mSA2 affinity-enhanced biotin-binding CAR T cells for universal tumor targeting

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ABSTRACT
Chimeric antigen receptor T cells (CAR-Ts) are promising cancer therapeutics. However, since cancer cells can lose the CAR-targeted antigen and avoid destruction, targeting multiple antigens with multiple CARs has been proposed. We illustrate here a less cumbersome alternative, anti-tag CARs (AT-CARs) that bind to tags on tumor-targeting antibodies. We have created novel AT-CARs, using the affinity-enhanced monomeric streptavidin 2 (mSA2) biotin-binding domain that when expressed on T cells can target cancer cells coated with biotinylated antibodies. Human T cells expressing mSA2 CARs with CD28/CD3ζ and 4-1BB/CD3ζ signaling domains were activated by plate-immobilized biotin and by tumor cells coated with biotinylated antibodies against the tumor-associated antigens CD19 and CD20. Furthermore, mSA2 CAR T cells were capable of mediating cancer cell lysis and IFNγ production in an antibody dose-dependent manner. The mSA2 CAR is a universal AT-CAR that can be combined with biotinylated tumor-specific antibodies to potentially target many different tumor types.

Despite this success and interest, many challenges remain to the application of CAR therapy. The single antigen specificity of CARs can render them ineffective against tumors that lack expression of the target antigen due to antigen loss or tumor heterogeneity. Additionally, the unregulated persistence of CAR activity can cause cytokine release syndrome and other toxicities. For targeting new cancers, the creation of new CARs is needed, which is technically challenging and requires extensive engineering and safety testing to rule out off-tumor toxicities.

One alternative approach that addresses some of these challenges is the creation of CARs that bind to common tag molecules – such as fluorescein isothiocyanate (FITC), peptide neo-epitopes (PNE), Fcy, and biotin – that are conjugated to TAA-specific antibodies. So-called anti-tag CAR (AT-CAR) therapy would be designed so that patients are infused with a tagged, TAA-specific antibody that binds to tumor cells, followed by T cells expressing AT-CARs that react with the tagged antibodies on tumor cells (Fig. 1A). This approach has the potential to allow for sequential or simultaneous targeting of multiple tumor antigens with different antibodies. Additionally, the activity of AT-CARs can be regulated by altering the concentration of tagged antibodies or halting antibody administration for better control over potential toxicities.

Here we report the construction and characterization of a new AT-CAR with potent activity composed of the affinity-enhanced monomeric streptavidin (mSA2) protein, engineered to have high affinity for biotin compared to other monomeric avidins (Kd = 5.5 × 10⁻⁹ at 37°C). Previous studies have shown that higher affinity can lead to greater T cell activation and antitumor response in the AT-CAR format. We find that mSA2 CAR T cells are efficiently stimulated by plate-immobilized biotin and that they are capable of potent target cell lysis and cytokine production when combined with biotinylated TAA-specific antibodies.

We first constructed lentiviral vectors encoding two mSA2 CAR signaling domain variants, mSA2-41BBζ and mSA2-CD28ζ (Fig. 1B). Driven by the EF1α promoter, CAR-coding regions consisted of the murine Igκ leader sequence, the codon-optimized mSA2 protein domain, the CD8α-hinge spacer domain, the CD28 transmembrane domain, either the CD28 or the 4-1BB cytoplasmic domain and the CD3ζ cytoplasmic
We found that the mSA2 CARs were efficiently expressed on the cell surface (Fig 2A) and that the mSA2 CAR T cells could be activated by plate-bound biotin (Fig 2B). Following one stimulation cycle, transduced T cells were sorted for TagBFP expression and then stained with biotin-FITC. The staining with biotin-FITC was specific to mSA2 CAR T cells and correlated with TagBFP expression (Fig 2A). T cells were also evaluated for helper and cytotoxic populations based on CD4 and CD8 expression. Next, we found that the mSA2 CAR T cells could be efficiently activated by plate-immobilized biotin (Fig 2B). Specifically, we incubated CAR T cells or control cells on plates coated with biotinylated antibodies for 18 hours and then assayed cells for T cell activation markers by flow cytometry. Incubation with plate immobilized biotin led to the upregulation of T cell activation markers CD69 and CD107a as well as the downregulation of CD62 L. We found that FMC63-CD28CAR T cells were activated by the plate immobilized anti-hlgG4 antibody that can bind to the IgG4 extracellular spacer but not by a biotinylated antibody against an irrelevant target (anti-MUC1 antibody H15K6).

