DNAM-1-based chimeric antigen receptors enhance T cell effector function and exhibit in vivo efficacy against melanoma

Ming-Ru Wu · Tong Zhang · Andre Alcon ·
Charles L. Sentman

Received: 5 June 2014 / Accepted: 17 December 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract Chimeric antigen receptor (CAR) T cell therapies hold great potential for treating cancers, and new CARs that can target multiple tumor types and have the potential to target non-hematological malignancies are needed. In this study, the tumor recognition ability of a natural killer cell-activating receptor, DNAM-1 was harnessed to design CARs that target multiple tumor types. DNAM-1 ligands, PVR and nectin-2, are expressed on primary human leukemia, myeloma, ovarian cancer, melanoma, neuroblastoma, and Ewing sarcoma. DNAM-1 CARs exhibit high tumor cell cytotoxicity but low IFN- γ secretion in vitro. In contrast to other CAR designs, costimulatory domains did not improve the expression and function of DNAM-1 CARs. A DNAM-1/CD3zeta CAR reduced tumor burden in a murine melanoma model in vivo. In conclusion, DNAM-1-based CARs may have the potential to treat PVR and nectin-2 expressing hematological and solid tumors.

Keywords CAR · Gene therapy · Immunotherapy · Adoptive T cell therapy · Melanoma

Abbreviations

B6	C57BL/6
CAR	Chimeric antigen receptor
^{51}Cr	$^{51}\text{Chromium}$
CYP	Cytoplasmic
DC	Dendritic cell
DNAM-1	DNAX accessory molecule-1
EC	Extracellular

MFI	Mean fluorescent intensity
NK	Natural killer
PI-3 kinase	Phosphoinositide-3 kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
scFv	Single-chain variable fragment
TM	Transmembrane
TME	Tumor microenvironment
UnTd	Untransduced

Introduction

Immunotherapy holds great potential to treat cancer. Immunotherapy strategies which harness T cell effector functions, such as chimeric antigen receptor (CAR) T cell therapies, have shown promising therapeutic efficacies in clinical trials [1–4]. Although studies evaluating CAR T cell therapies are encouraging, most clinical studies have focused on targeting hematological malignancies [5]. Some CARs are designed to target solid tumors and have the potential to target multiple tumor types, such as CARs targeting α -folate receptor, EGFR, ERBB2, EGP-2, Lewis Y, and GD2 [5]. Since targeted therapy can potentially select for ligand-negative tumor escape variants [3], new CAR designs with novel anti-tumor specificities and with ability to target many different types of cancer are needed. These new CARs will increase the types of tumors that can be targeted by CAR T cell therapy and may also provide therapeutic approaches for tumor escape variants.

To design CARs that can recognize multiple tumor types, we utilized the tumor recognition ability of NK cells. NK cells employ activating receptors, such as NKG2D, NKp30, and DNAX accessory molecule-1 (DNAM-1), to recognize stress-induced ligands expressed on various tumor types [6, 7]. In this study, to expand the NK cell receptor-based

M.-R. Wu · T. Zhang · A. Alcon · C. L. Sentman (✉)
Department of Microbiology and Immunology, Geisel School
of Medicine at Dartmouth, One Medical Center Drive, Lebanon,
NH 03756, USA
e-mail: Charles.Sentman@dartmouth.edu;
Charles.L.Sentman@dartmouth.edu

CAR repertoire, we harnessed DNAM-1 receptor specificity. In humans, DNAM-1 is expressed on most NK cells, $\alpha\beta$ T cells, $\gamma\delta$ T cells, and monocytes [8]. In mice, DNAM-1 is expressed on most peripheral NK cells, $\alpha\beta$ T cells, $\gamma\delta$ T cells, and NKT cells [9, 10]. DNAM-1 is up-regulated on CD4⁺ T cells and expressed at high amounts on all CD8⁺ T cells after T cell activation [10, 11].

DNAM-1 ligands, PVR and nectin-2, are immunoglobulin-like cell adhesion molecules [12, 13]. They belong to the nectin-like and nectin protein family and are responsible for various essential cell functions [14]. PVR enhances cell movement and proliferation [15], and nectin-2 regulates the formation of various types of cell–cell junctions [14]. These molecules are also known to be over-expressed on human tumor cell lines representing ovarian cancer, colon cancer, breast cancer, cervical cancer, hematological malignancies [8, 12], and sarcomas [16, 17]. Furthermore, several primary human tumor types, such as neuroblastoma [18], leukemias [19], myeloma [20], ovarian carcinoma [21], melanoma [22], and Ewing sarcoma [16], express DNAM-1 ligands. In addition, ovarian carcinoma and pre-AML malignancy myelodysplastic syndrome blasts may down-regulate DNAM-1 expression on NK cells as a means to evade NK cell tumor surveillance [21, 23]. All these findings suggest a potential to treat multiple tumor types by targeting DNAM-1 ligands.

