Published in final edited form as:

Cancer Immunol Res. 2016 June ; 4(6): 541–551. doi:10.1158/2326-6066.CIR-15-0263.

Augmentation of CAR T cell trafficking and antitumor efficacy by blocking protein kinase A (PKA) localization

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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 had enhanced killing of tumor cells compared to CAR T cells. When injected into tumor-bearing mice, murine and human CAR-RIAD T cells antitumor efficacy was enhanced compared to CAR T cells, due to resistance to tumor-induced hypofunction and increased T cell infiltration of established tumors. Subsequent *in vitro* assays showed that both mouse and human CAR-RIAD cells migrated more efficiently than CAR cells in response to the chemokine CXCL10 and also adhered better 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.

Keywords

Chimeric antigen receptor T cells; protein kinase A; adoptive T cell transfer; tumor microenvironment; immunotherapy

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Competing Interests: SMA has received a commercial research grant from Novartis. KN and SMA have applied for a patent covering the use of RISR-RIAD in adoptive T cell transfer. No other potential conflicts of interest were disclosed.

Introduction

The adoptive transfer of T cells transfected with chimeric antigen receptor (CAR) genes is showing great promise in treating blood-borne tumors (¹⁻³). However, in the case of solid tumors, many immunosuppressive factors exist within the tumor microenvironment 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 adenylyl cyclase. This spatial regulation is mediated by so called “A-kinase anchoring proteins (AKAPs)” that serve as a platform where cAMP and PKA signaling converge (²⁹⁻³¹). Over 50 distinct AKAPs have been identified (reviewed in (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 interleukin 2 (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) (³⁴) into T cells also expressing a CAR would enhance their function within the tumor microenvironment and result in superior tumoricidal ability as compared to CAR T cells alone.

Materials and Methods

Overall experimental design

The peptide blocking the localization of PKA to the immunologic synapse (RISRRIAD) 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 pIDT.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 Supplemental 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 was always checked after transduction and before each experiment. Equal numbers of mesoCAR vs 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/IL2r^{-/-} (NSG) mice bred at the Children's Hospital of Philadelphia. All test animals used were females at 10-12 weeks of age.

Cell lines

All cell lines used were cultured as described (¹⁰), and routinely examined for *Mycoplasma* infection. AE17ova murine mesothelioma cells were obtained from the University of Western Australia (³⁷), whereas EM human mesothelioma cells were derived from a patient's tumor (called EMParental, or EMP) (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 *Kras*^{G12D}:*Tip53*^{R172H}:*Pdx-1* Cre (KPC) mouse (¹³). Cell lines were not authenticated.

Antibodies

The following conjugated antibodies for flow cytometric analysis of murine cells were purchased from Biolegend: CD8 (#100762), CD4 (#100406), IFN γ (#505825), IL2 (#503808), and anti-GFP (#338008). For human cells, the following were purchased from Biolegend: FOXP3 (#320106); BD Biosciences: CD45 (#555483), CD8 (#555367), IL2 (#340448), CD69 (#555530), IFN γ (#562016), and TNF α (#340511); and R&D Biosystems: human mesothelin (FAB32652P). For the detection of live 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 #ab133021).

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 interferon- γ (IFN γ) ELISA**—Triplicate wells of 5000 luciferase-expressing parental and antigen-expressing cells were co-cultured 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 IFN γ 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, 2×10^6 AE17meso or PDA4662 cells were subcutaneously inoculated (^{35, 36}). Similarly, for mesoCAR studies in immunodeficient NSG mice, 2×10^6 EMmeso cells were subcutaneously inoculated (¹⁰). When tumors were approximately 200 mm³, mice were given 10^7 CAR-expressing T cells via intravenous administration (^{10, 35, 36}). Tumor

volume was monitored by caliper measurements twice weekly, and at different time points, tumor-bearing mice were sacrificed for mechanistic studies using immunoblotting and/or flow cytometry. All experiments had a minimum of 5 mice per group and were performed at least three times.

Immunoblotting—The phosphorylation status of various proximal T cell signaling entities were assessed by immunoblotting (pERK, pAkt, pLck) using CAR T cells and plate-bound CD3/CD28 antibodies (³⁵). T cells were also exposed to mesothelin-coated beads as described (³⁵) and harvested after 20 minutes. Additionally, antibodies for the detection of pLck^{Y505} were purchased from Cell Signaling Technologies (#2751S), and pCsk^{S364} from Abcam (#ab61782).

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}).

