently observed small numbers of residual tumor cells that appeared to express the targeted antigens (as detected by

immunohistochemistry) but were resistant to repetitive T-cell treatment, likely because the intensity of antigen expression also determines tumor susceptibility to CAR T cells. To model this effect, we generated an artificial tumor model in which expression of both target antigens was linked, by an internal ribosome entry site sequence, with distinct fluorescent tags. Thus, fluorescence intensity became a surrogate for target antigen expression, and we observed a linear correlation between antigen expression and sensitivity to T-cell treatment. This effect is a potential obstacle that may be surmounted by combining CAR T-cell therapy with epigenetic modulators, such as azacitidine, which can increase TAA expression by demethylating DNA.[22] Indeed, Goodyear *et al.*[23] recently demonstrated that administration of demethylating agents to patients with acute myeloid leukemia or myelodysplasia resulted in an increase in the frequency of endogenous T cells targeting a range of tumor-expressed antigens in 10 of 21 patients, likely due to the upregulation of these antigens in tumor cells. Remarkably, this correlated with major clinical responses in eight patients. Indeed, we have seen similar effects *in vitro* where the treatment of CAR-MUC1–resistant tumor cells with the demethylating agent decitabine resulted in MUC1 antigen upregulation, thus resensitizing tumor cells to repeat CAR-MUC1 T-cell therapy (**Supplementary Figure 3**). This finding suggests that the administration of epigenetic modulators could improve the therapeutic potency of CAR T cells by increasing the intensity of antigen expression on target cells, thereby preventing immune escape of tumor cells expressing levels of antigen below the threshold required for T-cell recognition.

Although in the current study we have used an immunodeficient mouse model to assess the potency of our CAR T cells, it is possible that these effector T cells, once transferred to an immunocompetent host, will have a positive bystander effect on the endogenous immune system by (i) producing proinflammatory cytokines, (ii) recruiting additional immune cells such as natural killer cells and antigen-presenting cells to the tumor site, and (iii) inducing epitope spreading. Thus, adoptive transfer of these CAR T cells has the potential to trigger a cascade of events *in vivo* that could amplify the antitumor activity. Evidence of such immune-mediated bystander effects has been previously reported by Brossart *et al.* who demonstrated that cancer patients vaccinated with either HER-2/neu or MUC1 peptides developed cytotoxic T lymphocyte against additional TAAs.[24] Thus, even in the absence of epigenetic therapy, dual-targeted CAR T cells may suffice to reactivate a potent endogenous tumor-targeted immune response that will produce tumor elimination; however, this remains to be tested clinically.

CAR T-cell therapy may be associated with "on-target but off-organ" adverse effects since the targeted antigens, in most instances, are not exclusively expressed on the tumor. For example, when Lamers *et al.* [25] treated patients with renal cell carcinoma using CAR T cells targeting carbonic anhydrase, they observed severe cholestasis, likely due to the recognition of normal biliary epithelial cells expressing the target antigen. Similarly, a fatal outcome in a patient following the infusion of Her2-targeted T cells was attributed to targeting of Her2-positive pulmonary endothelium.[26] Thus, dual-targeted CAR therapy may reduce the risk of immune escape, but correspondingly increase the risk of "off-tumor" toxicity, even when target antigens with a restricted expression profile are used. Efforts to reduce these risks have included the development of CAR receptors that require complementation by additional antigens present on the tumor ("AND" operator) or that are inhibited when they encounter both the TAA and an antigen present only on normal tissues ("NOT" operator).[27] The feasibility of this approach has recently been demonstrated. For example, Wilkie *et al.*[28] modified T cells with two CARs targeting the breast cancer–expressed TAAs Her2 (coupled with the CD3ζ endodomain) and MUC1 (coupled to CD28) and showed that complementary signals were produced only in the presence of tumor cells expressing both target antigens, leading to proliferation and potent cytotoxicity. The limitation of this approach is that it negates the major benefit of dual targeting, namely the ability to recognize and kill even those tumor subsets that have lost one of the targeted antigens. Although these possibilities are yet to be formally tested, other investigators have developed and clinically validated suicide or safety systems that can be triggered by small molecule drugs in the event of severe adverse effects, and these may mitigate the risks associated with dual receptors while retaining their potential benefits.[29,30]

In conclusion, our data demonstrate that while individually CAR-MUC1 and CAR-PSCA T cells have specific antitumor activity, this can be enhanced by cotargeting two tumor-expressed antigens. Even a dual-targeted approach such as this could lead to therapy failure due to the emergence of a "low" antigen-expressing tumor cell subpopulations that are resistant to CAR T cells, a phenomenon which could be overcome by the combination with epigenetic modulators. Although this preliminary observation supports the combination of these two therapies, additional preclinical and clinical studies will be required to confirm this finding.

