Cancer Therapy: Preclinical

Clinical Cancer Research

Multifactorial T-cell Hypofunction That Is Reversible Can Limit the Efficacy of Chimeric Antigen Receptor–Transduced Human T cells in Solid Tumors

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Abstract

Purpose: Immunotherapy using vaccines or adoptively transferred tumor-infiltrating lymphocytes (TIL) is limited by T-cell functional inactivation within the solid tumor microenvironment. The purpose of this study was to determine whether a similar tumor-induced inhibition occurred with genetically modified cytotoxic T cells expressing chimeric antigen receptors (CAR) targeting tumor-associated antigens.

Experimental Design: Human T cells expressing CAR targeting mesothelin or fibroblast activation protein and containing CD3ζ and 4–1BB cytoplasmic domains were intravenously injected into immunodeficient mice bearing large, established human mesothelin-expressing flank tumors. CAR TILs were isolated from tumors at various time points and evaluated for effector functions and status of inhibitory pathways.

Results: CAR T cells were able to traffic into tumors with varying efficiency and proliferate. They were able to slow tumor growth, but did not cause regressions or cures. The CAR TILs underwent rapid loss of functional activity that limited their therapeutic efficacy. This hypofunction was reversible when the T cells were isolated away from the tumor. The cause of the hypofunction seemed to be multifactorial and was associated with upregulation of intrinsic T-cell inhibitory enzymes (diacylglycerol kinase and SHP-1) and the expression of surface inhibitory receptors (PD1, LAG3, TIM3, and 2B4).

Conclusions: Advanced-generation human CAR T cells are reversibly inactivated within the solid tumor microenvironment of some tumors by multiple mechanisms. The model described here will be an important tool for testing T cell–based strategies or systemic approaches to overcome this tumor-induced inhibition. Our results suggest that PD1 pathway antagonism may augment human CAR T-cell function. *Clin Cancer Res; 20(16); 4262–73. ©2014 AACR.*

Introduction

Adoptive T-cell transfer (ACT) is a form of immunotherapy that has demonstrated increasing promise as a therapeutic option for cancer (1–3). ACT using cytotoxic T cells that have been genetically modified to express a chimeric antigen receptor (CAR) specifically targeting a tumor-associated antigen (TAA) or a cancer stromal antigen offers the advantages of specific, high-affinity binding of target cells in a major histocompatibility class (MHC)–independent fashion, optimization of T-cell activation via incorporation of different internal costimulatory domains (so-called "advanced generation" CARs), and relatively straightforward and efficient *ex vivo* preparation (4).

Recently, some dramatic tumor regressions in patients with hematologic malignancies using CARs targeting the B-cell antigen CD19 have been reported (3). This has spurred a growing interest in using this approach for a variety of solid tumors (5, 6). However, if CAR T cells behave similarly to endogenous T cells [or to *ex vivo* expanded tumor-infiltrating lymphocytes (TIL); refs. 7–10], it is likely that the efficacy of the infused T cells will be limited by a number of factors, including (i) inhibitory effects of tumor-derived cytokines, (ii) metabolic challenges (i.e., lack of arginine or tryptophan), (iii) a microenvironment characterized by hypoxia and low pH, (iv) negative effects of intratumoral immune-suppressor cells (5, 6, 11–13), (v) intrinsic inhibitory pathways mediated by upregulated inhibitory receptors reacting with their cognate ligands within the tumor (14, 15), and (vi) intracellular inhibitory pathways that are engaged after T-cell activation, which

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Translational Relevance

Adoptive T cell transfer (ACT) using T cells genetically modified to express chimeric antigen receptors (CAR) against tumor-associated antigens (TAA) has shown great promise in the treatment of blood-borne malignant disease, but may be limited by the strong immunosuppressive environment within solid tumors. We present a novel model demonstrating that reversible tumor-induced hypofunction of CAR T cells does occur in solid tumors. This model will be important in understanding the mechanisms of this effect and developing strategies to reduce or eliminate this hypofunction, paving the way for future clinical trials.

function to inhibit T-cell receptor (TCR) pathways and effector functions (16). Examples of surface-inhibitory receptors on TILs include CTLA4, PD1, LAG3, 2B4, and TIM3 (17, 18). Examples of upregulated intracellular inhibitors in TILs are phosphatases (i.e., SHP1 that dephosphorylates TCR kinases such as Lck and ZAP70; ref. 19), ubiquitin-ligases (i.e., cbl-b; ref. 20), and kinases [i.e., diacylglycerol kinase (DGK), which inactivates diacylglycerol; ref. 21]

Because advanced-generation CAR T cells have intrinsic costimulatory activity [i.e., cytoplasmic domains from CD28 and/or 4–1BB (CD137)], it is possible that they are more resistant to these inhibitory forces. For example, there are data supporting the ability of 4–1BB costimulation to blunt the anergy response (22–24). However, there are no data studying the same protective ability of 4–1BB in CAR-modified T cells. Furthermore, a significant portion of these data was from research in murine T cells (23, 25). The purpose of this study was to develop a model in which suppression of T-cell function using advanced-generation human CAR T cells could be studied.

Materials and Methods

Generation of mesoCAR construct and lentivirus vector preparation

The single-chain Fv domain of the anti-mesothelin antibody (scFv SS1), originally provided by Dr. Ira Pastan (26) (National Cancer Institute/NIH, Bethesda, MD) was previously subcloned into the lentiviral vector pELNS bearing the EF1α promoter and incorporated the CD3ζ and 4–1BB intracellular TCR signaling domains (27). A variant of the mesoCAR construct incorporating a myc-tag between the scFv SS1 and the CD8 hinge was generated to allow for clearer detection of surface mesoCAR expression on TILs harvested from mouse flank tumors. Construction of a similar CAR, but targeting murine fibroblast activation protein (FAP), has been described previously (28).

Cell lines

For mesoCAR studies, a human mesothelioma cell line derived from a patient's tumor was used––EMP (parental). Because EMP did not have baseline expression of the TAA mesothelin, it was transduced with a lentivirus to stably express human mesothelin (the transduced cell line was named EMMESO). Mesothelin expression level is shown in Supplementary Fig. S1.

Mouse 3T3Balb/C cells (3T3p) were purchased from the American Type Culture Collection. Mouse FAP-expressing 3T3Balb/C cells (3T3mFAP) were created by lentiviral transduction of the parental line with murine FAP (28).

All lines were also transduced to stably express firefly luciferase (called EMPffluc, EMMESOffluc, 3T3p-ffluc, and 3T3mFAP-ffluc). The culture conditions are described in Supplementary Methods.

Isolation, bead activation, transduction, expansion of primary human T lymphocytes, and T-cell effector assays

These protocols are described in the Supplementary Methods.

Animals

All animal experiment protocols were approved and conducted in accordance with the Institutional Animal Care and Use Committee. NOD/scid/IL2rγ$^{-/-}$ (NSG) mice were bred in the Animal Services Unit of the Wistar Institute and Children's Hospital of Philadelphia. Female mice were used for experiments at 10 to 16 weeks of age.