T-cell stimulation and flow cytometry (FACS)—Single-cell suspensions were stained for surface and intracellular markers using the previously listed antibodies based on the manufacturers' recommendations. For intracellular cytokine staining, cells were stimulated for 4-6 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 µM ionomycin. Acquisition was performed on a CyAn-ADP Analyzer (Beckman Coulter) or a BD LSRFortessa (BD Biosciences). Data was analyzed using FlowJo (TreeStar).

Transwell migration studies—Equal number of transduced mesoCAR-GFP and mesoCAR-RIAD-mCherry cells were placed in 0.5 µm polycarbonate transwell membranes, and allowed to migrate toward PBS, cell culture medium alone, or with different concentrations of the chemokine CXCL10 in tissue culture plates. After 4 hours, migrated cells in the bottom wells were collected and counted based on GFP and mCherry expression.

Adhesion assays—Non-treated 24-well tissue culture plates were coated with 5 µg/ml fibronectin, ICAM1, and VCAM1 in 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. Equal numbers 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 comparing 2 groups, the Student *t* test was used, whereas for studies comparing more than 2 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 CD38 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 tumor microenvironment (10). To disarm one of the possible 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 (Supplemental Fig. S2). The transduction efficiency with either mesoCAR or mesoCAR-RIAD was checked after transduction and before each experiment. For human T cells, this was between 35-60%. 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 CD4:CD8 ratio, differentiation phenotype, or expression of activation or inhibitory receptors. Details are provided in Supplemental Figs. S3 and S4.

In order to assess the effector functions of these T cells, transduced T cells were cocultured overnight with human and murine mesothelioma cells expressing mesothelin. Compared to T cells transduced with mesoCAR alone, both human and murine transduced mesoCAR-RIAD T cells generally showed enhanced killing ability (Fig. 1A) and higher IFN γ 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 mesoCAR-RIAD T cells at baseline, we stimulated human T cells with human mesothelin protein-coated on beads or PMA/ionomycin (Supplemental 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 stimulation, we saw 8.2% of mesoCAR-RIAD cells making IFN γ vs only 2.4% of mesoCAR T cells). However, in similar experiments using murine T cells transduced with mesoCAR vs mesoCAR-RIAD, we saw no differences in cytokine release after mesothelin bead exposure (data not shown).

MesoCAR-RIAD T cells were more resistant to immunosuppression mediated by adenosine and PGE $_2$. An overnight coculture assay was performed with both human (Fig. 1C; left panels) and murine (Fig. 1C; right panels) T cells in the presence/absence of varying doses of adenosine and PGE $_2$. As expected, we observed a dose-dependent inhibition of tumor-cell killing by mesoCAR T cells in the presence of adenosine or PGE $_2$. However, killing by the mesoCAR-RIAD T cells was virtually unaffected by these inhibitory molecules (data shown at 5:1 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 panel), mesoCAR-RIAD T cells showed

some evidence of basal activation with increased phosphorylation of Lck^{Y394} (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, Lck^{Y394}, and Akt phosphorylation. In mesoCAR-RIAD T cells, slightly higher levels of phosphorylation of these molecules were observed. Densitometry analyses are shown in Supplemental Fig. S6.

With human T cells, we were also able to obtain immunoblots after stimulating the cells with mesothelin-coated beads to specifically assess CAR-mediated pERK generation (Fig. 2A, right panel). 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 inactivation was operative in our cells, we assessed the phosphorylation status of Csk^{S364} and Lck^{Y505} 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 phosphorylation at both these residues at baseline in mesoCAR-RIAD T cells compared to mesoCAR T cells.

Enhanced tumor killing by mesoCAR-RIAD T cells in vivo

To compare the ability of mesoCAR-RIAD T cells versus mesoCAR T cells to control tumor burden, we used two tumor models that have produce more PGE₂ compared to normal lung or liver tissue (Supplemental Fig. S7A). Human EMmeso cells growing in the flanks of immunodeficient NSG mice were administered a single intravenous dose of 10⁷ mesoCAR- or mesoCAR-RIAD-expressing primary human T cells when the tumors were ~200 mm³ in size. Animals were sacrificed 32 days after CAR T cell injection to allow analysis of tumor infiltrating lymphocytes (TILs) (below). 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% ($P = 0.01$) compared to untreated tumors; however, injection of mesoCAR-RIAD T cells significantly enhanced the mesoCAR antitumor effect ($P = 0.0001$), resulting in tumors that were 70% smaller compared to untreated tumors (Fig. 3A). mesoCAR-RIAD T cells significantly reduced the growth of EMmeso tumors compared to mesoCAR T cells, $P = 0.05$ (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 10⁷ mesoCAR or mesoCAR-RIAD murine T cells were administered intravenously. Tumor

growth was monitored for the next 10-14 days. Again, we observed some tumor slowing by mesoCAR T cells, but mesoCAR-RIAD T cells significantly reduced the growth of AE17meso tumors compared to mesoCAR T cells, $P = 0.05$ (Fig. 3B).

