Augmentation of CAR T-cell Trafficking and Antitumor Efficacy by Blocking Protein Kinase A Localization

Kheng Newick, Shaun O’Brien, Jing Sun, Veena Kapoor, Steven Maceyko, Albert Lo, Ellen Puré, Edmund Moon, and Steven M. Albeda

Abstract

Antitumor treatments based on the infusion of T cells expressing chimeric antigen receptors (CAR T cells) are still relatively ineffective for solid tumors, due to the presence of immunosuppressive mediators such as prostaglandin E2 (PGE2) and adenosine and poor T-cell trafficking. PGE2 and adenosine activate protein kinase A (PKA), which then inhibits T-cell receptor (TCR) activation. This inhibition process requires PKA to localize to the immune synapse via binding to the membrane protein ezrin. We generated CAR T cells that expressed a small peptide called the "regulatory subunit I anchoring disruptor" (RIAD) that inhibits the association of PKA with ezrin, thus blunting the negative effects of PKA on TCR activation. After exposure to PGE2 or adenosine in vitro, CAR-RIAD T cells showed increased TCR signaling, released more cytokines, and showed enhanced killing of tumor cells compared with CAR T cells. When injected into tumor-bearing mice, the antitumor efficacy of murine and human CAR-RIAD T cells was enhanced compared with that of CAR T cells, due to resistance to tumor-induced hypofunction and increased T-cell infiltration of established tumors. Subsequent in vivo assays showed that both mouse and human CAR-RIAD cells migrated more efficiently than CAR cells did in response to the chemokine CXCL10 and also had better adhesion to various matrices. Thus, the intracellular addition of the RIAD peptide to adoptively transferred CAR T cells augments their efficacy by increasing their effector function and by improving trafficking into tumor sites. This treatment strategy, therefore, shows potential clinical application for treating solid tumors.

Introduction

The adoptive transfer of T cells transfected with chimeric antigen receptor (CAR) genes is showing great promise in treating bloodborne tumors (1–3). However, in the case of solid tumors, many immunosuppressive factors exist within the tumor microenvironment (TME) that appear to render these CAR T cells ineffective (4–10). Adenosine, a purine nucleoside present at high concentrations during hypoxia (11), and prostaglandin E2 (PGE2), a small molecule derivative of arachidonic acid produced by the inducible cyclooxygenase 2 enzyme (COX2), are potent inhibitors of T-cell proliferation and activity via signaling through their own G-coupled receptors to activate protein kinase A (PKA) in a cyclic AMP (cAMP)–dependent manner (12–17). PKA localizes to the immune synapse and then affects multiple proteins in the T-cell signaling cascade (18–20). One of the most important and proximal effects is the phosphorylation of serine-364 on the kinase Csk, resulting in activation. Activated Csk then phosphorylates the key signaling molecule Lck on tyrosine-505, which inhibits its activity. This leads to the subsequent inhibition of T-cell signaling and impaired T-cell receptor (TCR)–induced T-cell proliferation and cytotoxic ability. The activation state of Csk appears to be important in setting the TCR signaling threshold and affinity recognition (21).

The PKA holoenzyme is a heterotetramer consisting of two regulatory (R) subunits that can further be categorized into type I PKA (consisting of RI subunits) and type II PKA (consisting of RII subunits), and two catalytic (C) subunits (19). After binding to CAMP, the R subunits dissociate, and the C subunits proceed to phosphorylate a myriad of target substrates. Given the negative effects of PKA activation described above, much effort has been invested in manipulating the PKA pathway in T cells to improve tumor killing, including the generation of dominant-negative PKA constructs (22–24), the use of receptor agonists/antagonists (25–27), and PKA inhibitors (28), as well as genetic manipulation of the PKA system in various mouse models (12).

An alternative inhibitory approach takes advantage of the fact that in order for PKA to elicit its functions, it must be tethered to lipid rafts in close proximity to the CAMP-generating enzyme adenyl cyclase. This spatial regulation is mediated by so-called "A-kinase anchoring proteins (AKAP)" that serve as a platform where CAMP and PKA signaling converge (29–31). Over 50 distinct AKAPs have been identified (reviewed in ref. 32). In 2007, Ruppelt and colleagues described the requirement of the AKAP ezrin in tethering PKA to the lipid rafts in T cells (30).

Based on this observation, peptides that bind to the RI subunit of PKA with high affinity and disrupt PKA–AKAP associations,
including the ezrin–PKA association, were designed (29, 31). One peptide, called ‘regulatory subunit I anchoring disruptor’ (RIAD), displaced PKA from lipid rafts and ultimately diminished phosphorylation of Y505 on Lck, leading to upregulated T-cell signaling (31). Later, an endogenous element upstream in the traditional A-kinase binding domain of ezrin that enhanced RIAD binding to PKA called the ‘RI specifier region,’ or RISR was identified (29). A transgenic mouse model in which RISR–RIAD was expressed in T cells under the control of the distal lck promoter was generated, and these mice exhibited heightened TCR signaling and IL2 secretion, and resistance to PGE2 and murine AIDS (33).