In vivo xenograft experiments

A total of 5×10^6 EMMESO tumor cells were injected in the flanks of NSG mice in a solution of X-Vivo media (Lonza) and Matrigel (BD Biosciences). After tumors were established (100–200 mm^3), the mice were randomly assigned to one of three intravenous (tail-vein) treatment groups: (i) 20×10^6 nontransduced (NT) T cells (Dynabeads activated T cells), (ii) 20×10^6 mesoCAR T cells (Dynabeads activated T cells transduced with mesoCAR), and (iii) saline. Tumors were measured using calipers and tumor volumes were calculated using the formula $(\pi/6) \times (\text{length}) \times (\text{width})^2$. An additional experiment was performed in which mice bearing EMMESO flank tumors were injected with 20×10^6 FAPCAR T cells. FAP is highly expressed on the tumor fibroblasts, which make up about 6.3% of the digested EMMESO tumors.

At different time points, tumors were harvested, microdissected, and digested in a solution of 1:2 DNAse:collagenase with rotation at 37°C for 1 hour. Digested tumors were then filtered through 70-μm nylon mesh cell strainers, and red blood cells were lysed if needed (BD Pharm Lyse; BD Biosciences). After single-cell suspensions were achieved, 1×10^6 cells were placed in standard FACS tubes and were stained with fluorochrome-conjugated anti-human CD45 or CD3 antibodies.

Groups contained 10 mice each. The *in vivo* experiments were repeated three times in independent fashion.

Ex vivo TIL analysis

After digestion, TILs were isolated by using an anti-human CD45 PE antibody (BD Biosciences) with the

EasySEP PE Selection Kit (STEMCELL Technologies; #18551). Once isolated, TILs were analyzed in three different ways: (i) luciferase-based killing assays, (ii) intracellular cytokine expression, and/or (iii) measurement of antigen-induced T-cell IFNγ secretion (refer to Supplementary Methods for detailed protocols).

Antibodies

Refer to Supplementary Methods for details.

Inhibitors

The SHP1 inhibitor sodium stibogluconate (SSG) was purchased from EMD Millipore (567565). Two different inhibitors of DGK were purchased from Sigma [DGKinh1, (nonspecific inhibitor), D5919; DGKinh2 (α isoform specific), D5794]. Functional antibody against PDL1 was a generous gift from Dr. Gordan Freeman (29). Human IL2 (Proleukin; Prometheus Laboratories Inc. Dana Farber Cancer Institute, Boston, MA) was acquired through the Hospital of the University of Pennsylvania pharmacy. Dose–response curves were performed for both DGK inhibitors and for SSG, and the highest doses that did not induce direct tumor cell killing were used. Both DGK inhibitors (1 μmol/L) and SSG (25 μg/mL) were also demonstrated to be appropriate in other published investigations (30, 31). All inhibitory studies were done twice in independent fashion with comparable results.

Immunoblotting

Lysates of T cells (40 μg) before and/or after activation with beads were run on SDS-PAGE gels, transferred, and immunoblotted using standard approaches. Primary and secondary antibodies used are described in Supplementary Methods.

Statistical analysis

All results were expressed as means ± SEM as indicated. For studies comparing two groups, the student *t* test was used. For comparisons of more than two groups, we used one-way ANOVA with appropriate *post hoc* testing. Differences were considered significant when $P < 0.05$.

Results

Intravenous injection of human mesoCAR T cells slows but does not eradicate human tumors

The human mesothelin–expressing mesothelioma tumor cell line EMMESO was injected into the flanks of NSG mice and allowed to grow to a size between 100 and 200 mm^3. At that time, tumor-bearing mice were given one intravenous injection of 20 million T cells [mesoCAR expression was approximately 50% (data not shown)]. Significant slowing of tumor growth was seen after a delay of 14 days (Fig. 1A); however, unlike our experience with another mesothelioma cell line (27, 32), no tumor regression or cures were noted. Injection of NT T cells had minimal antitumor effects when compared with the saline-treated control (Fig. 1A), indicating that the reduction in tumor growth observed in animals treated with T cells expressing mesoCAR was specifically a result of the mesoCAR.

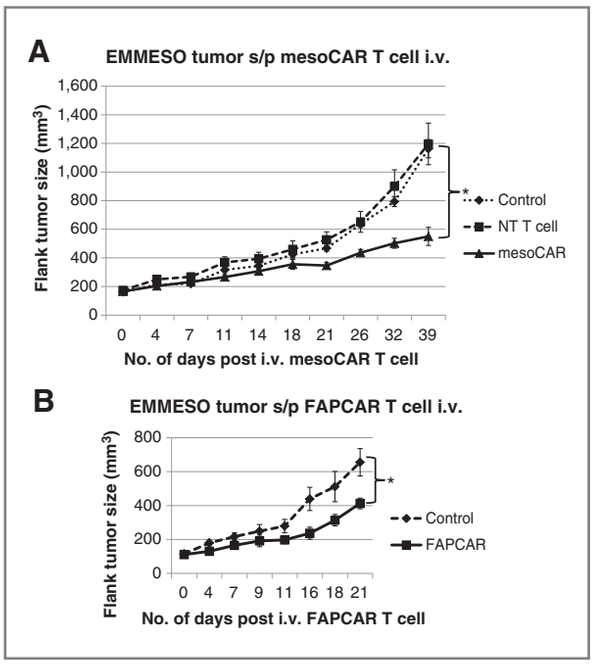

Figure 1. CAR T cells slow tumor growth but do not cause regression. A, 5×10^6 EMMESO tumor cells were injected into the flanks of NSG mice. After they grew to approximately 200 mm^3 in size, 20×10^6 mesoCAR T cells were injected via tail vein and tumors were measured for 39 days after injection. T cells were able to slow growth by day 18 but were unable to eradicate flank tumors. B, 5×10^6 EMMESO tumor cells were injected into the flanks of NSG mice. After they grew to approximately 150 mm^3 in size, 20×10^6 FAPCAR T cells were injected via tail vein and tumors were measured for 41 days after injection. T cells were able to slow growth by day 11 but were unable to eradicate flank tumors; *, $P < 0.05$.

Human mesoCAR T cells traffic into tumors and proliferate

To understand why we did not see tumor regression, we first evaluated whether our tumor antigen had been lost. Interestingly, immunohistochemistry of EMMESO tumors at 40 days after T-cell injection showed robust and uniform expression of mesothelin (Supplementary Methods and Supplementary Fig. S2).

We next evaluated the number and phenotype of the human T cells approximately 40 days after CAR T-cell injections by removing and digesting the tumors and spleens and identifying human $CD3^+$ cells by flow cytometry. In EMMESO tumors from mice injected with mesoCAR T cells, we noted that approximately 10% of the digested cells (8.2×10^6) were human T cells. In contrast, in EMMESO tumors injected with NT T cells, only 0.1% (2.5×10^5) of the digest was human T cells (Fig. 2A and B). In contrast with our findings in the tumors, CAR T-cell persistence was much lower than NT T cells in spleens (Fig. 2C and D). We also examined tumors at earlier time points (Fig. 2E and F). The percentage and number of intratumoral CAR T cells increased steadily over time, starting from very small numbers detectable at day 5.