DNAM-1 can play an important role in tumor immunosurveillance. In NK cells, DNAM-1 confers primary activation signals to enhance NK cell killing of tumor cells, but in T cells, DNAM-1 provides co-stimulatory signals and adhesive function [9, 24]. Whether adhesion molecules such as DNAM-1 can be engineered to efficiently confer primary T cell activation signals for CAR T cell therapy is unknown. This opens up the possibility of a panel of adhesion molecule-based CAR designs. In this study, we utilized the first- and the second-generation CAR design principles, with CD3 ζ signaling alone and with co-stimulation molecule signaling to harness DNAM-1 for CAR design. We designed DNAM-1-based CARs which direct T cells to mediate cytotoxicity and cytokine secretion upon encountering DNAM-1 ligand-expressing tumor cells. DNAM-1 CAR T cells also mediated in vivo therapeutic efficacy in a syngeneic melanoma model.

Materials and methods

Mice

C57BL/6 (B6) mice were purchased from the National Cancer Institute (Frederick, MD) and were used at the age of 8–12 weeks. All experiments were conducted under the approval of Dartmouth College's Center for Comparative Medicine and Research.

Cell lines and cell culture

PT67 and GP+E86 were obtained from American Type Cell culture (Rockville, MD). MC-38 cell line was obtained from Dr. Richard J. Barth (Geisel School of Medicine, Lebanon, NH). ID8 cell lines were generated as described previously [25]. PT67, GP+E86, MC-38, B16F10, and ID8 cells were cultured in DMEM supplemented with 10 % heat-inactive FBS (Atlanta Biologicals, Lawrenceville, GA), 10 mM HEPES, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 50 μ M 2-ME. Primary T cells and RMA cells were cultured in RPMI 1640 containing the same supplements as for DMEM.

Construction of DNAM-1-based chimeric antigen receptors (CARs)

Six DNAM-1-based CARs were constructed. Full-length murine *Dnam-1*, *Cd28*, *Ox40*, and *4-1bb* cDNAs were cloned by RT-PCR using RNA from activated mouse splenocytes. Wild type (WT) represents full-length *Dnam-1*. DZ was constructed by fusing full-length *Dnam-1* to murine *Cd3 ζ* cytoplasmic (CYP) domain [aa 52–164]. D28Z, D40Z, and DBBZ were constructed by inserting murine *Cd28* CYP [aa 178–218], *Ox-40* CYP [aa 237–272], or *4-1bb* CYP [aa 209–256], respectively, into DZ construct at the junction of *Dnam-1* and *Cd3 ζ* CYP. Dtm28Z was constructed by fusing *Dnam-1* extracellular domain [aa 1–254] with *Cd28* TM-CYP [aa 151–218], followed by *Cd3 ζ* cytoplasmic (CYP) domain [aa 52–164]. All PCR reactions were performed using high-fidelity DNA polymerase Phusion (New England BioLabs, Ipswich, MA). All constructs were further cloned into pFB-neo vector (Stratagene, Palo Alto, CA) with standard molecular biology techniques.

Retrovirus production and transduction

CAR plasmids were used to transfect PT67 cells, and stable PT67 packaging cell lines were generated by G418 (1 mg/mL) selection. Virus harvested from stable PT67 cells was then used to transduce GP+E86 packaging cell lines. Stable GP+E86 packaging cell lines were generated by G418 (1 mg/mL) selection. Retroviral transduction of T cells was performed as previously described [26].

Flow cytometry

Analysis of DNAM-1 expression on CAR T cells was done by staining with Alexa Fluor 647-conjugated anti-mouse DNAM-1 mAbs (clone: 10E5; Biolegend, San Diego, CA). DNAM-1 ligand PVR was stained with PE-conjugated

anti-mouse PVR mAbs (clone: 4.24.1; Biolegend). Nectin-2 on tumor cells was stained with anti-nectin-2 mAbs (clone: 6A606; Santa Cruz Biotechnology, Dallas, Texas) followed by secondary staining using DyLight 649-conjugated goat anti-rat IgG Abs (clone: Poly4054; Biolegend). For staining of mouse cells, the samples were pre-incubated with Fc receptor block antibody (anti-mouse CD16/CD32, clone: 2.4G2; Bio X Cell, West Lebanon, NH). Samples were analyzed by Accuri C6 cytometer (BD Biosciences, San Jose, CA). Flow cytometry data was analyzed by Accuri or FlowJo software (TreeStar Inc, Ashland, OR).

Cytotoxicity assay

Cytotoxicity was determined by a 4-h ^{51}Cr (^{51}Cr) release assay or LDH release assay. For ^{51}Cr release assays, tumor cells were labeled with ^{51}Cr (1 mCi/mL) in 37 °C for 1 h and then washed and diluted to a suitable concentration with RPMI 1640 complete media before co-culturing with DNAM-1 CAR T cells. Co-culture was performed at effector to target ratio (E/T ratio) 25:1, 5:1, and 1:1 in triplicate wells. Supernatant was harvested, and ^{51}Cr release was measured by a gamma counter. Specific lysis was calculated by the following equation: percentage of specific lysis = [(experimental release – target spontaneous release)/(target maximal release – target spontaneous release)] $\times$ 100. LDH release assays were done using CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI) following the manufacturer's protocol. T cells and tumor cells were co-cultured at an E/T ratio 1:1 in triplicates in 96-well flat-bottom plates for 4 h. Specific lysis was calculated by the following equation: percentage of specific lysis = [(experimental release – effector spontaneous release – target spontaneous release)/target maximal release – target spontaneous release] $\times$ 100. To verify that cytotoxicity was dependent on DNAM-1, experiments were done by pre-incubating CAR T cells with anti-DNAM-1 mAbs (clone: 10E5; eBioscience, San Diego, CA) at 10 $\mu\text{g/mL}$ for 30 min.