Given the key role of PKA signaling in the inhibition of T-cell function in tumors and our ability to genetically manipulate T cells for adoptive transfer, we hypothesized that cloning the RISR–RIAD transgene (referred to as RIAD henceforth; ref. 34) into T cells also expressing a CAR would enhance their function within the TME and result in superior tumoricidal ability as compared with CAR T cells alone.

Materials and Methods

Overall experimental design

The peptide blocking the localization of PKA to the immunologic synapse (RISR–RIAD) was cloned into retroviral and lentiviral vectors encoding CARs directed against human mesothelin (mesoCAR) or murine fibroblast activation protein (FAP). Murine and human T cells expressing human CAR (mmesoCAR and hmesoCAR, respectively) and CAR-RIAD (mmesoCAR-RIAD and hmesoCAR-RIAD, respectively) constructs were evaluated for their in vitro and in vivo functions. All experiments were performed at least thrice in independent fashions, unless otherwise indicated.

Generation of RIAD-expressing mesoCAR and FAPCAR and T-cell production

The RISR–RIAD construct (32–34), into which Myc and DDK (FLAG) tags were incorporated, was synthesized by Integrated DNA Technologies in the pDT SMART cloning plasmid. The insert was subcloned into CAR constructs in retroviral vectors that were used to transduce mouse T cells (35) and into lentiviral vectors (36) for use in human T cells as previously described. The structure of these constructs is shown in Supplementary Fig. S1. The isolation, bead activation, transduction and subsequent expansion of primary human or mouse T cells were carried out as previously described (10, 35).

The transduction efficiency with either mesoCAR or mesoCAR-RIAD cells was always checked after transduction and before each experiment. Equal numbers of mesoCAR versus mesoCAR-RIAD cells were always used for each in vitro killing/cytokine experiment (Fig. 1), in vitro migration assays (Fig. 5), and in vivo experiments (Fig. 3).

Animals

All animal protocols were approved and carried out in compliance with the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania. Studies using retroviral MigR1-transduced T cells were carried out in wild-type C57BL/6 (strain CD45.2) mice obtained from Charles River Laboratories. In some studies, murine T cells were prepared from CD45.1 C57BL/6 mice. Studies using lentiviral pTRPE-transduced human T cells were conducted in NOD/scid/IL2rg−/− (NSG) mice bred at the Children’s Hospital of Philadelphia. All test animals used were females at 10 to 12 weeks of age.

Cell lines

All cell lines used were cultured as described (10) and were routinely examined for Mycoplasma infection. AE17ova murine mesothelioma cells were obtained from the University of Western Australia (37), whereas epithelial mesothelioma (EM) human mesothelioma cells were derived from a patient’s tumor (called EMParental, or EMP; ref. 10). The SS1 scfV used in the mesothelin CAR is specific against human mesothelin, and therefore human mesothelin was stably transduced into the murine AE17ova (AE17meso) and human EM (Emmeso) mesothelioma cancer cell lines (10, 35). The murine 4662 pancreatic ductal carcinoma cell line (PDA4662) was derived from an autochthonous pancreatic tumor isolated from a fully backcrossed C57BL/6 KrasG12D;Trp53R172H;Pdx-1 Cre (RPC) mouse (13). Cell lines were not authenticated.

Antibodies

The following conjugated antibodies for flow cytometric analysis of murine cells were purchased from Biologend: CD8 (#100762), CD4 (#100406), IFNγ (#505825), IL2 (#503808), and anti-GFP (#338008). For human cells, the following were purchased from BD Biosciences: CD45 (#555483), CD8 (#555367), IL2 (#340448), CD69 (#555530), IFNγ (#562016), and TNFα (#340511); and R&D Biosystems: human mesothelin (FAB32652P). For the detection of five cells, Aqua Live/Dead (Life Technologies #L34957) was used.

PGE2 ELISA

Tumors and normal lung and liver were dissected from mice, snap frozen in liquid nitrogen, and homogenized in cold 1X PBS containing a cocktail tablet of protease inhibitors (Roche #04693116001). Homogenates were sonicated and centrifuged, and supernatants were used for PGE2 ELISA (Abcam #ab113021).

Assessment of T-cell effector functions

Functional assays performed to characterize persistence and activity of endogenous and genetically modified T cells are outlined below.