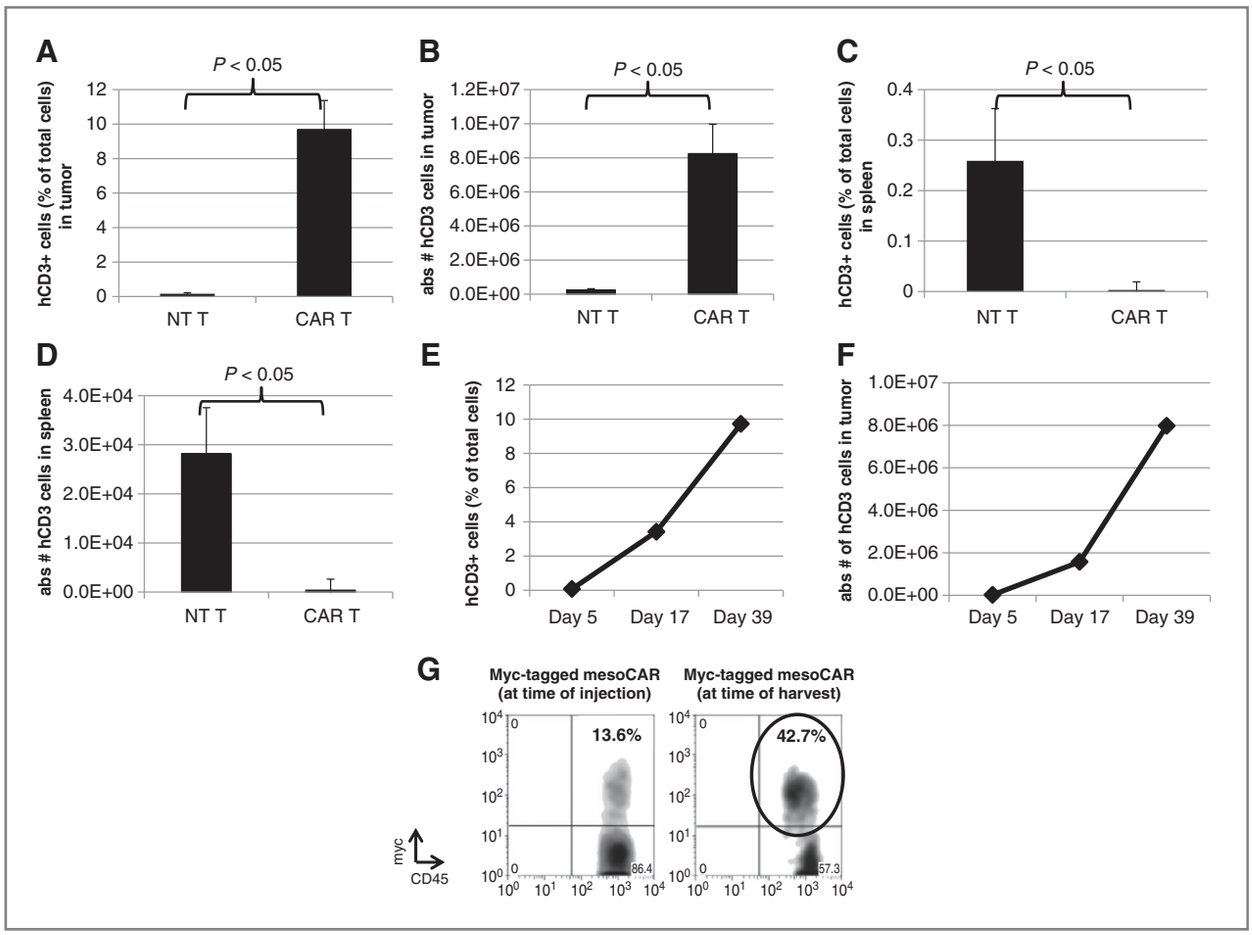

Figure 2. CAR T cells infiltrate, survive, proliferate, and retain transgene expression in tumors. 39 days after tail vein T-cell injection, flank tumors were harvested and digested and the quantity of human $CD3^+$ cells was assessed. The percentage (A) and absolute number (B) of intratumoral CAR T cells were much higher than NT T cells [9.7% (8.2×10^6) CAR T cells vs. 0.16% (2.5×10^5) NT T cells ($P < 0.05$)]. The percentage (C) and absolute number (D) of intrasplenic CAR T cells were much lower than NT T cells [<0.05% (3.89×10^2) CAR T cells vs. 0.25% (2.82×10^4) NT T cells ($P < 0.05$)]. The percentage (E) and absolute number (F) of intratumoral CAR T cells were also assessed at earlier time points and demonstrated a marked increase over time [0.07% (2.16×10^4) in the first week after T-cell injection to 9.7% (7.98×10^6) in the sixth week after T-cell injection]. G, when myc-tagged mesoCAR TILs were isolated from flank tumors 40 days after injection, they retained surface expression of myc-tagged mesoCAR after infiltration into tumors. Percentage of Myc-tag–expressing T cells after infiltration was actually higher than at the time of injection (42.7% vs. 13.6%).

Tumor-infiltrating human mesoCAR T cells continue to express CAR receptors on their surface

To evaluate loss or downregulation of the surface expression of the CARs, we injected mice bearing EMMESO tumors with T cells expressing a CAR that had been engineered to express a myc-tag in the extracellular domain. CAR expression (using an anti-myc antibody) on human T cells from tumors 40 days after injection was compared with the expression on CAR T cells that had been originally injected (Fig. 2G). In this experiment, the percentage of TILs expressing CAR on their surface increased to more than 42% compared with 13% at the time of injection.

Tumor-infiltrating human CAR T cells become hypofunctional

The data above suggested that although the human T cells were present in large numbers, they had become hypofunctional. Given that the level of CAR expression on the CAR TILs was equal to or greater than that of the cells before injection (Fig. 2G), we compared their functional activity. We isolated and analyzed the mesoCAR TILs from EMMESO tumors 40 days after injection (all studies were started immediately after isolation) and compared them with the same batch of mesoCAR T cells that had been used for the original injection and frozen away ("cryo mesoCAR"). These cells were studied after thawing and incubating at 37°C in 5% CO_2 for 18 hours to mirror handling before injection.

When we added cryo mesoCAR T cells and flank mesoCAR TILs to cultured EMMESO cells expressing firefly luciferase (EMMESOffluc) at a 20:1 ratio for 18 hours, the cryo mesoCAR T cells were highly efficient in killing tumor cells (>95%), whereas the mesoCAR TILs killed only about 10% (Fig. 3A; $P < 0.001$). Similarly, whereas cryo mesoCAR