Cytokine production by CAR T cells

DNAM-1 CAR T cells were co-cultured with tumor cells at an E/T ratio 4:1 in triplicate wells in RPMI 1640 complete media. Culture supernatant was harvested 24 h later, and IFN- γ concentration was determined by DuoSet ELISA kits (R&D systems, Minneapolis, MN).

DNAM-1 CAR T cell therapy in a B16F10 melanoma model

B6 mice were injected with B16F10 subcutaneously (2×10^5 cells) in the shaved right flank on day 0. DZ CAR

T cells (2×10^6) were injected intra-tumorally at day 7 and 9 in 50 μL of HBSS. Tumor area was measured by a caliper every other day. Mice were euthanized when the tumor area reached 150 mm^2 .

Statistical analysis

One-way ANOVA and Student's *t* test were used to analyze differences between groups, as indicated in each figure legend. *P* < 0.05 was considered statistically significant. Statistical analysis was done using GraphPad Prism software (GraphPad, La Jolla, CA).

Results

Structure and expression profile of DNAM-1-based chimeric antigen receptors (CARs)

To utilize DNAM-1 to recognize tumor cells and directly activate T cells, a DNAM-1-based CAR was constructed by fusing the full-length DNAM-1 with CD3 ζ cytoplasmic (CYP) domain, which is abbreviated as DZ (Fig. 1a). By utilizing the full-length DNAM-1 protein, this CAR maintained its native DNAM-1 transmembrane (TM) and cytoplasmic domains so that it should localize at the cell surface in a similar manner as native DNAM-1. In addition, to test whether co-stimulation signaling in DNAM-1-based CAR design enhanced cytotoxicity and cytokine production, three other DNAM-1-based CARs, D28Z, D40Z, and DBBZ were constructed by inserting CD28, OX-40, or 4-1BB CYP domains, respectively, into the DZ construct at the junction of DNAM-1 and CD3 ζ CYP. Another DNAM-1-based CAR that used an intracellular truncated DNAM-1, Dtm28Z was also constructed by fusing the extracellular domain (EC) of DNAM-1 with CD28 TM and CYP, followed by CD3 ζ CYP domain (Fig. 1a). Because T cells express endogenous DNAM-1, staining intensity of DNAM-1 on the DNAM-1 CAR T cells is contributed by both endogenous DNAM-1 and the CAR (Fig. 1b). The DNAM-1 staining mean fluorescent intensity (MFI) of transduced T cells showed that the MFI of DZ was greatest compared to those containing co-stimulation domains (DZ > Dtm28Z > D28Z > D40Z > DBBZ). The expression efficiency of various CARs was confirmed by transfecting a DNAM-1-negative cell line 293T cells, a cell of embryonic kidney origin, with the CAR constructs, so the expression of DNAM-1 was only due to the CAR expression. In this case, the expression level of DNAM-1 was DZ > D28Z = D40Z > DBBZ (Fig. 1c). Thus, the difference in CAR expression in T cells likely reflects differences in protein translation, stability, or turnover of the DNAM-1 CARs.

Fig. 1 Structure and expression profiles of DNAM-1-based CARs. **a** Schematic diagram of wild-type DNAM-1 (WT) and various DNAM-1-based CARs. Extracellular (EC), transmembrane (TM), and cytoplasmic (CYP) domains are as indicated. DZ was constructed by fusing full-length DNAM-1 with CD3 ζ CYP. D28Z, D40Z, and DBBZ were constructed by inserting CD28, OX40, and 4-1BB CYP, respectively, into the DZ construct between the full-length DNAM-1 and CD3 ζ CYP. Dtm28Z was constructed by fusing DNAM-1 EC domain with CD28 TM-CYP, followed by CD3 ζ CYP. **b** Surface DNAM-1 expression on CAR T cells. T cells untransduced or transduced by various DNAM-1-based CARs or pFB-neo vector (Mock) were stained with anti-CD8 β mAbs and anti-DNAM-1 mAbs. CD8 β –cells are CD4 + T cells. Histograms are pre-gated on live cells and are representative of samples from two independent experiments. The values shown are mean fluorescent intensity (MFI) $\pm$ standard deviation (SD) of DNAM-1 staining on T cells and the normalized DNAM-1 MFI to vector control (Mock) T cells from three different batches of T cells. **c** 293T cells were co-transfected with CAR plasmid and EGFP plasmid, harvested 24 h after transfection and stained with anti-DNAM-1 mAbs. The histogram shows DNAM-1 expression pre-gated on EGFP + cells. Data are representative of two independent experiments