In vitro cytotoxicity and IFNγ ELISA

Triplicate wells of 5,000 luciferase-expressing parental and antigen-expressing cells were cocultured with differing ratios of CAR-expressing T cells to tumor cells as previously described (10). Cytotoxicity of T cells was evaluated the following day, and culture supernatants were collected for IFNγ ELISA (10, 35, 36). To evaluate the resistance of RIAD-expressing T cells to immunosuppression, in vitro cytotoxicity assays were also performed in the presence of PGE2 (Enzo Life Sciences; #BML-PG007) and adenosine (Sigma; #A9251).

In vivo studies

For mesoCAR and FAPCAR studies in wild-type C57BL/6 mice, 2×106 AE17meso or PDA4662 cells were subcutaneously inoculated (35, 36). Similarly, for mesoCAR
PKA Blockade Improves CAR Therapy

Figure 1.
Primary T cells transduced with mesoCAR-RIAD exhibit superior killing ability and robust IFNγ production in vitro and are resistant to adenosine and PGE2 suppression. A, human mesoCAR and mesoCAR-RIAD T cells were cocultured at various E:T ratios with parental EM (EMP) or mesothelin-expressing EM (EMmeso) cells (left). Murine mesoCAR and mesoCAR-RIAD T cells were cultured with ova- or mesothelin-expressing AE17 murine mesothelioma cells (right). These tumor cells are also stably transduced with luciferase. After overnight incubation, the number of live tumor cells was determined by quantifying luciferase activity.

B, cell culture supernatants from the assay described above were analyzed for IFNγ production via ELISA. Left, IFNγ production by human T cells; right, IFNγ production by murine T cells. C, mesoCAR and mesoCAR-RIAD human T cells were cocultured with EMmeso cells (Effectort:Target (E:T) 10:1) overnight in the presence of increasing doses of adenosine or PGE2 (left). Similarly, the coculture assay was performed for murine mesoCAR and mesoCAR-RIAD T cells and AE17meso tumor cells at an E:T ratio of 5:1 (right). Statistical analyses were performed using one-way ANOVA comparing mesoCAR and mesoCAR-RIAD cells. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. At least three independent experimental replicates were performed. Data represent means ± SEM; n = 3 replicates per condition.

Ex vivo T-cell analysis. Tumors were harvested from mice, micro-dissected, digested, and used in ex vivo tumor assays as described in detail in previous publications (10, 35, 36).

Fluorescence-activated cell sorting. Single-cell suspensions were stained for surface and intracellular markers using the previously listed antibodies based on the manufacturer’s recommendations. For intracellular cytokine staining, cells were stimulated for 4 to 6 hours at 37°C in the presence of 0.7 μg/mL GolgiStop (BD Biosciences #554724) with plate-bound 1 μg/mL anti-CD3 and/or 2 μg/mL anti-CD28 antibodies, and 30 ng/mL PMA and 1 μmol/L ionomycin. Acquisition was performed on a CyAn-ADP Analyzer (Beckman Coulter) or a BD LSRFortessa (BD Biosciences). Data were analyzed using FlowJo (TreeStar).

Transwell migration studies. An equal number of transduced mesoCAR-GFP and mesoCAR-RIAD-mCherry cells were placed in 0.5-μm polycarbonate Transwell membranes and allowed to...

Cancer Immunology Research

544

sible inhibitory elements (the cAMP 
36, 38, 39), but are limited by T-cell hypofunction induced by an 
along with the human CD3

similar experiments using murine T cells transduced with meso-

mCherry expression.

Adhesion assays. Nontreated 24-well tissue culture plates were 
were then blocked with 
PBS for 2 hours at room temperature. These substrates were 
removed prior to rinsing with PBS. Plates were then blocked with 
complete cell culture medium for 30 minutes at 37°C. An equal 
number of transduced mesoCAR-GFP and mesoCAR-RIAD-
mCherry T cells were cultured overnight with these substrates. The next day, cells were harvested and counted based on GFP and mCherry expression.

Statistical analysis

All results were reported as means ± SEM. For studies com-
paring more than two groups, one- or two-way ANOVA 
was used with the appropriate post hoc testing, with *, P ≤ 0.05; 
**, P ≤ 0.01; ****, P ≤ 0.001; and *****, P ≤ 0.0001.

Results

RIAD enhances in vitro CAR T-cell function

CAR T cells expressing the scFv from anti-human mesothelin, 
along with the human CD3ζ and 4-1BB cytoplasmic domains (mesoCAR), slow mesothelin-expressing tumors in mice (10, 35, 
36, 38, 39), but are limited by T-cell hypofunction induced by an 
immunosuppressive solid TME (10). To disarm one of the pos-
sible inhibitory elements (the cAMP–PKA pathway), we used 
modified lentiviruses (for human T cells) and retroviruses (for 
murine T cells) to express mesoCAR, with and without the RIAD 
transgene (Supplementary Fig. S2). The transduction efficiency 
with either mesoCAR or mesoCAR-RIAD was checked after trans-
duction and before each experiment. For human T cells, this 
was between 35% and 60%. For mouse T cells, this was between 60% 
and 88%.