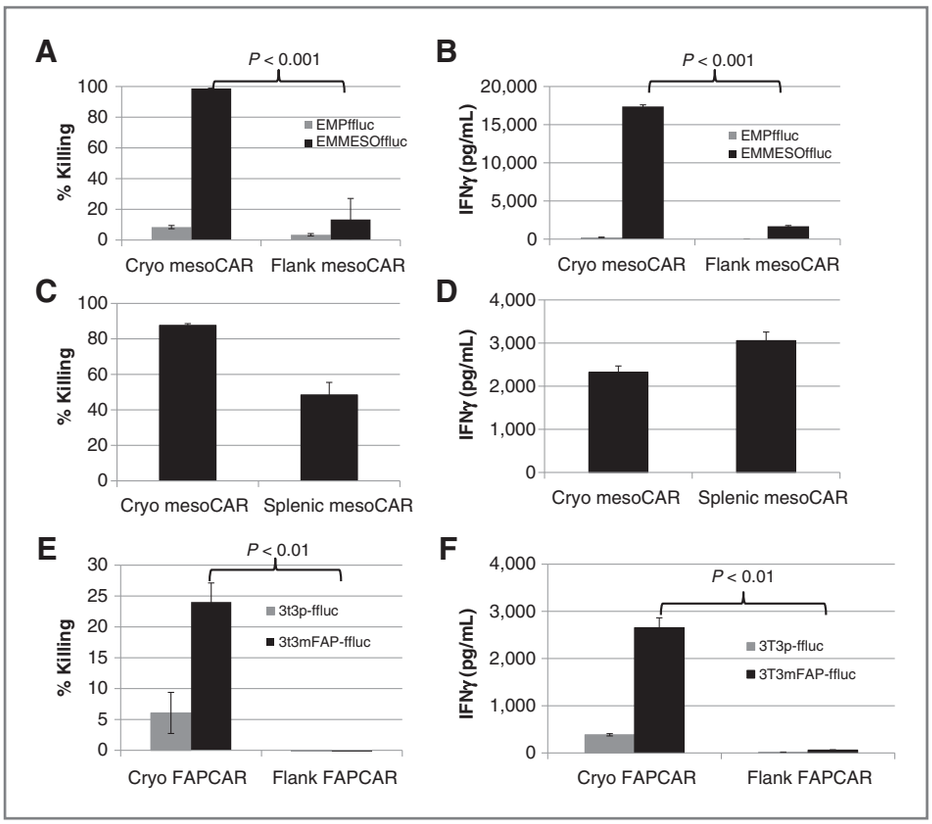

Figure 3. CAR T cells undergo tumor-induced hypofunction of cytolytic and cytokine secretion ability. A, cytotoxicity: flank mesoCAR TILs from day 39 demonstrated hypofunctional killing of EMMESOffluc compared with cryo mesoCAR T-cell controls (98.7% vs. 12.3%, $P < 0.001$). TILs/T cells were cocultured with firefly luciferase expressing EMMESO tumors at 20:1 E:T ratio for 18 hours. Measurement of luciferase activity of remaining tumor cells was used to calculate percentage killing. B, IFNγ secretion: flank mesoCAR TILs demonstrate hypofunctional secretion of IFNγ in response to EMMESOffluc tumor compared with cryo mesoCAR T-cell controls (17,387 vs. 1,689 pg/mL, $P < 0.001$). Coculture was performed at a ratio of 20:1 (effector to target) for 18 hours at 37°C, in 5% CO_2. EMPffluc targets were also tested to confirm antigen-specific tumor killing ability of both the cryo mesoCAR T cells and the mesoCAR TILs. C, isolated spleen-infiltrated T cells demonstrated much less loss of tumor killing function as compared with TILs (A) and (D) much less loss of tumor-induced IFNγ secretion as compared with TILs (B). E, flank FAPCAR TILs from day 28 demonstrated hypofunctional killing of 3T3mFAP-ffluc compared with cryo FAPCAR T-cell controls (24% vs. 0%, $P < 0.01$). TILs/T cells were cocultured with firefly luciferase expressing 3T3p and 3T3mFAP tumors at 10:1 E:T ratio for 18 hours. Measurement of luciferase activity of remaining tumor cells was used to calculate percentage killing. F, IFNγ secretion: flank FAPCAR TILs demonstrate hypofunction secretion of IFNγ in response to 3T3mFAP-ffluc tumor compared with cryo FAPCAR T-cell controls (2,653.7 vs. 60.85 pg/mL, $P < 0.01$).

T cells released large amounts of IFNγ into the supernatant, the mesoCAR TILs secreted very little (Fig. 3B; $P < 0.001$). Neither mesoCAR TILs nor cryo mesoCAR T cells had significant antitumor activity against EMPffluc, demonstrating that the response was specific to mesothelin TAA. The *ex vivo* killing ability of mesoCAR T cells isolated from the spleens of the same tumor-bearing mice demonstrated much less loss of function than the T cells isolated from the tumor, suggesting that these effects were specific to the tumor microenvironment (Fig. 3C and D).

We next examined the ability of the CAR T cells and CAR TILs to produce cytokines (using intracellular cytokine staining by flow cytometry; Fig. 4A). When exposed to albumin-coated beads (control), neither the cryo mesoCAR T cells nor fresh mesoCAR TILs (CD4 and CD8 cells) produced IFNγ. After exposure to mesothelin-coated beads, a clear subpopulation of CD4 (0.59%) and CD8 (2.07%) cryo mesoCAR T cells made IFNγ (Fig. 4A, left). In contrast, the mesoCAR TILs did not produce IFNγ after exposure to their surrogate antigen (Fig. 4A, right) despite having relatively high expression of mesoCAR on the surface (Fig. 2G). We saw very similar responses with IL2 production (Supplementary Fig. S3). We next exposed the same cells to PMA/ionomycin (Fig. 4B). Approximately 20% of the freshly thawed mesoCAR T cells made IFNγ (Fig. 4B, bottom left). In contrast with the results with the beads, a similar percentage of the mesoCAR TILs (22%) produced IFNγ after PMA/ionomycin (Fig. 4B, bottom right).

We also assessed the ability of the T cells to signal by assessing phospho-ERK expression using immunoblotting 20 minutes after exposure to beads (Fig. 4C). Phospho-ERK expression was minimal in both types of CAR T cells after exposure to control beads. Cryo mesoCAR T cells exhibited ERK activation via phosphorylation after exposure to both mesothelin and anti-CD3/CD28–coated beads (Fig. 4C, left). In contrast, no phospho-ERK was detected in the