DNAM-1 CAR T cells mediate robust and specific cytotoxicity against DNAM-1 ligand-expressing tumor cells

DNAM-1 ligands are expressed on primary human ovarian carcinomas [21], melanomas [22], and various hematological malignancies [19, 20]. In order to study DNAM-1 CAR T cell therapeutic efficacy against these clinically relevant tumor types in a mouse tumor model, the expression of two DNAM-1 ligands, PVR and nectin-2, on various mouse tumor cell lines representing ovarian cancer, melanoma, colon cancer, and lymphoma was determined. Ovarian carcinoma cell line ID8, melanoma cell line B16F10, and colon adenocarcinoma cell line MC-38 expressed both PVR and nectin-2 (Fig. 2a). To examine whether DNAM-1-based CAR T cells exhibit cellular cytotoxicity against DNAM-1 ligand + tumors, CAR T cells were co-cultured with RMA (ligand negative), ID8, or B16F10 tumor cell lines at E/T ratios of 1:1, 5:1, and 25:1. Specific killing of ligand-positive but not ligand-negative tumor cells occurred by all four different CAR T cells (Fig. 2b). Cytotoxicity assays also showed that simply over-expressing DNAM-1 receptor on T cells did not lead to tumor killing (Fig. 2c), so the addition of CD3 ζ cytoplasmic domain was needed for CAR activity. The capability to kill tumor cells correlated with the cell surface expression efficiency of individual CARs, which was DZ > Dtm28Z > D28Z > D40Z > DBBZ. To confirm that the killing was dependent upon DNAM-1, anti-DNAM-1 mAbs were used to block cytotoxicity of DZ CAR T cells (Fig. 2d).

DNAM-1 CAR T cells produce low amounts of cytokines against DNAM-1 ligand-expressing tumor cells

IFN- γ is known to play important roles in anti-tumor immunity and can be critical to CAR T cell therapeutic efficacy

[27, 28]. Specific but low IFN- γ production (up to 200 pg/ml) by DNAM-1 CAR T cells was observed upon co-culturing with MC-38, ID8, or B16F10 tumor cells (Fig. 3a). There was an increase in IFN- γ production when CAR T cells were co-cultured in the presence of IL-2 (Fig. 3b). However, this was still moderate compared to other CARs in similar assays, which often produce more than 5,000 pg/ml of IFN- γ [25, 26]. The high cytotoxicity but low IFN- γ secretion phenotype might be due to insufficient activation of CAR T cells by tumor cells, or that DNAM-1 CARs may not confer adequate signaling to elicit IFN- γ production. To test the potential of DNAM-1 CAR T cells to produce IFN- γ , plate-bound anti-DNAM-1 mAbs were used to cross-link the CARs on these T cells. Robust IFN- γ production from 45,000 to 200,000 pg/ml was observed upon CAR cross-linking (Fig. 3c). Thus, under conditions of sufficient CAR triggering, DNAM-1 CAR T cells produce robust amounts of cytokines even when the CAR is expressed at low amounts. No IFN- γ was produced by untransduced T cells that express endogenous DNAM-1, which lacks a primary signaling domain.

DNAM-1 CAR T cells reduced tumor burden in vivo in a syngeneic melanoma model

To test whether DNAM-1 CAR T cells mediate therapeutic efficacy in vivo, B16F10 cells (2×10^5 cells) were injected subcutaneously into B6 mice on day 0 and tumor growth was measured. DZ CAR T cells (2×10^6 cells) were administered intra-tumorally on day 7 and day 9. There was a decreased tumor area after DZ CAR T cell treatment. Delayed tumor growth and decreased overall tumor burden were observed, even after mice stopped receiving CAR T cell therapy (Fig. 4). Although the ligands are present on other normal tissues, the localized treatment with these DNAM-1 CAR T cells was effective and no signs of unwanted toxicity were observed. These results indicate that DNAM-1 CAR T cell therapy mediates therapeutic efficacy against melanoma in vivo.

Discussions

In this study, an adhesion molecule, DNAM-1, was used to create CAR T cells that specifically killed multiple tumor types. Rather than use single-chain variable fragment (scFv) derived from anti-PVR or anti-nectin-2 mAbs for the CAR designs, the full-length DNAM-1 protein was used. A unique feature of DNAM-1 CARs is that they induced robust T cell cytotoxicity but elicited low IFN- γ production compared to other CARs. There are several potential reasons that could explain this phenomenon. Since the threshold to activate T cells to secrete IFN- γ is