The baseline characteristics of the human and mouse mesoCAR 
T cells versus the mesoCAR-RIAD T cells showed no consistent 
differences with regard to the CD4:CD8 ratio, differentiation 
phenotype, or expression of activation or inhibitory receptors. 
Details are provided in Supplementary Figs. S3 and S4.

In order to assess the effector functions of these T cells, trans-
duced T cells were cocultured overnight with human and murine 
mesothelioma cells expressing mesothelin. Compared with T cells 
transduced with mesoCAR alone, both human- and murine-
transduced mesoCAR-RIAD T cells generally showed enhanced killing ability (Fig. 1A) and higher IFNγ production in a dose-
dependent manner (Fig. 1B). The constructs were target specific, as 
little to no killing of tumor cells that did not express the target 
mesothelin was observed.

To further investigate the functionality and activity of meso-
CAR-RIAD T cells at baseline, we stimulated human T cells with 
human mesothelin protein coated on beads or PMA/ionomycin 
(Supplementary Fig. S5) and performed flow cytometry. The 
cytokine release at baseline and after bead stimulation was slightly 
higher in mesoCAR-RIAD T cells versus mesoCAR T cells (after 
meso-bead simulation, we saw 8.2% of mesoCAR-RIAD cells 
making IFNγ vs. only 2.4% of mesoCAR T cells). However, in 
similar experiments using murine T cells transduced with meso-
CAR vs. mesoCAR-RIAD T cells, we saw no differences in cytokine 
release after mesothelin bead exposure (data not shown).

MesoCAR-RIAD T cells were more resistant to immunosup-
pression mediated by adenosine and PGE2. An overnight cocul-
ture assay was performed with both human (Fig. 1C, left) and 
and murine (Fig. 1C, right) T cells in the presence/absence of varying 
doses of adenosine and PGE2. As expected, we observed a dose-
dependent inhibition of tumor-cell killing by mesoCAR T cells 
in the presence of adenosine or PGE2. However, killing by the 
mesoCAR-RIAD T cells was virtually unaffected by these inhibi-
tory molecules [data shown at 5:1 effector-to-target ratio (E:T) for 
human cells, and 10:1 E:T for murine cells].

TCR signaling in mesoCAR-RIAD T cells

To evaluate signaling, both human and murine CAR T cells 
(50% transduced cells) were examined at baseline and after 20 
minutes of exposure to plate-bound anti-CD3 and anti-CD28 
antibodies. In human cells (Fig. 2A, left), mesoCAR-RIAD T 
cells showed some evidence of basal activation with increased 
phosphorylation of LckY505 (an activated form of Lck) and Akt 
compared with mesoCAR T cells. After stimulation, in both 
types of cells, we observed the expected increases in Erk, 
LckY505, and Akt phosphorylation. In mesoCAR-RIAD T cells, 
slightly higher levels of phosphorylation of these molecules 
were observed. Densitometry analyses are shown in Supple-
mentary Fig. S6.

With human T cells, we were also able to obtain immunoblots 
after stimulating the cells with mesothelin-coated beads to spe-
cifically assess CAR-mediated pERK generation (Fig. 2A, right). 
Again, we saw a small increase in basal pERK levels (compare lanes 
1 and 2). Exposure to the beads modestly increased pERK levels in 
mesoCAR T cells (compare lanes 1 and 3). However, higher pERK 
levels were seen after bead exposure of mesoCAR-RIAD T cells 
(compare lanes 2 and 4, and lanes 3 and 4).

In murine T cells (Fig. 2B), there appeared to be a slight 
upregulation of pAkt at baseline. After CD3/CD28 activation, 
pERK increased in mesoCAR-RIAD T cells versus mesoCAR T cells, 
but no increase in pAkt was observed. For unclear reasons, studies 
using mesothelin-coated beads were not successful in murine 
T cells.

PKA inhibitory signaling attenuated in mesoCAR-RIAD 
T cells

PKA regulates T-cell signaling by phosphorylating the kinase 
Csk at S364, which leads to phosphorylation of the key TCR 
proximal signaling molecule, Lck, at Y505, a change that inhibits 
the activity of Lck. To confirm that this mechanism of RIAD 
inectivation was operative in our cells, we assessed the phosho-
ylation status of CskS364 and LckY505 in both human and mouse 
CAR T cells (Fig. 2C); both groups consisted of ~50% transduced 
T cells. As predicted, we observed a reduction of phosphoryla-
tion at both these residues at baseline in mesoCAR-RIAD T cells 
compared with mesoCAR T cells.