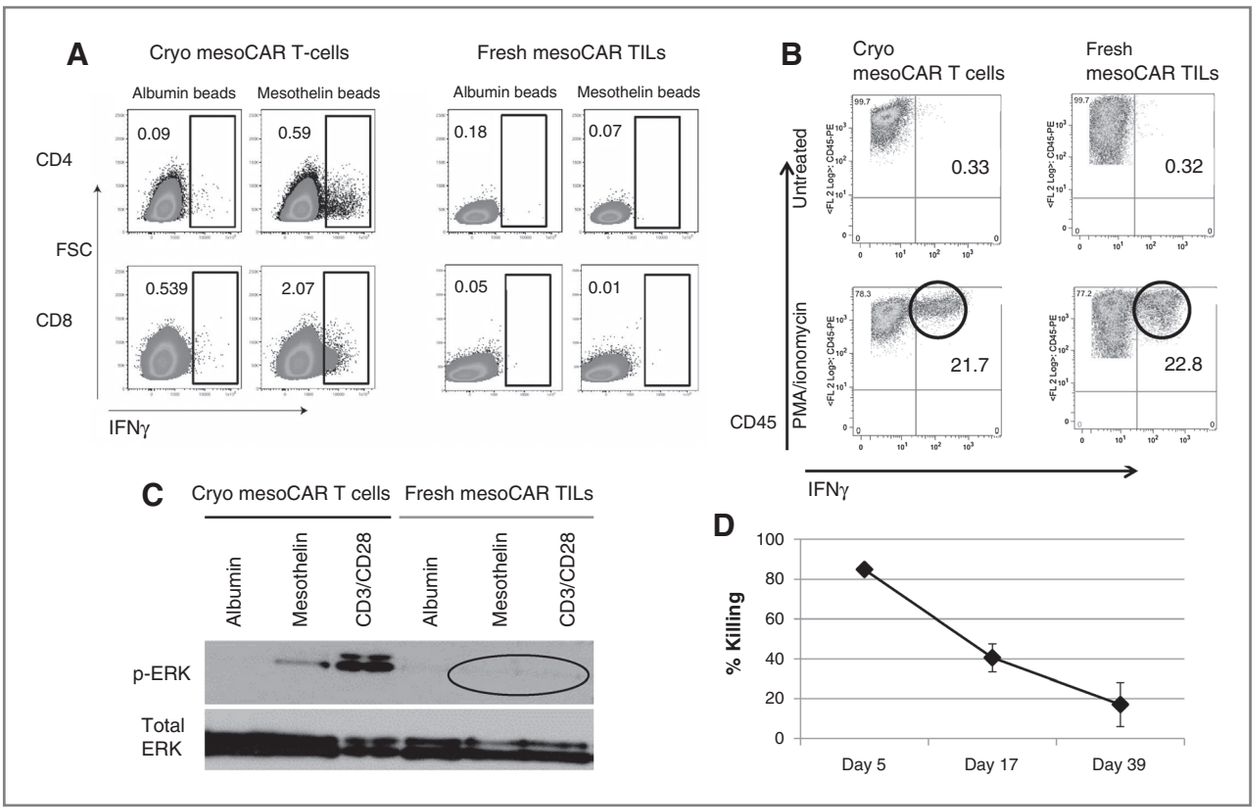

Figure 4. CAR TIL hypofunction progresses rapidly after injection and may be due to defects in proximal signaling. A, upon exposure to either albumin-labeled beads (negative control) or mesothelin-labeled beads, the mesoCAR TILs were found to be hypofunctional in their ability to produce IFNγ in response to the antigen-coated beads. B, upon exposure to 0.1 μg/mL PMA and 2 μg/mL ionomycin, the mesoCAR TILs demonstrated preserved ability to produce IFNγ as measured by intracellular cytokine staining by flow cytometry. C, upon exposure to either albumin-labeled beads (negative control), mesothelin-labeled beads, or CD3/CD28 beads (positive control) for 15 minutes, mesoCAR TILs were found to be hypofunctional in their ability to produce signal via phosphorylation of ERK in response to antigen or CD3/28 stimuli. D, when flank tumors were harvested at early (day 5), mid (day 17), and late (day 39) time points, isolated EMMESO TILs were cocultured with firefly luciferase expressing EMMESO tumor at 20:1 E:T ratio. After 18 hours, percentage killing was calculated after measuring luciferase activity of the remaining tumor cells. We demonstrated that EMMESO TILs undergo rapidly increasing tumor-induced hypofunction after i.v. injection.

mesoCAR TILs after exposure to mesothelin or anti-CD3/CD28–coated beads (Fig. 4C, right).

It was also of interest to determine the kinetics of inducing T-cell hypofunction. MesoCAR TILs were thus isolated from tumors at 5, 17, and 39 days after injection, and their ability to kill tumor cells *ex vivo* was determined (Fig. 4D). MesoCAR TILs isolated at day 5 were still highly active, killing 85% of EMMESO tumor cells as assessed by our *in vitro* killing assay. In contrast, mesoCAR TILs isolated on days 17 and 39 had progressive hypofunction.

The hypofunction seen in human mesoCAR TILs is reversible

To determine whether the hypofunction was reversible, mesoCAR TILs isolated from day 39 tumors were "rested" for 24 hours away from the tumor in either media alone or media plus low-dose IL2 and then had their effector functions assessed. As shown in Fig. 5A and B, substantial recovery of killing ability and IFNγ release was seen 24 hours at 37°C, 5% CO2 (away from tumor). The presence of IL2 in the resting media accelerated recovery, but was not required (Supplementary Fig. S4). We saw a similar recovery in the ability to produce cytokines (at the single-cell level using flow cytometry) for IFNγ and TNFα in response to EMMESO tumor (Supplementary Fig. S5).

The ability to phosphorylate ERK after surrogate antigen stimulation or ligation of the endogenous TCR was also restored after 24 hours of "rest" (Fig. 5C). Thus, a profound but reversible functional impairment exists in human CAR TILs in mice with progressive tumor growth.

Human mesoCAR TILs express increased levels of inhibitory receptors

We next evaluated the expression of four inhibitory receptors, that have been previously described in hypofunctional TILs isolated from humans, using flow cytometry on (i) the cryo mesoCAR T cells that were used for injection, (ii) freshly isolated mesoCAR TILs from EMMESO tumor at day 39, and (iii) "recovered" mesoCAR TILs that had been removed from EMMESO tumor and "rested" 24 hours (Supplementary Table S1). CAR TILs expressed high levels of inhibitory receptors. These levels were generally much