Fig. 2 DNAM-1 CAR T cells mediate robust cytotoxicity against DNAM-1 ligand + tumor cells. **a** DNAM-1 ligand expression on tumor cell lines. RMA, MC-38, ID8, and B16F10 cancer cell lines were stained for DNAM-1 ligand expression with anti-PVR mAbs or anti-nectin-2 mAbs and analyzed by flow cytometry. The filled and open histograms are isotype and DNAM-1 ligand staining, respectively. **b** Cytotoxicity assays were performed to measure the specific lysis of RMA, ID8, and B16F10 cells by DNAM-1 CAR T cells. Tumor cells were labeled with ^{51}Cr and cultured with untransduced or DNAM-1 CAR T cells at E/T ratio 1:1, 5:1, and 25:1 for 4 h. Data shown are mean + SD of triplicate wells and are representative of two independent experiments. Statistical analysis was performed

using one-way ANOVA to compare DZ, D28Z, and D40Z CAR T cells to DBBZ T cells (* $p < 0.05$). **c** CAR T cells and tumor cells were co-cultured at E/T ratio 1:1 for 4 h. LDH release cytotoxicity assays measure specific lysis of tumor cells by CAR T cells. Data shown are mean + SD of triplicate wells and are representative of two independent experiments. **d** DZ CAR T cells were pre-incubated with anti-DNAM-1 mAbs or rat IgG (10 $\mu\text{g}/\text{mL}$) for 30 min before co-culturing with B16F10 cells at an E/T ratio of 5:1. Experiments were performed in triplicate wells. Data shown are mean + SD of triplicate wells and are representative of two independent experiments. Statistical analysis was performed using one-way ANOVA (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.0001$)

Fig. 3 DNAM-1 CAR T cells mediate IFN- γ production upon culturing with DNAM-1 ligand + tumor cells. Untransduced (UnTd) T cells, Mock, WT, DZ, D28Z, D40Z, DBBZ, or Dtm28Z CAR T cells were co-cultured with tumor cells at an E/T ratio 4:1 without IL-2 (**a**) or with IL-2 (25 U/mL) (**b**). **c** DNAM-1 CAR T cells were cultured with or without IL-2 (25 U/mL) in 96-well plates pre-coated with 1 μ g anti-DNAM-1 mAbs or control Abs. Cell-free medium was collected after 24 h, and the amount of IFN- γ was measured by ELISA. Data shown are mean + SD of triplicate wells and are representative of two independent experiments. Statistical analysis was performed using one-way ANOVA (* p < 0.05; ** p < 0.005; *** p < 0.0001)

100- to 1,000-fold higher than the threshold to activate cytotoxicity [29], it may be that the ligand density on tumor cells tested was not high enough to trigger sufficient cross-linking of the CAR to induce robust IFN- γ production. However, this was unlikely given that the tumor cells

Fig. 4 DNAM-1 CAR T cells reduced tumor burden in a B16F10 melanoma model in vivo. B16F10 melanoma cells (2×10^5 cells) were injected subcutaneously into B6 mice on day 0. On days 7 and 9, 2×10^6 untransduced T cells or DZ T cells were injected intratumorally, and tumor area was measured. Shaded region indicates the time period of the T cell injections. Data are pooled from two independent experiments (untransduced group, $n = 9$; DZ group $n = 10$). Error bars represent SEM. Statistical analysis was performed using Mann-Whitney U test for each time-point (* p < 0.05)

expressed high amounts of DNAM-1 ligands (Fig. 2a). Alternatively, the DNAM-1 CAR T cells may not have the signaling capacity to elicit robust IFN- γ secretion. This was also unlikely because anti-DNAM-1 mAbs were able to cross-link CARs and elicit robust IFN- γ secretion (Fig. 3c). Instead, it is possible that steric hindrance of DNAM-1 and its ligand interaction may lead to weak signaling within the T cells. The DNAM-1 ligands, PVR and nectin-2, exhibit a unique triple-immunoglobulin-fold structure [14], along with the double immunoglobulin-fold structure of DNAM-1. This overall structure of the ligand-receptor complex is relatively bulky and may hinder the CAR-activating signals enough to impair IFN- γ secretion. It has been shown that the distance between a CAR and its ligand on an opposing target cell is important for optimal T cell activation [30].

To overcome the potential suboptimal DNAM-1 CAR T cell activation and cytokine secretion, co-stimulatory molecules were incorporated into several of the CAR designs. However, the cytotoxicity and IFN- γ production was not enhanced by these second-generation CAR designs. Decreased cytotoxicity and IFN- γ secretion by DNAM-1 CARs bearing co-stimulatory molecules compared to CD3 ζ alone was observed. This was likely due to the decreased cell surface expression of these CARs containing co-stimulatory molecules (D28Z, D40Z, and DBBZ). These data support the hypothesis that the amount of CAR cell surface expression correlates to CAR-induced cell activation. The co-stimulatory domains attached to these DNAM-1 CAR constructs were in a unique position since they were not directly adjacent to the cell membrane. They were inserted after the full-length DNAM-1 protein. The full-length DNAM-1 molecule was used to preserve the intrinsic signaling motifs of the DNAM-1 cytoplasmic