Enhanced tumor killing by mesoCAR-RIAD T cells in vivo

To compare the ability of mesoCAR-RIAD T cells versus meso-
CAR T cells to control tumor burden, we used two tumor models 
that have produce more PGE2 compared with normal lung or liver 
tissue (Supplementary Fig. S7A). Human EMMeso cells growing 
in the flanks of immunodeficient NSG mice were administered in

Published OnlineFirst April 4, 2016; DOI: 10.1158/2326-6066.CIR-15-0263

Downloaded from cancerimmunolres.aacrjournals.org on August 17, 2021. © 2016 American Association for Cancer Research.
a single intravenous dose of $10^7$ mesoCAR- or mesoCAR-RIAD–expressing primary human T cells when the tumors were $\sim 200$ mm$^3$ in size. Animals were sacrificed 32 days after CAR T-cell injection to allow analysis of tumor-infiltrating lymphocytes (TIL; see later). Although we saw clear antitumor activity, neither type of CAR T cell could cure these established tumors (Fig. 3).

EMmeso-bearing NSG mice treated with mesoCAR T cells showed significantly slower tumor progression by approximately 40% ($P \leq 0.01$) compared with untreated tumors; however, injection of mesoCAR-RIAD T cells significantly enhanced the mesoCAR antitumor effect ($P \leq 0.0001$), resulting in tumors that were 70% smaller compared with untreated tumors (Fig. 3A). mesoCAR-RIAD T cells significantly reduced the growth of Emmeso tumors compared with mesoCAR T cells, $P \leq 0.05$ (Fig. 3A).

Murine AE17meso cells were injected subcutaneously in the flanks of wild-type C57BL/6 mice, and after tumors were established (about 7–10 days after inoculation), a single dose of $10^7$ mesoCAR or mesoCAR-RIAD murine T cells was administered intravenously. Tumor growth was monitored for the next 10 to 14 days. Again, we observed some tumor slowing by mesoCAR T cells, but mesoCAR-RIAD T cells significantly reduced the growth of AE17 meso tumors compared with mesoCAR T cells, $P \leq 0.05$ (Fig. 3B).

The use of adoptively transferred RIAD-expressing cells did not nonspecifically boost endogenous antitumor activity of mouse T cells, as mesoCAR-RIAD T cells were ineffective in controlling AE17ova tumor (which do not express the cognate antigen mesothelin) progression in wild-type mice (Supplementary Fig. S7B).

**Figure 2.** RIAD attenuates PKA signaling and enhances TCR signaling in mesoCAR T cells. A (left), equal numbers of mesoCAR– and mesoCAR-RIAD–expressing human T cells were examined at baseline or exposed to immobilized CD3 and CD28 antibodies for 20 minutes. Lysates were prepared, equal amounts of protein were run on an SDS gel, and then immunoblotted for phospho-ERK (pERK), phospho-Lck at tyrosine-394 (pLck-Y394), and phospho-Akt (pAkt), along with their respective loading controls, including actin. Right, equal numbers of human mesoCAR– and mesoCAR-RIAD–expressing T cells were examined at baseline or exposed to mesothelin protein immobilized on beads for 20 minutes. Lysates were prepared, equal amounts of protein were run on an SDS gel, and then immunoblotted for phospho-ERK and actin as a loading control. B, equal numbers of murine mesoCAR– and mesoCAR-RIAD-expressing T cells were examined at baseline or exposed to immobilized CD3 and CD28 antibodies for 20 minutes. Lysates were prepared, equal amounts of protein were run on an SDS gel, and then immunoblotted for phospho-Akt (pAkt) or phospho-ERK. C, lysates from murine mesoCAR– and mesoCAR-RIAD-expressing T cells at baseline were immunoblotted for phospho-Csk at serine-364 (pCsk-S364) and pLck at tyrosine-505 (pLck-Y505). Total Erk or actin were used as loading controls (left, human T cells; right, murine T cells).
To evaluate the efficacy of RIAD in another CAR system, we utilized a construct that targets fibroblast activation protein (FAP) present on stromal cells surrounding the tumor (36). We inoculated C57BL/6 wild-type mice with the pancreatic cell line PDA4662 (13). PDA4662-bearing mice were subsequently treated with a single dose of murine $10^7$ FAPCAR or FAPCAR-RIAD T cells when the tumor burden was approximately 200 mm$^3$. At this dosage of T cells, 14 days after adoptive transfer, only FAPCAR-RIAD T cells showed statistically significant antitumor activity in this model (Fig. 3C).