MATERIALS AND METHODS

Donor and cell lines. The pancreatic cancer cell line, CAPAN1, which naturally expresses PSCA and MUC1, and the human embryonic kidney cell line, 293T, were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in complete Iscove's modified Dulbecco's medium (IMDM) media (Gibco by Life Technologies Corporation, Grand Island, NY), 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) and 2 mmol/ L-glutaMAX (Gibco by Life Technologies Corporation) and a humidified atmosphere containing 5% carbon dioxide (CO_2) at 37 °C. Peripheral blood mononuclear cells from healthy volunteers were obtained with informed consent on protocols approved by the Baylor College of Medicine Institutional Review Board.

OKT3/CD28 blast generation. Peripheral blood mononuclear cells obtained from healthy donors were activated with OKT3 (1 mg/ml) (Ortho Biotech, Bridgewater, NJ) and CD28 antibodies (1 mg/ml) (Becton Dickinson, Mountain View, CA), plated in a nontissue culture-treated 24-well plate at 1×10^6 peripheral blood mononuclear cells per 2 ml in complete media (Roswell Park Memorial Institute (RPMI) 1640; Hyclone Laboratories) containing 45% Clicks medium (Irvine Scientific, Santa Ana, CA), 10% fetal bovine serum (Hyclone Laboratories), and 2 mmol/l L-glutaMAX (Gibco by Life Technologies Corporation), and subsequently split and fed with fresh media plus interleukin-2 (IL-2) (50 U/ml).

Generation of retroviral constructs and retroviral transduction. We synthesized (DNA 2.0, Menlo Park, CA) a codon-optimized single-chain variable fragment (scFV) of MUC1 and a humanized, codon-optimized

scFV of PSCA based on published sequences.[31,32] The scFV fragments were cloned in-frame with the human immunoglobin G1-CH2CH3 domain and with the ζ-chain of the T-cell receptor (TCR)/CD3 complex in the SFG retroviral backbone, to make first-generation CAR-PSCA and CAR-MUC1 retroviral constructs. To distinguish CAR-modified T cells, ΔCD19[17] was incorporated into the CAR-MUC1 retroviral vector using an internal ribosome entry site element. To generate second- and third-generation CAR-PSCA constructs, the CD28 endodomain or CD28 and 41BB costimulatory endodomains were added to the first-generation CAR between the immunoglobin G1-CH2CH3 domain and the TCR/CD3ζ endodomain. We also synthesized (DNA 2.0) the TAAs MUC1 and PSCA, based on published sequences.[33,34] The fluorescent markers mOrange and GFP were incorporated into the MUC1 antigen and the PSCA antigen vectors, respectively, again using an internal ribosome entry site element. Retroviral supernatant was produced as previously described,[31,32] filtered (using a 0.45-mm filter) and stored at −80 °C.

T-cell transduction. For T-cell transduction, CAR-MUC1 or CAR-PSCA retroviral supernatant was plated in a nontissue culture-treated 24-well plate (1 ml/well), which was precoated with a recombinant fibronectin fragment (FN CH-296; Retronectin; Takara Shuzo, Otsu, Japan). OKT3/CD28-activated T cells (0.2×10^6/ml in complete media with IL-2 100 U/ml) were added to the plates (1 ml/well) and then transferred to a 37 °C, 5% CO_2 incubator. CAR T-cell expansion was performed in a G-Rex 100M (Wilson Wolf Manufacturing, New Brighton, MN) with 1 l of complete media supplemented with IL-2 (50 U/ml).[35]

293T transduction. For transduction, MUC1-mOrange or PSCA-GFP viral supernatant was plated in a retronectin precoated 24-well plate (1 ml/well). 0.2×10^5/ml 293T cells were added to the supernatant (1 ml/well), then the cells were spun at 1,000*g* for 30 minutes at room temperature and transferred to a 37 °C, 5% CO_2 incubator. Expression of MUC1-mOrange or PSCA-GFP was measured 72 hours posttransduction by flow cytometry and using a fluorescence microscope to detect the fluorescent markers mOrange and GFP. Cells were maintained or expanded in complete IMDM media every 3–4 days.

Cell sorting. 293T cells were sorted, based on mOrange and GFP expression, using a MoFlo flow cytometer (Cytomation, Fort Collins, CO). Sorted cells were cultured in complete IMDM media supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (25 μg/ml) (Gibco by Life Technologies Corporation) for 1 week in a six-well plate, then further expanded in a T175 flask using complete IMDM media, which was replenished every 3–4 days.