domain that can relay activation signals from LFA-1 and T cell receptors [31, 32]. However, CD28, OX-40, and 4-1BB co-stimulation are known to activate phosphoinositide-3 kinase (PI-3 kinase) [33], whose substrate is the membrane-bound phospholipid, phosphatidylinositol-4,5-bisphosphate (PIP2). Thus, placing the co-stimulation domains away from the cell membrane may compromise co-stimulation signaling through PI-3 kinase. However, the Dtm28Z CAR T cells, which contain CD28 CYP directly adjacent to cell membrane, also secreted similar amount of IFN- γ upon co-culturing with tumor cells (Fig. 3a, b). The inability of co-stimulatory molecules to enhance DNAM-1 CAR T cell activation further supported the idea that the discordant cytotoxicity and IFN- γ secretion were due to the unique structure of DNAM-1. The bulky receptor–ligand complex may limit CAR clustering upon engagement and thereby dampen overall signaling [34]. Alternatively, the ligand–receptor interface may increase the inter-membrane distance between interacting T cells and tumor cells. This increased distance could cause the immunological synapse to exclude membrane-bound phosphatase CD45 less effectively, thus making it more difficult to surpass the threshold for T cell activation [35]. Another potential reason is that DNAM-1 associates with LFA-1 on the cell membrane [31]. This association may lead to microclusters of CARs that confer T cells with a unique signaling capacity [36]. The inability of co-stimulation signals to enhance DNAM-1 CARs T cell function also highlighted the fact that each CAR design is unique on its own, and it is necessary to customize CAR design for each target.

Toxicity is a major concern for all CAR T cell therapies. On-target off-tumor self-reactivity of CAR T cell therapy can cause manageable side effects, such as B cell aplasia [1–3], to challenging inflammatory responses [37]. PVR is expressed on epithelium, endothelial cells and dendritic cells (DCs), and nectin-2 is expressed on epithelium, neurons, and fibroblasts. Thus, DNAM-1 CAR T cells may exhibit self-reactivity upon systemic administration. Just like CARs targeting α -folate receptor, ERBB2 and EGFR, which are over-expressed on tumor cells but also expressed on certain epithelial tissues, a careful titration of dosage to identify a safe and effective therapeutic window is necessary. In this study, CAR T cells were given locally to avoid potential systemic toxicity but still allow anti-tumor efficacy. Other CAR designs may be used to avoid self-reactivity when the ligands are expressed on normal cells [38, 39]. On the other hand, one might be able to utilize the self-reactive feature of DNAM-1 to enhance therapeutic efficacy in certain disease settings. There might be benefits from targeting ovarian cancer and the tumor microenvironment (TME) simultaneously because immature DCs express PVR and nectin-2 [40] and are the major players contributing to the immunosuppressive TME of ovarian

cancer [41]. Furthermore, since DNAM-1 plays an important role in enhancing monocyte transendothelial migration [42], one might be able to use the potential interaction of a DNAM-1 CAR to endothelial cells to enhance CAR T cell extravasation at the endothelial junction and enhance CAR T cell trafficking to the tumor site.

In conclusion, the data in this study showed that DNAM-1 CAR T cells mediate robust cytotoxicity and low IFN- γ secretion against DNAM-1 ligand-positive tumor cells. DNAM-1 CAR T cells also decreased the tumor burden of melanoma-bearing mice. These data also suggest that there may be therapeutic potential in targeting other over-expressed adhesion molecules on tumor cells.

Acknowledgments The authors thank the National Cancer Institute Biological Resource Branch for providing recombinant human IL-2, Dartmouth College Molecular biology Core for technical support, and the Center for Comparative Medicine and Research for providing animal care. This work was supported by a National Institute of Health grant CA130911.

Conflict of interest The authors declare that they have no conflict of interest.

References

- Porter DL, Levine BL, Kalos M, Bagg A, June CH (2011) Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med* 365(8):725–733
- Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, June CH (2011) T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med* 3(95):95ra73
- Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, Teachey DT, Chew A, Hauck B, Wright JF, Milone MC, Levine BL, June CH (2013) Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *N Engl J Med* 368(16):1509–1518
- Brentjens RJ, Davila ML, Riviere I, Park J, Wang X, Cowell LG, Bartido S, Stefanski J, Taylor C, Olszewska M, Borquez-Ojeda O, Qu J, Wasielewska T, He Q, Bernal Y, Rijo IV, Hedvat C, Kobos R, Curran K, Steinherz P, Jurcic J, Rosenblatt T, Maslak P, Frattini M, Sadelain M (2013) CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. *Sci Transl Med* 5(177):177
- Sadelain M, Brentjens R, Riviere I (2013) The basic principles of chimeric antigen receptor design. *Cancer Discov* 3(4):388–398
- Lanier LL (2005) NK cell recognition. *Annu Rev Immunol* 23:225–274
- Kruse PH, Matta J, Ugolini S, Vivier E (2014) Natural cytotoxicity receptors and their ligands. *Immunol Cell Biol* 92(3):221–229
- Shibuya A, Campbell D, Hannum C, Yssel H, Franz-Bacon K, McClanahan T, Kitamura T, Nicholl J, Sutherland GR, Lanier LL, Phillips JH (1996) DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes. *Immunity* 4(6):573–581
- Tahara-Hanaoka S, Miyamoto A, Hara A, Honda S, Shibuya K, Shibuya A (2005) Identification and characterization of murine DNAM-1 (CD226) and its poliovirus receptor family ligands. *Biochem Biophys Res Commun* 329(3):996–1000