To better understand the in vivo mechanisms of the enhanced antitumor effects observed with mesoCAR-RIAD T cells, EMmeso-bearing mice were sacrificed 32 days after T-cell administration. Statistical analyses were performed using one-way ANOVA comparing mesoCAR and mesoCAR-RIAD tumors at the final time point. $^*\ P < 0.05; ^{**}\ P < 0.01; ^{***}\ P < 0.001; ^{****}\ P < 0.0001$. At least three independent experimental replicates were performed. Data represent means ± SEM; $n = 5–7$ mice per group.

MesoCAR-RIAD increased the numbers and activity of tumor-infiltrating T cells

To better understand the in vivo mechanisms of the enhanced antitumor effects observed with mesoCAR-RIAD T cells, EMmeso-bearing mice were sacrificed 32 days after T-cell administration, and their tumors were pooled and processed as described in Materials and Methods. The percentage of human CD3+, CD8+, and CD4+ cells (from total live tumor cells) was significantly higher ($P < 0.05$) within the tumors of mesoCAR-RIAD–treated mice compared with mesoCAR-treated
mice (Fig. 4A; Supplementary Fig. S8A). The majority of these T cells were CD8\(^+\), with only a small percentage being CD4\(^+\) cells (an approximately 10:1 ratio of CD8\(^+\) to CD4\(^+\) T cells).

To examine a model in which T cells can be measured at an earlier time point, we used the murine AE17meso model. Three days after adoptive transfer, we analyzed the murine TIL population by flow cytometry. As with the human model, we also observed a greater influx of murine mesoCAR-RIAD CD8\(^+\) T cells into the tumors by flow cytometry (\(P \leq 0.05\); Fig. 4B). Again, CD8\(^+\) TILs outnumbered CD4\(^+\) TILs at an approximately 10:1 ratio (Supplementary Fig. S8B).

Figure 4. RIAD transgene enhances mesoCAR T-cell persistence and activity in vivo. A, EMmeso tumors harvested at day 32 after T-cell transfer were digested, pooled, and analyzed using flow cytometry analysis. Cells were stained with anti-human CD8, CD3, and a live/dead stain. Left, representative flow tracings with increased CD8 T cells in the mesoCAR-RIAD-treated tumors. Right, bar chart shows quantification of tumor digests from 7 mice per group. The frequency of live CD4\(^+\) T cells was calculated from the difference between the total frequency of live CD3\(^+\), CD8\(^+\), and CD4\(^+\) T cells in the mesoCAR-RIAD-treated tumors, although the number of CD4\(^+\) T cells was low. B, AE17meso tumors harvested at day 3 after T-cell transfer were digested and analyzed. Total tumor digests were stained with anti-murine CD45.1 to recognize adoptively transferred cells (left). Bar chart shows frequencies by treatment group (representative of 5 mice per group). C, TILs were freshly isolated from human mesoCAR- and mesoCAR-RIAD–treated tumors (from A) or allowed to rest overnight in media. The cells were subjected to an overnight luciferase-based cytolysis assay against EMmeso cells to determine the percentage of killing of tumor cells (left). Supernatants from the assay were also measured for IFN\(\gamma\) production (right). Freshly harvested mesoCAR TILs were hypofunctional with respect to both killing and IFN\(\gamma\) secretion and had some recovery after "rest." In contrast, the freshly harvested mesoCAR-RIAD TILs retained almost full cytolytic capability and enhanced IFN\(\gamma\) secretion compared with mesoCAR TILs.

To study the function of the adoptively transferred CARs from EMmeso tumors, we isolated human T cells and measured the ex vivo ability of these TILs (<i>"at harvest"</i>) to kill tumor cells and secrete IFN\(\gamma\) compared with CAR TILs that had been allowed to "rest" overnight (Fig. 4C). In agreement with our previous studies (10), freshly harvested mesoCAR TILs were hypofunctional with respect to both killing and IFN\(\gamma\) secretion and had some recovery after "rest." In contrast, the freshly harvested mesoCAR-RIAD TILs retained almost full cytolytic capability and enhanced IFN\(\gamma\) secretion compared with mesoCAR TILs.
MesoCAR-RIAD cells show enhanced migratory ability

Because one explanation for the increased numbers of mesoCAR-RIAD TILs was enhanced migration to tumor sites, we performed in vitro transmigration assays in which transduced T cells marked by GFP or mCherry (see Supplementary Fig. S1) were allowed to migrate toward the indicated stimulants placed in the bottom well. After 4 hours, migrated cells were collected and counted. Data represent means ± SEM; n = 3 replicates per condition. B, the CAR T cells used for the transmigration assay in A were stained for baseline surface expression of CXCR3. C, overnight adhesion assays to the indicated substrates were performed using human T cells (left) and murine T cells (right) overnight. The number of adherent cells is plotted. Data represent means ± SEM; n = 3 replicates per condition. D, the CAR T cells used for the overnight adhesion assay in C were stained for baseline surface expression of adhesion receptors. Statistical analyses were performed using one-way ANOVA as indicated. *, P ≤ 0.05; **, P ≤ 0.01; ****, P ≤ 0.001; *****, P ≤ 0.0001. At least three independent experimental replicates were performed.