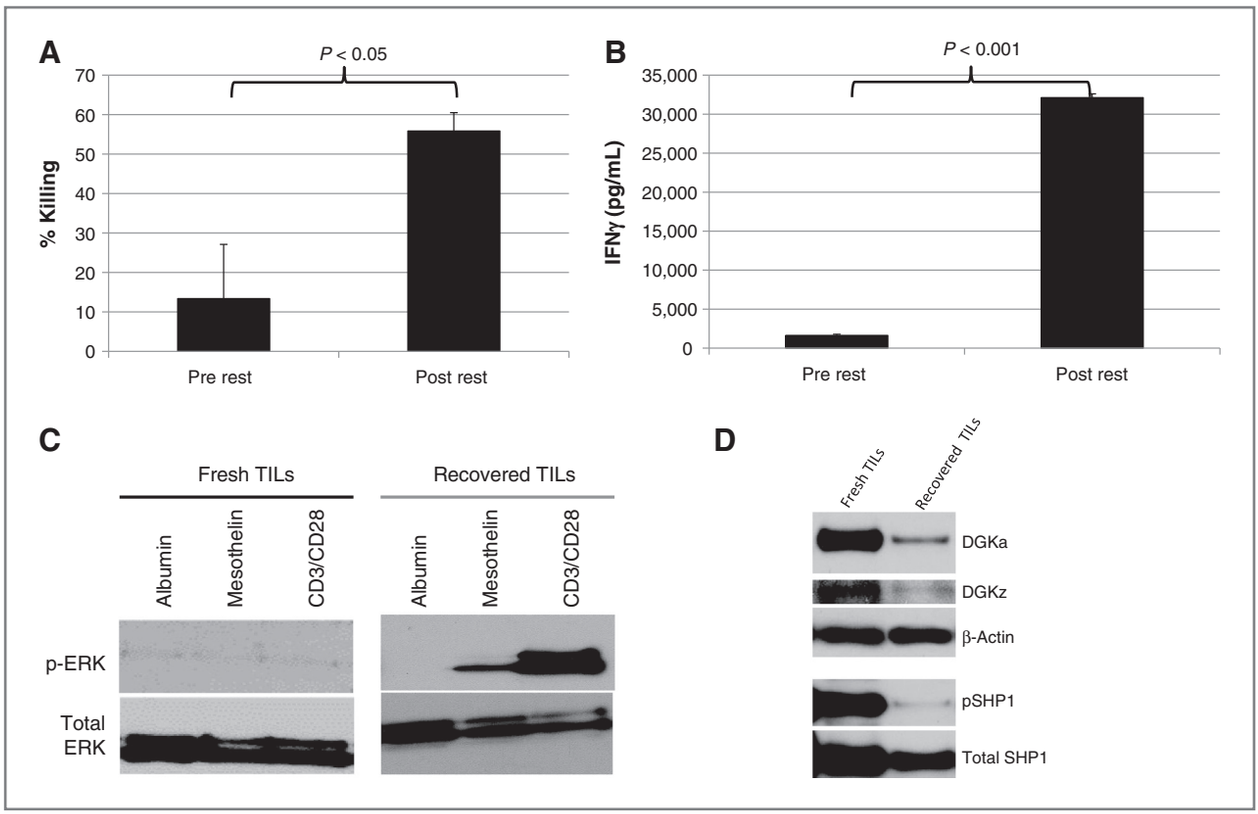

Figure 5. CAR TIL cytolytic and cytokine secreting functions recover after rest away from tumor. DGK and pSHP1 expression is increased in hypofunctional CAR TILs. Human TILs isolated from flank EMMESO tumors at day 39 and "rested" away from tumor for 24 hours demonstrated significant recovery in their EMMESOffluc tumor killing ability (A) and their ability to secrete IFNγ in response to EMMESOffluc tumor (B). C, compared with freshly harvested EMMESO TILs, resting away from tumor led to recovery of antigen-specific signaling function of EMMESO TILs as measured by detection of phosphorylated ERK. D, DGKa and DGKz and pSHP1 significantly elevated in TILs freshly isolated from EMMESO. Expression of both isoforms of DGK as well as pSHP1 decreased dramatically after overnight rest of TILs.

lower after 24 hours of recovery away from the tumor microenvironment. For the CD4 CAR TILs, PD1 went from 73% to 53%, LAG3 went from 63% to 3%, and TIM3 went from 24% to 1%. 2B4 expression was high and remained elevated after rest (67% to 88%). For the CD8 CAR TILs, PD1 went from 26% to 21%, LAG3 went from 48% to 13%, and TIM3 went from 56% to 1%. 2B4 expression was high and remained elevated after rest (96% to 98%).

We also evaluated three of these inhibitory receptors on the human T cells that could be isolated from the spleens of the EMMESO mice. Interestingly, the expression levels of PD1, TIM3, and LAG3 were all lower on the splenic T cells compared with the TILs (Supplementary Table S2), supporting the hypothesis that the tumor microenvironment induces the upregulation of inhibitory receptors.

Human mesoCAR TILs express increased levels of intracellular inhibitory enzymes

We also explored the expression levels of two intrinsic inhibitors of T-cell function that have been implicated in TIL dysfunction, SHP1 and DGK, using immunoblotting (Fig. 5D). The levels of both isoforms of DGK (α and ζ), as well as the phosphorylated form of SHP1 (pSHP1), were significantly elevated in mesoCAR TILs that were freshly isolated from EMMESO flank tumor compared with rested TILs. This was also confirmed for DGKα using flow cytometry in which 23% of fresh EMMESO TILs expressed DGKα. Expression was undetectable after overnight rest (data not shown).

Blockade of inhibitors in human mesoCAR T cells enhances their *ex vivo* killing function

Given these expression data, we studied the potential functional importance of specific inhibitory pathways in mesoCAR TILs by introducing available blocking agents during the *ex vivo* killing and cytokine release assays. Addition of an anti-PDL1 antibody significantly restored the killing activity and ability to secrete IFNγ by the mesoCAR TILs (Fig. 6A and B). The relatively high dose of 10 μg/mL anti-PDL1 antibody was based on previously published investigations in cancer immunotherapy (33). Addition of either a type I or type II DGK inhibitor also significantly increased the killing ability (Fig. 6C), but without significantly increasing tumor-induced IFNγ secretion (Fig. 6D). Addition of the SHP1 inhibitor, SSG, slightly inhibited the killing ability of cryo mesoCAR T cells, but significantly increased that of the