10. Seth S, Georgoudaki AM, Chambers BJ, Qiu Q, Kremmer E, Maier MK, Czeloth N, Ravens I, Foerster R, Bernhardt G (2009) Heterogeneous expression of the adhesion receptor CD226 on murine NK and T cells and its function in NK-mediated killing of immature dendritic cells. *J Leukoc Biol* 86(1):91–101
11. Dardalhon V, Schubart AS, Reddy J, Meyers JH, Monney L, Sabatos CA, Ahuja R, Nguyen K, Freeman GJ, Greenfield EA, Sobel RA, Kuchroo VK (2005) CD226 is specifically expressed on the surface of Th1 cells and regulates their expansion and effector functions. *J Immunol* 175(3):1558–1565
12. Bottino C, Castriconi R, Pende D, Rivera P, Nanni M, Carnemolla B, Cantoni C, Grassi J, Marcenaro S, Reymond N, Vitale M, Moretta L, Lopez M, Moretta A (2003) Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. *J Exp Med* 198(4):557–567
13. Tahara-Hanaoka S, Shibuya K, Onoda Y, Zhang H, Yamazaki S, Miyamoto A, Honda S, Lanier LL, Shibuya A (2004) Functional characterization of DNAM-1 (CD226) interaction with its ligands PVR (CD155) and nectin-2 (PRR-2/CD112). *Int Immunol* 16(4):533–538
14. Takai Y, Miyoshi J, Ikeda W, Ogita H (2008) Nectins and nectin-like molecules: roles in contact inhibition of cell movement and proliferation. *Nat Rev Mol Cell Biol* 9(8):603–615
15. Fujito T, Ikeda W, Kakunaga S, Minami Y, Kajita M, Sakamoto Y, Monden M, Takai Y (2005) Inhibition of cell movement and proliferation by cell-cell contact-induced interaction of Nectin-5 with nectin-3. *J Cell Biol* 171(1):165–173
16. Verhoeven DH, de Hooze AS, Mooiman EC, Santos SJ, ten Dam MM, Gelderblom H, Melief CJ, Hogendoorn PC, Egeler RM, van Tol MJ, Schilham MW, Lankester AC (2008) NK cells recognize and lyse Ewing sarcoma cells through NKG2D and DNAM-1 receptor dependent pathways. *Mol Immunol* 45(15):3917–3925
17. Cho D, Shook DR, Shimasaki N, Chang YH, Fujisaki H, Campana D (2010) Cytotoxicity of activated natural killer cells against pediatric solid tumors. *Clin Cancer Res* 16(15):3901–3909
18. Castriconi R, Dondero A, Corrias MV, Lanino E, Pende D, Moretta L, Bottino C, Moretta A (2004) Natural killer cell-mediated killing of freshly isolated neuroblastoma cells: critical role of DNAX accessory molecule-1-poliovirus receptor interaction. *Cancer Res* 64(24):9180–9184
19. Pende D, Spaggiari GM, Marcenaro S, Martini S, Rivera P, Capobianco A, Falco M, Lanino E, Pierri I, Zambello R, Bacigalupo A, Mingari MC, Moretta A, Moretta L (2005) Analysis of the receptor-ligand interactions in the natural killer-mediated lysis of freshly isolated myeloid or lymphoblastic leukemias: evidence for the involvement of the Poliovirus receptor (CD155) and Nectin-2 (CD112). *Blood* 105(5):2066–2073
20. El-Sherbiny YM, Meade JL, Holmes TD, McGonagle D, Mackie SL, Morgan AW, Cook G, Feyler S, Richards SJ, Davies FE, Morgan GJ, Cook GP (2007) The requirement for DNAM-1, NKG2D, and NKp46 in the natural killer cell-mediated killing of myeloma cells. *Cancer Res* 67(18):8444–8449
21. Carlsten M, Norell H, Bryceson YT, Poschke I, Schedvins K, Ljunggren HG, Kiessling R, Malmberg KJ (2009) Primary human tumor cells expressing CD155 impair tumor targeting by down-regulating DNAM-1 on NK cells. *J Immunol* 183(8):4921–4930
22. Lakshminanth T, Burke S, Ali TH, Kimpfler S, Ursini F, Ruggeri L, Capanni M, Umansky V, Paschen A, Sucker A, Pende D, Groh V, Biassoni R, Hoglund P, Kato M, Shibuya K, Schadendorf D, Anichini A, Ferrone S, Velardi A, Karre K, Shibuya A, Carbone E, Colucci F (2009) NCRs and DNAM-1 mediate NK cell recognition and lysis of human and mouse melanoma cell lines in vitro and in vivo. *J Clin Invest* 119(5):1251–1263
23. Carlsten M, Baumann BC, Simonsson M, Jadersten M, Forsblom AM, Hammarstedt C, Bryceson YT, Ljunggren HG, Hellstrom-Lindberg E, Malmberg KJ (2010) Reduced DNAM-1 expression on bone marrow NK cells associated with impaired killing of CD34 + blasts in myelodysplastic syndrome. *Leukemia* 24(9):1607–1616