Discussion

Although adoptive T-cell therapy using CARs for cancer has shown great promise in the treatment of bloodborne malignancies (1–3), their application in the treatment in solid tumors has not yet been as successful. One reason for this is the inefficiency of trafficking of CARs into solid tumors. A second cause appears to be
the rapid loss of secretory and cytolytic function of the TILs induced by factors within the TME (such as TGFβ) and by the upregulation of intrinsic negative regulators [such as PD-1 and diacylglycerol kinase (DGK); refs. 14, 35], a phenomenon also documented in endogenous TILs (4, 5). Attempts to overcome these limitations include introducing chemokine receptors into CAR cells (39), reducing expression levels of DGK (35), or using dominant-negative TGFβ receptors to prevent TGFβ-mediated inactivation of cytotoxic T lymphocytes in the TME (40–42).

Soluble mediators also likely play a role. Two major inhibitors of T-cell function present to varying degrees in the TME are adenosine and PGE2 (reviewed in refs. 43, 44). Extracellular adenosine produced by tumors cells and regulatory T cells may be an especially prominent trigger of TGFβ generation in hypoxic TMEs (reviewed in ref. 15). In this study, we demonstrated the importance of adenosine and PGE2 in CAR dysfunction by showing that the presence of either agent during coculture of CART cells with tumor cells results in a decrease in tumor cell killing. We also know that high concentrations of PGE2 are present in our experimental tumor models.

The peptides RISR and RIAD (31, 33) can block PKA activity at the TCR level in transgenic mice, suggesting that the coexpression of these transgenes with our CAR constructs in T cells might protect them from TGFβ-mediated immunosuppression and thus render them resistant to both adenosine- and PGE2-mediated inhibition.

This strategy appeared highly effective. Consistent with our proposed mechanism, we saw a decrease in the baseline phosphorylation of Csk at S364 and reduced phosphorylation of Csk at Y305 in the CAR-RIAD cells. Both human and mouse T cells expressing the CAR-RIAD constructs were almost completely resistant to the in vitro inhibitory effects of adenosine and PGE2.

In addition to their resistance to adenosine and PGE2-mediated inhibition, mesoCAR-RIAD T cells were more tumoricidal and released more IFNγ, even in the absence of added adenosine or PGE2. This may be due to the endogenous secretion of these mediators by the tumor cells in culture. However, the basal activation state of mesoCAR-RIAD T cells, especially the human cells, was increased in the absence of tumor cells. In addition, exposure of cells to mesothelin-coated beads resulted in increased cytokine secretion in the mesoCAR-RIAD cells compared with the mesoCAR T cells. Because these studies were done in a tumor-free setting, these data are consistent with the concept that a certain level of intrinsic “tonic” PKA activity exists in our CART T cells that may function to set a basal signaling tone. It is likely that this “tone” is set via the effect of PKA on Csk, as the observation that Csk activity levels establish the TCR threshold is well established (21, 45).

Furthermore, a basal level of PKA activity exists in human peripheral blood T cells, especially in CD8+CD45RO+ cells, the phenotype of our human CART cells (46). It is unclear at this time whether this basal activity is cell intrinsic or if there is some endogenous secretion of PGE2 or adenosine that feeds back into the cells through cognate receptors.

In agreement with our in vitro data, mesoCAR-RIAD T cells were more efficacious in a number of tumor models compared with mesoCAR T cells, including immunodeficient NSG mice bearing Emmeso human mesothelioma tumors treated with human T cells, wild-type C57BL/6 mice bearing established AE17meso mouse mesothelioma tumors treated with mouse T cells, and wild-type C57BL/6 mice bearing established PDA4662 mouse pancreatic tumors treated with mouse T cells. Most of this effect was probably due to resistance to the inhibitory influences of PGE2 and/or adenosine in the TME, because freshly isolated human mesoCAR-RIAD TILs had much more ex vivo antitumor activity than did similarly processed CAR TILs. However, it is possible that the increased basal and stimulated activity of the mesoCAR-RIAD T cells may also be playing a role. Despite their enhanced reactivity, no nonspecific activity against non-antigen-expressing cells in vitro or in vivo was detected.