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

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 dose of T cells, fourteen days post-adoptive transfer, only FAPCAR-RIAD T cells showed significant antitumor activity in this model (Fig. 3C).

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 on 32 days after T cell administration, and their tumors were pooled and processed as described in Methods. The percent 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 to mesoCAR-treated mice (Fig. 4A and Supplemental Fig. S8A). The majority of these T cells were CD8^+ , with only a small percentage being CD4^+ cells (an approximate 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 ($P = 0.05$) (Fig. 4B). Again, CD8^+ TILs outnumbered CD4^+ TILs at an approximate 10:1 ratio (Supplemental Fig. S8B).

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 (“at harvest”) to kill tumor cells and secrete $\text{IFN}\gamma$ compared to 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 $\text{IFN}\gamma$ secretion and had some recovery after “rest”. In contrast, the freshly harvested mesoCAR-RIAD TIL retained almost full cytolytic capability and enhanced $\text{IFN}\gamma$ secretion compared to mesoCAR TILs.

MesoCAR-RIAD cells show enhanced migratory ability

Since 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 Supplemental Fig. S1) were allowed to traffic toward

the T cell chemokine CXCL10. We observed higher CXCL10-mediated migration rate in mesoCAR-RIAD T cells compared to mesoCAR T cells in a dose-dependent fashion in both human and mouse T cells (Fig. 5A). To further explore this augmentation of chemotactic ability, we assessed the expression of the key chemokine receptor responsible for CXCL10 transmigration (that is, CXCR3). As shown in Fig. 5B, both human and mouse mesoCAR-RIAD cells expressed significantly ($P = 0.05$) higher CXCR3 than did mesoCAR T cells. We also studied the ability of these T cells to adhere to relevant substrates (fibronectin, VCAM-1, and ICAM-1) that might be expressed on tumor endothelium or tumor cells. Overnight adhesion assays showed that both human and murine mesoCAR-RIAD cells adhered significantly better to these substrates than mesoCAR T cells (Fig. 5C). At baseline, mesoCAR T cells did not show enhanced expression of CD11a (LFA-1) or CD18. However, mesoCAR-RIAD T cells did show significantly increased expression of the subunits of the VLA4 receptor (CD49a and CD29) that bind VCAM-1 and fibronectin (Fig. 5D).

Discussion

Although adoptive T cell therapy using chimeric antigen receptors (CARs) for cancer has shown great promise in the treatment of blood-borne 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 tumor microenvironment (such as transforming growth factor (TGF) β) and by the upregulation of intrinsic negative regulators (such as PD1 and diacylglycerol kinase (DGK) (^{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 by using dominant negative TGF β receptors to prevent TGF β -mediated inactivation of cytotoxic T lymphocytes in the tumor microenvironment (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 (^{43, 44})). Extracellular adenosine produced by tumors cells and regulatory T cells may be an especially prominent trigger of cAMP generation in hypoxic tumor microenvironments (reviewed in (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 CAR T 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 co-expression of these transgenes with our CAR constructs in T cells might protect them from cAMP/PKA-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 Lck at Y505 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 IFN γ , 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 to the mesoCAR cells. Since 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 CAR T cells that may function to set a basal signaling tone. It is likely 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 CAR T 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, since 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 non-specific 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 IFN γ 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 to the mesoCAR T cell-treated mice in both the NSG and syngeneic models (Figs. 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 CAR T-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 post-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 adhered significantly better 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 (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*.

There are a number of potential limitations of our study. 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 employed should be considered since no animal model can completely predict the response that might occur in patients. The syngeneic mouse models using mouse CAR T 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 that 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 tumor microenvironment (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 the potential applicability of our approach to human CAR 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Omkar Kawalekar for providing mesothelin-coated beads. We also would like to thank Dr. Astero Klabatsa, Soyeon Kim, and Naomi Saint Jean for helpful input and discussion.

Financial support: This project was supported by NCI (National Cancer Institute) grants P01 CA 66726-07 (to SMA), R21 CA169741, R01 CA141144 and R01 CA 172921 (to SMA and EP), a research grant from Novartis Pharmaceuticals Corporation, and funding from the Lung Cancer Translational Center of Excellence at UPenn. KN was funded by T32 HL07586. SO was funded by T32 CA09140.