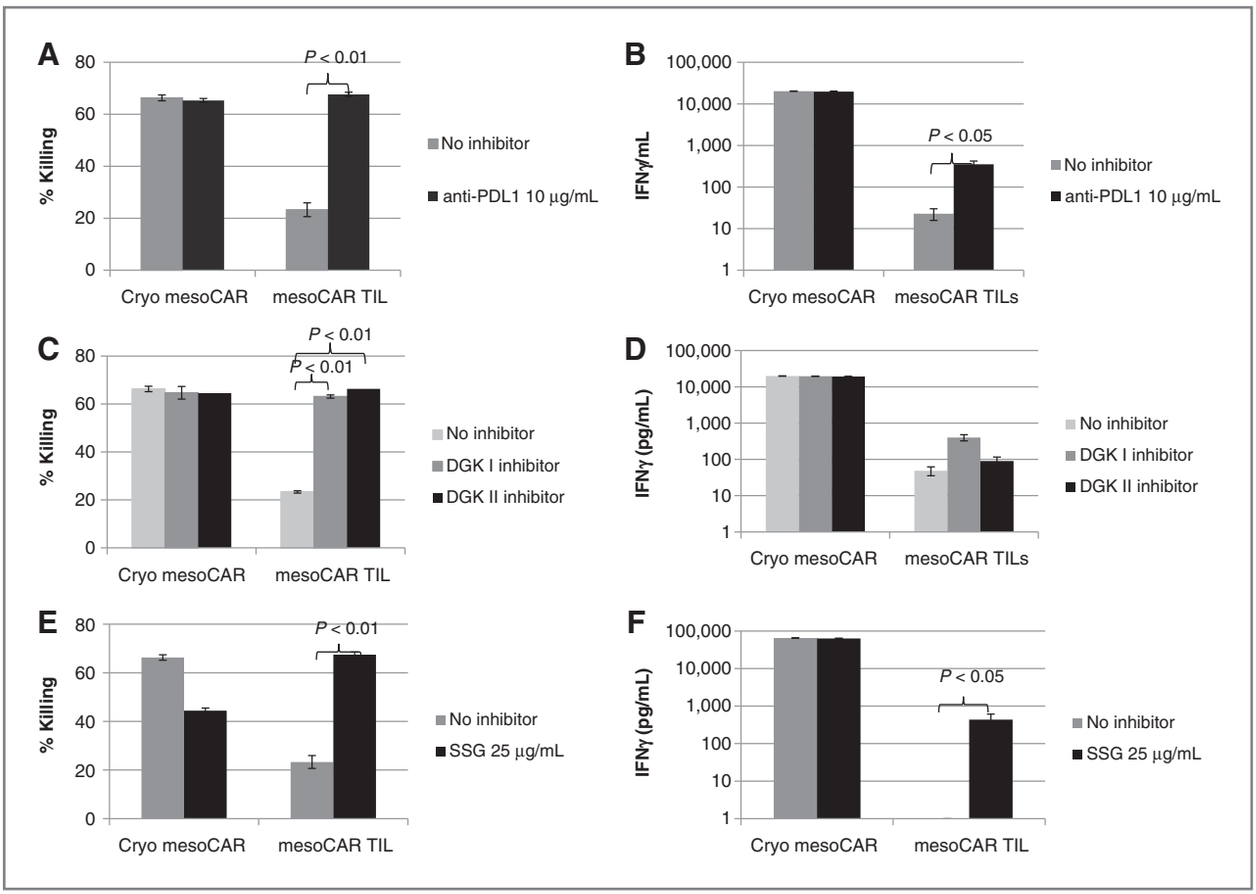

Figure 6. PDL1 blockade and DGK inhibition are able to restore CAR TIL function. 10 μg/mL of anti-PDL1 antibody was added to the coculture killing assay and was able to restore mesoCAR TIL killing of EMMESOffluc (A) and tumor-induced mesoCAR TIL secretion of IFNγ after fresh isolation from flank tumor (B). C, 1 μmol/L of type I and II DGK inhibitor was added to the coculture killing assay and was able to restore mesoCAR TIL killing of EMMESOffluc with minimal increase in tumor-induced mesoCAR TIL secretion of IFNγ after fresh isolation from flank tumor (D). 25 μg/mL of SSG was added to the coculture killing assay and was able to restore mesoCAR TIL killing of EMMESOffluc (E) and tumor-induced mesoCAR TIL secretion of IFNγ after fresh isolation from flank tumor (F).

mesoCAR TILs (Fig. 6E), as well as significantly increasing tumor-induced IFNγ secretion (Fig. 6F).

Tumor-infiltrating human T cells against other tumors and other TAAs also become hypofunctional

To investigate the generalizability of our findings, we studied mesoCAR T cells in an additional human mesothelioma model (M30 cells) and saw virtually identical induction of hypofunction as we did with EMMESO (data not shown). To demonstrate that these effects were independent of the specific TAA, we evaluated T cells modified with a CAR directed against murine FAP, which is expressed on the mouse fibroblasts in the stroma formed in the EMMESO flank tumors. Similar to our findings using murine T cells and mouse tumors (28), injection of 10^7 FAPCAR T cells significantly slowed the growth of EMMESO tumors, but did not eradicate them by day 41 after injection (Fig. 1B). FAPCAR TILs isolated from flank tumors at day 28 post injection (by methods described above) also demonstrated profound hypofunction in cytolytic activity and IFNγ secretion similar to that seen in the mesoCAR TILs (Fig. 3E and F).

Discussion

Adoptive T cell transfer (ACT) using chimeric antigen receptor–transduced T cells has demonstrated increasing promise as a therapeutic option for cancer, especially in hematopoietic tumors (3, 34). Given that T-cell inactivation has been reported in solid tumors (5, 6, 11), the purpose of this study was to develop a model in which this process could be assessed using advanced-generation human CAR T cells. We found that a single intravenous injection of human mesoCAR T cells or FAPCAR T cells into immunodeficient mice induced a significant decrease in the tumor growth rate compared with that of controls, but did not result in tumor regressions or cures. By harvesting and analyzing the tumors at multiple time points, we were able to show that this failure to cure the tumors was not due to loss of tumor antigen, loss of the CAR on the surface of the T cells, nor a failure of the CAR T cells to accumulate within the tumor. Instead, our data show that loss of antitumor efficacy was due to a progressive loss of CAR T-cell effector function primarily caused by the tumor microenvironment. This T-cell hypofunction is very similar to that described previously

in mouse and human TILs (7–9, 18, 35) and in nongenetically modified adoptively transferred T cells in mouse models (10, 36, 37).

Interestingly, although the CAR TILs isolated from tumors had a profound but reversible functional defect, they accumulated to high numbers in the tumor microenvironment. This suggests that the dysfunctional CAR TILs survive and retain proliferative capacity, and is consistent with the hypothesis that the 4–1BB cytoplasmic domain in the CAR supports T-cell survival, but does not protect T cells from hypofunction. Our animal data, thus, predict that even though human CAR T cells may accumulate within solid tumors in patients, their efficacy may be limited by a progressive inactivation within certain solid tumors. The relative kinetics of CAR T-cell accumulation versus their rate of inactivation within tumors will ultimately determine the overall antitumor efficacy, and this balance will likely be tumor specific.

Given these observations, we evaluated the nature of this tumor-induced CAR T-cell hypofunction. One feature was that the hypofunction was rapidly and almost fully reversible when the CAR TILs were removed from the tumor microenvironment. Although supplemental IL2 accelerated the recovery, it was not necessary. We believe that this reversibility was due to a general recovery of T-cell function due to the removal of inhibitory factors that reside in the tumor microenvironment. However, it is formally possible that our observations were due to a selection and expansion of the most functional TILs, with a concomitant loss of the hypofunctional TILs during the rest period. This possibility is difficult to exclude and may require advanced molecular strategies like "bar-coding" the T cells to track them during the rest period. However, we find the explanation of recovery of antitumor function more likely, based on published work from other groups' investigations (38–40). If one assumes that the CAR TILs are not irreversibly inactivated, it may be best to describe their phenotype as "hypofunctional" rather than "exhausted" or "anergic," which infer a permanent state of dysfunction (41, 42).

A second feature of our hypofunctional CAR TILs was that although TIL hypofunction was evident by 17 days postinjection, tumor volume did not progress dramatically at that point but rather "plateaued." This was likely due to a balance between progressively decreasing TIL function (Fig. 4D) and increasing number of TILs in the tumor (Fig. 2E) as a result of the proliferative 4–1BB signaling incorporated into the CAR construct. Potentially, combining strategies that increase the rate or duration of TIL proliferation and decrease TIL hypofunction would widen the "therapeutic sweet spot," leading to tumor regression.

A third feature of our hypofunctional CAR TILs was that the block seemed to be "proximal" in the T-cell signaling pathway. After stimulation by antigen or by CD3/CD28 crosslinking, CAR TILs failed to secrete cytokines or to phosphorylate ERK. CAR TILs also had defects in the phosphorylation of Lck, ZAP70, and SLP76 (data not shown). However, when the CAR TILs were exposed to PMA/ionomycin, bypassing the early TCR activation steps, the cells were fully capable of making cytokines. Although these features of reversibility and defects in proximal signaling have been well documented previously in mouse (17, 43) and to some extent, human TILs (44, 45), the exact mechanisms responsible for this phenotype remain elusive. Both cytoplasmic and cell-surface candidates have been implicated. For instance, Rappl and colleagues elucidated one possible mechanism of terminally differentiated, late-stage T-cell hypofunction as being due to impaired TCR synapse formation from immobility of TCR membrane surface components, which can be bypassed by introducing a first-generation CAR construct that confers tumor reactivity by signaling through normal T-cell signaling components (46). The mechanism of the hypofunction described here seems to be different as demonstrated by dysfunctional proximal T-cell signaling. The described *in vivo* model is inadequate to assess TCR function as it lacks human antigen-presenting cells.