Using these models, at least two mechanisms responsible for the enhanced efficacy of mesoCAR-RIAD T cells were identified. Consistent with our in vitro data, mesoCAR-RIAD TILs were more functional than mesoCAR TILs. As previously reported (10), freshly isolated human TILs from mesoCAR T cell–treated mice had reduced ability to kill tumor cells or release IFNγ in our ex vivo assay. In contrast, freshly isolated TILs from mesoCAR-RIAD T cell–treated mice were nearly equivalent in function to the infused product.

We also observed (somewhat unexpectedly) that the number of CD8 mesoCAR T cells infiltrating the tumors was significantly higher in the mesoCAR-RIAD T cell–treated mice compared with the mesoCAR T cell–treated mice in both the NSG and syngeneic models (Fig. 4A and B). Although both CD4+ and CD8+ T cells increased, the CD8+ cells outnumbered the CD4+ by a ratio of 10:1. We did not directly study the relative importance of CD8+ versus CD4+ T cells in our model, but there is good evidence that both types of cells are needed for full CART-cell efficacy (47, 48).

In the NSG model, this could be due to enhanced persistence and/or increased trafficking of mesoCAR-RIAD T cells. However, seeing increased infiltration of adoptively transferred T cells in tumors in the syngeneic mouse model at only 3 days after adoptive transfer suggests that the increased infiltration was due to enhanced trafficking. Transmigration assays using the chemokine CXCL10 (which binds to the CXCR3 chemokine receptor that is highly expressed on CAR T cells) revealed significantly better mesoCAR-RIAD T-cell migration. Overnight adhesion assays also showed that mesoCAR-RIAD T cells showed significantly better adhesion to fibronectin, ICAM, and VCAM ligands, thus potentially contributing to their enhanced tumor trafficking. These changes were associated with increased expression of the chemokine receptor CXCR3 and the CD49d integrin (VLA-4). The differential baseline expression of these adhesion receptors is reflective of the complexity of leukocyte mobilization during an immune response, as previously reported (16).

There is abundant literature describing the effects of PKA on leukocyte cell migration and adhesion, although the reported effects are complex and somewhat cell type specific (reviewed in ref. 17). With regard to lymphocytes, elevated intracellular cAMP inhibits RhoA activation and integrin-dependent leukocyte adhesion induced by chemoattractants (49). This observation fits with our data showing that inhibition of PKA by RIAD augmented T-cell migration and adhesion in vitro and T-cell migration in vivo.

Our study has a number of potential limitations. In our models, PGE2 concentrations were relatively high, but different tumors likely vary in their expression of PGE2 or adenosine, which could affect the degree of RIAD-induced augmentation seen. The potential immunogenicity of the RISR–RIAD peptide may also be an issue for clinical translation; however, analysis of RISR/RIAD in silico showed no immunogenic neoepitopes for common HLA types. Lastly, the intrinsic limitations in the animal models used should be considered because no animal model can completely...
predict the response that might occur in patients. The syngeneic mouse models using mouse CART cells have an intact immune system and complete species compatibility, but the behavior and persistence of adoptively transferred mouse T cells are clearly different than those of human T cells. The NSG model we used allows the study of human T cells and human tumors, but lacks many elements of an actual human TME (i.e., regulatory T cells, tumor-associated macrophages, etc.), and is a chimeric system in which not all mouse cytokines are cross-reactive with human cells and vice versa. However, by showing similar effects using both types of models (with human and mouse T cells) and different CARs and CAR targets, we feel that the potential applicability of our approach to human CART T-cell clinical trials has been shown.

There is a clear need for approaches to overcome the many potential limitations of CARs in the treatment of solid tumors. The genetic addition of the RIAD transgene (which is rather small) should be feasible for virtually any CAR (or T cells with transgenic TCRs) in a bicistronic fashion. We thus propose that the addition of RIAD could be an important strategy to augment CAR efficacy in the treatment of solid tumors where an immunosuppressive milieu exists, trafficking is limited, and antitumor responses have been suboptimal.

Disclosure of Potential Conflicts of Interest
S.M. Albelda reports receiving commercial research support from Novartis.

No other potential conflicts of interest were disclosed by the other authors.

References

Augmentation of CAR T-cell Trafficking and Antitumor Efficacy by Blocking Protein Kinase A Localization

Kheng Newick, Shaun O’Brien, Jing Sun, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-15-0263

Supplementary Material
Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2016/04/02/2326-6066.CIR-15-0263.DC1

Cited articles
This article cites 48 articles, 26 of which you can access for free at:
http://cancerimmunolres.aacrjournals.org/content/4/6/541.full#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
http://cancerimmunolres.aacrjournals.org/content/4/6/541.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerimmunolres.aacrjournals.org/content/4/6/541.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.