24. Gilfillan S, Chan CJ, Cella M, Haynes NM, Rapaport AS, Boles KS, Andrews DM, Smyth MJ, Colonna M (2008) DNAM-1 promotes activation of cytotoxic lymphocytes by nonprofessional antigen-presenting cells and tumors. *J Exp Med* 205(13):2965–2973
25. Barber A, Zhang T, DeMars LR, Conejo-Garcia J, Roby KF, Sentman CL (2007) Chimeric NKG2D receptor-bearing T cells as immunotherapy for ovarian cancer. *Cancer Res* 67(10):5003–5008
26. Zhang T, Wu MR, Sentman CL (2012) An NKp30-based chimeric antigen receptor promotes T cell effector functions and antitumor efficacy in vivo. *J Immunol* 189(5):2290–2299
27. Barth RJ Jr, Mule JJ, Spiess PJ, Rosenberg SA (1991) Interferon gamma and tumor necrosis factor have a role in tumor regressions mediated by murine CD8 + tumor-infiltrating lymphocytes. *J Exp Med* 173(3):647–658
28. Zhang T, Barber A, Sentman CL (2007) Chimeric NKG2D modified T cells inhibit systemic T-cell lymphoma growth in a manner involving multiple cytokines and cytotoxic pathways. *Cancer Res* 67(22):11029–11036
29. Faroudi M, Utzny C, Salio M, Cerundolo V, Guiraud M, Muller S, Valitutti S (2003) Lytic versus stimulatory synapse in cytotoxic T lymphocyte/target cell interaction: manifestation of a dual activation threshold. *Proc Natl Acad Sci USA* 100(24):14145–14150
30. Hudecek M, Lupo-Stanghellini MT, Kosasih PL, Sommermeyer D, Jensen MC, Rader C, Riddell SR (2013) Receptor affinity and extracellular domain modifications affect tumor recognition by ROR1-specific chimeric antigen receptor T cells. *Clin Cancer Res* 19(12):3153–3164
31. Shibuya K, Lanier LL, Phillips JH, Ochs HD, Shimizu K, Nakayama E, Nakauchi H, Shibuya A (1999) Physical and functional association of LFA-1 with DNAM-1 adhesion molecule. *Immunity* 11(5):615–623
32. Shibuya K, Shirakawa J, Kameyama T, Honda S, Tahara-Hanaoka S, Miyamoto A, Onodera M, Sumida T, Nakauchi H, Miyoshi H, Shibuya A (2003) CD226 (DNAM-1) is involved in lymphocyte function-associated antigen 1 costimulatory signal for naive T cell differentiation and proliferation. *J Exp Med* 198(12):1829–1839
33. Cai YC, Cefai D, Schneider H, Raab M, Nabavi N, Rudd CE (1995) Selective CD28pYMN mutations implicate phosphatidylinositol 3-kinase in CD86-CD28-mediated costimulation. *Immunity* 3(4):417–426
34. Germain RN (1997) T-cell signaling: the importance of receptor clustering. *Curr Biol* 7(10):R640–R644
35. Choudhuri K, Wiseman D, Brown MH, Gould K, van der Merwe PA (2005) T-cell receptor triggering is critically dependent on the dimensions of its peptide-MHC ligand. *Nature* 436(7050):578–582
36. Alarcon B, Swamy M, van Santen HM, Schamel WW (2006) T-cell antigen-receptor stoichiometry: pre-clustering for sensitivity. *EMBO Rep* 7(5):490–495
37. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA (2010) Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther* 18(4):843–851
38. Kloss CC, Condomines M, Cartellieri M, Bachmann M, Sadelain M (2013) Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells. *Nat Biotechnol* 31(1):71–75
39. Fedorov VD, Themeli M, Sadelain M (2013) PD-1- and CTLA-4-based inhibitory chimeric antigen receptors. *Sci Transl Med* 5(215):215ra172

40. Pende D, Castriconi R, Romagnani P, Spaggiari GM, Marcenaro S, Dondero A, Lazzeri E, Lasagni L, Martini S, Rivera P, Capobianco A, Moretta L, Moretta A, Bottino C (2006) Expression of the DNAM-1 ligands, Nectin-2 (CD112) and poliovirus receptor (CD155), on dendritic cells: relevance for natural killer-dendritic cell interaction. *Blood* 107(5):2030–2036
41. Huarte E, Cubillos-Ruiz JR, Nesbeth YC, Scarlett UK, Martinez DG, Buckanovich RJ, Benencia F, Stan RV, Keler T, Sarobe P, Sentman CL, Conejo-Garcia JR (2008) Depletion of dendritic cells delays ovarian cancer progression by boosting antitumor immunity. *Cancer Res* 68(18):7684–7691
42. Reymond N, Imbert AM, Devilard E, Fabre S, Chabanon C, Xerri L, Farnarier C, Cantoni C, Bottino C, Moretta A, Dubreuil P, Lopez M (2004) DNAM-1 and PVR regulate monocyte migration through endothelial junctions. *J Exp Med* 199(10):1331–1341