With regard to the mechanisms of hypofunction, we evaluated two intrinsic inhibitory enzymes previously identified in hypofunctional TILs. The first candidate was the phosphatase SHP1, which can inactivate a number of the kinases in the early TCR signaling cascade (19, 47, 48). Investigators have shown in mice that TILs have elevated SHP1 activity and that inhibition of SHP1 in nonlytic TILs *in vitro* restored their tumor-cytolytic ability (43). Conversely, mice deficient in SHP1 showed increased effector T-cell activity (48). The second candidate was DGK, a key enzyme that inactivates diacylglycerol, a downstream messenger necessary for translating the TCR signal into T-cell stimulation (21, 44, 49). Loss of DGK leads to resistance to T-cell anergy and increased CD8 T-cell function (21, 44, 49). Our group has recently shown that loss of the α or ζ isoform of DGK augments murine and human CAR T-cell effector function (31, 50). Both SHP1 and DGK have been shown to be upregulated in hypofunctional TILs (21, 44, 49, 51).

Our data support the importance of both SHP1 and DGK in the induction of hypofunction of the human CAR TILs. First, CAR TILs had high expression of both SHP1 and DGK, which rapidly declined 24 hours after the TILs were removed from the tumors. Second, when we blocked DGK or SHP1 activity using chemical inhibitors in our *ex vivo* killing assay, we were able to reduce the defects in tumor killing, with lesser effects on IFNγ secretion. Consistent with these data, Prinz and colleagues demonstrated increased CD8 activity in human TILs from renal cell cancers after blocking DGK (44). The factors within the tumor microenvironment that upregulate these inhibitory enzymes, as well as approaches to inhibit these enzymes *in vivo*, are active areas of research in our laboratory. In addition to cytoplasmic inhibitory pathways, it has become increasingly recognized that expression of some cell-surface inhibitory receptors on T cells can induce dysfunction (18). Overall, we observed a similar phenotype as that described by previous studies in human TILs, with increased expression of PD1, TIM3, Lag3, and 2B4 (52–55). Interestingly, our data suggest that the tumor microenvironment seemed to play an important role in "shaping" the expression pattern

of specific inhibitory receptors on the T cells. First, the expression of inhibitory receptors was much higher on TILs, rather than on human T cells isolated from spleens of the same animals. Second, after overnight "rest" away from the tumor microenvironment, expression levels of most of these receptors decreased dramatically. Finally, we were able to test the functional significance of the PD1/PDL1 interaction in the EMMESO model. Addition of a blocking PDL1 antibody to our *ex vivo* CAR TIL killing assay was able to restore the defect in tumor cell killing.

We believe the model that we have described here has many potential advantages and possible uses. First, it allows study of human (not mouse) CAR T cells. This is important as we have found the behavior of human CAR T cells to be very different that of murine CAR T cells with regard to key parameters such as persistence, sensitivity to activation-induced cell death, and the activation state at the time of injection. Furthermore, it allows the study of CAR T cells that are prepared identically to those being injected into patients. This should enhance the generalizability of the findings to clinical trials. Second, the use of a uniform preparation of CAR T cells prepared from one donor allows one to focus on microenvironmental differences rather than intrinsic T-cell variability. Third, the use of CAR T cells targeted to specific targets allows the study of antigen-specific T-cell interactions rather than just general TCR activation induced by CD3/CD28 beads or PMA/ionomycin stimulation, as has been used in most studies of human TILs. Fourth, the model uses well-characterized human solid tumor cells that can be genetically modified, if needed. This allows the systematic study of different tumor microenvironments. Although we have used flank tumors for convenience, orthotopic tumor cell placement is possible (and probably desirable). Most importantly, the CAR T cells studied in the model seem to very closely resemble naturally occurring human TILs in all of our assays. Finally, the CAR T-cell hypofunction in this model seems to be generalizable across different tumors and CARs recognizing different targets. The *in vivo* experiment detailed above was repeated using other MPM cell lines in the NSG xenograft model. After a single intravenous dose of mesoCAR T cells in NSG mice bearing flank tumors from another human MPM cell line (M30), we observed slowing of tumor growth (with no cures), and the mesoCAR TILs also demonstrated tumor-induced hypofunction that improved with overnight rest away from tumor. The hypofunction was also demonstrated in T cells redirected against a nonmesothelin antigen (mFAP) after infiltration in our NSG xenograft model. Whether the immunosuppression occurred because of EMMESO tumor or the murine fibroblasts within the tumor stroma will be investigated. Other groups have demonstrated that tumor-associated fibroblasts also play a significant immunosuppressive role (56).

Limitations of the preclinical model should also be acknowledged. The NSG mice have a hybrid immune system, thus limiting the ability to study the effects of important tumor immune cells such at T-regulatory cells, natural killer (NK) cells, dendritic cells, B cells, and myeloid cells. The innate immune cells present in NSG mice are not isologous with the human T cells and tumor cells. The use of mice with more fully "humanized" immune systems may be advantageous. Finally, there may be some species incompatibility issues (i.e., some ligands in murine microenvironment might not react with human TCRs). Despite these potential problems, the CAR TILs isolated from the tumors do seem to strongly resemble human TILs, suggesting that the key interactions that induce T-cell hypofunction are present.

In addition to studying mechanisms of tumor-induced T-cell hypofunction, models such as ours will allow testing of a wide variety of therapeutic approaches in an *in vivo* setting where anti-human reagents can be used. One strategy would be systemic administration of agents that might affect the tumor microenvironment. These could include agents like (i) inhibitors of soluble inhibitory mediators such as TGFβ or PGE2, (ii) activating cytokines such as IL2, IL12, IL15, or type I interferons, (iii) small molecular inhibitors of intrinsic inhibitors like DGK and SHP1, (iv) agents that might alter tumor pH or oxidative status, and (v) anti–T-cell inhibitory receptor antibodies like anti-PD1, anti-PDL1, anti-TIM3, etc. Another promising strategy will be to introduce genetic changes into the human T cells along with the CAR to prevent or modulate hypofunction. This approach would allow highly specific alterations and avoid any systemic toxicity. There are a wide variety of possibilities that include introduction of new gene genes such as cytokines (i.e., IL12; refs. 57) or stimulatory proteins, such as constitutively active AKT (58). It should also be possible to express shRNA, dominant-negative constructs, or intracellular antibodies to reduce or block the expression or activities of inhibitory surface receptors (such as the TGFβ receptor; ref. 59), PD1 and Tim3 or intrinsic inhibitors (such as DGK and SHP1). On the basis of our present findings, we plan to test anti-PD1 antagonists with CAR T cells in future clinical trials.

Disclosure of Potential Conflicts of Interest

M.C. Milone reports receiving commercial research support from and is a consultant/advisory board member for Novartis. C.H. June reports receiving a commercial research grant and speakers' bureau honoraria from Novartis. E.J. Wherry reports receiving commercial research grants from Bristol-Myers Squibb, Janssen Pharmaceuticals, and Ono Pharmaceutical, and has ownership interest (including patents) in Genentech. No potential conflicts of interest were disclosed by the other authors.

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