Abbreviations Used

CAR	Chimeric antigen receptor
TCR	T cell receptor
PGE2	prostaglandin E2
PKA	protein kinase A
RIAD	regulatory subunit I anchoring disruptor
TILs	tumor-infiltrating lymphocytes
mmesoCAR	murine mesothelin CAR T cells
hmesoCAR	human mesothelin CAR T cells

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Fig. 1. Primary T cells transduced with mesoCAR-RIAD exhibit superior killing ability and robust IFN γ production in vitro and are resistant to adenosine and PGE₂ suppression

A. Human mesoCAR and mesoCAR-RIAD T cells were cocultured at various effector-to-target (E:T) ratios with parental EM (EMP) or mesothelin-expressing EM (EMmeso) cells (left panel). Murine mesoCAR and mesoCAR-RIAD T cells were cultured with ova- or mesothelin-expressing AE17 murine mesothelioma cells (right panel). 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 IFN γ production via ELISA. Left panel shows IFN γ production by human T cells and the right panel shows IFN γ production by murine T cells.

C. mesoCAR and mesoCAR-RIAD human T cells were co-cultured with EMmeso cells (E:T ratio of 10:1) overnight in the presence of increasing doses of adenosine or PGE₂ (left panels). 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 panels).

Statistics 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 shown are means $\pm$ SEM, n = 3 replicates per condition.

Fig. 2. RIAD attenuates PKA signaling and enhances TCR signaling in mesoCAR T cells

A. In the left panel, 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 a 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.

In the right panel, 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 a 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 a 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 panel showing human T cells, and right panel showing murine T cells).

Fig. 3. Enhancement of tumor control by mesoCAR-RIAD T cells in vivo

A. EMMeso human tumor cells (2×10^6 cells) were injected subcutaneously into the flanks of immunodeficient NSG mice and after tumors were established (~ 150 - 200 mm^3 in volume), 10 million mesoCAR- or mesoCAR-RIAD-expressing human T cells were administered via tail vein injections. Tumor development was monitored using calipers for the next 32 days after T cell administration, at which time animals were sacrificed for *ex vivo* analyses.

B. AE17meso murine tumors cells (2×10^6 cells) were injected subcutaneously into the flanks of C57BL/6 mice, and after they were established ($\sim 150 \text{ mm}^3$ in volume), mesoCAR- or mesoCAR-RIAD-expressing murine T cells (10^7) were administered via tail vein injections. Tumors were measured and mice were sacrificed 10 days after T cells administration.

C. PDA4662 murine pancreatic cancer cells (10^6 cells) were subcutaneously injected into C57BL/6 mice, and FAPCAR- and FAPCAR-RIAD-expressing T cells (10^7 cells) were injected via the tail vein. Mice were monitored and sacrificed at Day 23 post-T cell administration.

Statistics were performed using one-way ANOVA comparing mesoCAR and mesoCAR-RIAD tumors at the final time point. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. At least three independent experimental replicates were performed. Data shown are means $\pm$ SEM, $n = 5$ - 7 mice per group.

Fig. 4. RIAD transgene enhances mesoCAR T cell persistence and activity in vivo

A. EMmeso tumors harvested at Day 32 post 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. The left panel shows representative flow tracings with increased CD8 T cells in the mesoCAR-RIAD-treated tumors. The right panel 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⁺ and CD8⁺ T cells. There were significant increases in 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 post T-cell transfer were digested and analyzed. Total tumor digests were stained with anti-murine CD45.1 to recognize adoptively transferred cells (left panel). 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 4A) or allowed to rest overnight in media. The cells were subjected to an overnight luciferase-based cytotoxicity assay against EMmeso cells to determine the percent killing of tumor cells (left panel). Supernatants from the assay were also measured for IFN γ production (right panel). Freshly harvested mesoCAR TIL were hypofunctional with respect to both killing and IFN γ secretion and had some recovery after “rest”. In contrast, the freshly harvested mesoCAR-RIAD TIL retained almost full cytolytic capability and enhanced IFN γ secretion compared to mesoCAR TIL.

Fig. 5. RIAD transgene enhances mesoCAR T cell chemotaxis and adhesion in vitro

A. Equal numbers of transduced human (left panel) and murine (right panel) mesoCAR or mesoCAR-RIAD T cells were placed in transwell inserts, and allowed to migrate toward the indicated stimulants placed in the bottom well. After 4 hours, migrated cells were collected and counted. Data shown are means $\pm$ SEM, $n = 3$ replicates per condition.

B. The CAR T cells used for the transmigration assay above 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 panel) and murine T cells (right panel) overnight. The number of adherent cells is plotted. Data shown are means $\pm$ 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.

Statistics were performed using one-way ANOVA as indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. At least three independent experimental replicates were performed.