Immunohistochemistry. CAPAN1 cells were stained as described previously[31,32] with either mouse antihuman MUC1 antibody or rabbit antihuman PSCA antibody (AbCam, Cambridge, MA) diluted to 1:200 and 1:80, respectively, in phosphate-buffered saline/1% bovine serum albumin for 1 hour at room temperature and costained with antimouse horseradish peroxidase or antirabbit horseradish peroxidase (AbCam).

Cytotoxicity

Chromium release assay. The cytotoxicity specificity of effector T-cell populations was measured in a standard 6-hour ^{51}Cr release assay as previously described,[31,32] using E:T ratios ranging from 40:1 to 5:1 and using CAPAN1 and 293T cells as targets.

Coculture experiment. CAPAN1, 293T, 293T-MUC1-mOrange, or 293T-PSCA-GFP were used as targets. In brief, GFP/CAPAN1 cells were mixed with either OKT3/CD28 blasts or CAR-modified T cells at a 1:5 ratio in the presence of IL-2 (50 U/ml) in complete media. For our engineered tumor model, 293T-MUC1-mOrange and 293T-PSCA-GFP (or control 293T cells alone) were mixed at 1:1 ratio, then OKT3/CD28 blasts or CAR-modified T cells were added to the mixture at a ratio of 10:1 (T cells:tumor cell), in the presence of IL-2 (50 U/ml) in complete media. After 72 hours, all residual cells were collected, counted, stained, and then analyzed by flow cytometry (Gallios; Beckman Coulter, Brea, CA).

Flow cytometry

Immunophenotyping. T cells were analyzed 3–4 weeks after the generation of the culture by surface stained with monoclonal antibodies to: CD3, CD4, CD8, CD19, CD56, CD27, CD28, CD45RO, and CD62L (Becton Dickinson, Franklin Lakes, NJ). Cells were washed once with phosphate-buffered saline supplemented with 2% fetal bovine serum, pelleted, and antibodies added in saturating amounts (10 μl). To detect CAR-transduced cells, T cells were stained with a monoclonal antibody Fc-specific cyanine-Cy5-conjugated antibody (Jackson Immuno Research Laboratories, West Grove, PA), which recognizes the immunoglobin G1-CH2CH3 component of the receptor. Cells were analyzed using a Gallios Flow cytometer, and the data were analyzed using Kaluza software (Beckman Coulter).

MUC1 antigen staining. One million CAPAN1 cells were fixed with 80% methanol and washed with 0.1% tween-phosphate-buffered saline. One microgram of anti-MUC1 antibody (AbCam) was added and incubated at room temperature for 30 minutes. Then cultures were washed and incubated with 0.4 μg of a goat antimouse immunoglobin G antigen-presenting cell antibody (BD Pharmingen, San Jose, CA) for 20 minutes at 4 °C in the dark. Cells were then washed twice and analyzed.

In vivo *study.* One million CAPAN1 cells, which were engineered to express eGFP-FFLuc, were inoculated intraperitoneally into severe combined immunodeficiency mice mice. Bioluminescence images were recorded once a week using Lumina In Vivo Imaging System (Caliper Life Sciences, Hopkinton, MA) and analyzed by Living image software. After engraftment, defined as an increase in tumor signal in at least two consecutive bioluminescence measurements, mice were treated intraperitoneally with CAR-modified T cells (30×10^6 cells/animal). All treated groups received IL-2 (4,000 U/animal) intraperitoneally three times per week and bioluminescence imaging was done once a week.

Decitabine treatment. CAPAN1 cells were cultured in a T175 flask using complete IMDM media containing 1 μmol/l 5-Aza-2′-deoxycytidine—decitabine—(Sigma-Aldrich, St. Louis, MO) for 4 days, with fresh media and decitabine replenished daily.[36] Subsequently, decitabine-treated CAPAN1 cells were rested for 2 days in complete IMDM and then cocultured with CAR-MUC1 T cells.

SUPPLEMENTARY MATERIAL

Figure S1. Tumor immune escape occurs irrespective of whether T cells are modified with a 1st, 2nd or 3rd generation CAR construct.
Figure S2. Fluorescence intensity of mOrange was correlated with MUC1 antigen expression on the engineered 293T cells expressing MUC1-mOrange.
Figure S3. Decitabine treatment upregulates MUC1 expression of CAR-resistant T cells and resensitizes them to T cell treatment.

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