Next, we tested if mSA2 CAR T cells could be combined with anti-tumor antibodies to mediate specific T cell effector functions. We co-incubated mSA2–41BB or mSA2–CD28 CAR T cells with CD20+ Raji target cells (Fig S1) in the presence of varying amounts of biotinylated Rituximab (anti-CD20). The mSA2 CAR T cells responded by up-regulating T cell activation markers in a dose-responsive manner to the biotinylated Rituximab (Fig 3A). They also produced IFNγ (Fig 3B) and performed target cell lysis (Fig 3C) in a dose-responsive manner. We found that mSA2–CD28 CAR T cells produced vastly more IFNγ compared to the mSA2–41BB CAR T cells, however, both showed comparable levels of specific target cell lysis. This result is consistent with previous findings for traditional CARs with CD28 versus 4–1BB co-signaling domains from several research groups. It is likely the result of established differences in CD28 and 4–1BB signaling pathways which signal via Akt and TRAFs, respectively. Notably, incubating the mSA2 CAR T cells with biotinylated antibody and off-target cells lead neither to T cell activation nor any significant induction of T cell effector functions, indicating that immobilizing the antibodies on the surface of the target cells was necessary to initiate CAR T cell receptor signaling and that soluble antibody alone could not induce activation. As an additional control, we found that MOCK transduced T cells, when combined with biotinylated antibodies, were not activated nor induced to produce cytokines or lyse tumor cells (Fig S2).

We then tested the activity of mSA2–41BB and mSA2–CD28 CAR T cells when combined with various antigen-positive and antigen-negative target cell lines, in the presence of biotinylated Rituximab, FMC63 (anti-CD19), or biotinylated Cetuximab (anti-EGFR) antibodies. Biotinylated Cetuximab served as a negative control as its target antigen, EGFR, is not expressed on any of the targeted cell lines. Target cell lines included Jurkat cells which are negative for both CD19 and CD20, K562 cells which are negative for both CD19 and CD20, K562 cells which are engineered to express CD19 (K562+CD19), and Raji cells which are naturally positive for both CD19 and CD20 (Fig S1). Following co-incubation of T cells and target cells with the different biotinylated antibodies, we found that the mSA2-CAR T cells were induced to express...
activation markers (Fig. 4A), produce IFN-γ (Fig. 4B) and lyse target cells (Fig. 4C). Importantly, the presence of both the biotinylated antibody and the targeted antigen on the cancer cells was required for mSA2 CAR cells to be activated and functional. We found that the lytic abilities of mSA2-CD28z CAR cells were comparable to the FMC63-CD28z positive control CAR.

Future studies will include in vivo testing of mSA2–41BBz and mSA2-CD28z CAR T cells in mice bearing human tumor xenografts with different biotinylated antibody doses and schedules. The potency of the biotinylated antibodies and mSA2 CAR T cells could potentially be further improved by performing site-specific biotinylation on the antibodies at various amino acid positions and selecting for sites that give the most potent lysis, likely optimizing the formation of the T cell synapse.16

For future therapeutic applications one concern is the potential immunogenicity of the mSA2 protein. While it is known that tetrameric avidin can elicit antibody and cellular responses, additional studies will be necessary to determine immunogenicity of the monomeric mSA2.25 The monomeric form with fewer repeating structures could be expected to elicit a more diminished antibody response. Nevertheless, mutations have been reported that can make tetrameric avidin less immunogenic, which could be applied to the mSA2 protein domain.26 As many CARs in the clinic have immunogenic mouse antibody domains including the most common CAR FMC63, there could be a similar therapeutic window in which the mSA2-CAR T cells could function before being rejected.

In comparison to other tag-CARs, the biotin tag may be better tolerated than PNE or FITC, which are entirely foreign molecules.

The mSA2-CAR is a new AT-CAR with potential for targeting several different antigens by T cells engineered to express a single CAR. Complementary to previously developed tag-CARs, the mSA2 avidin binding domain has a unique compact structure (122 amino acids in length) that may make it suitable to targeting particular new antigens. This CAR has great promise for use in combination with the ever-increasing list of FDA-approved tumor-targeting antibodies and antibodies in clinical development.7 It is also ripe for combining with antibodies that display tumor-specificity but lack anti-tumor therapeutic activities on their own. In addition to future promise as an off-the-shelf therapeutic, which will be tested in preclinical animal models, the mSA2-CAR can already be used as an off-the-shelf reagent for preselecting in vitro the best candidate antibodies for antigen binding domains of traditional CARs before proceeding with their construction.

Materials and methods

**Lentiviral vector construction and virus production**

The CAR coding regions listed in Supplementary Table S1 were synthesized (Integrated DNA Technologies) and cloned into the pSICO-EF1 vector using Gibson Assembly.18 Virus
was generated using the above described transfer vectors following methods described previously in detail.18

**Antibody biotinylation**

Antibodies FMC63, Rituximab and Cetuximab were purchased (Absolute Antibody) and biotinylated using the EZ-Link NHS biotin kit (ThermoFisher Scientific) and were determined by HABA assay to contain an average of 3–4 molecules of biotin per antibody.

**Cell line culture**

Human tumor cell lines Jurkat Clone E6–1 (TIB-152), K562 (CCL-243), and Raji (CCL-86) were obtained from American Type Culture Collection (ATCC) and cultured at 37°C in RPMI medium supplemented with 1X MEM amino acids solution, 10 mM Sodium Pyruvate, 10% fetal bovine serum (FBS) and Penicillin-Streptomycin (Life Technologies). K562+CD19 cells that stably express full-length CD19 were generated by transducing K562 cells with CD19-expressing lentivirus and sorting for cells positive for CD19 expression. HEK293 T (human embryonic kidney) cells (ATCC) were cultured at 37°C in DMEM supplemented with 10% FBS, and Penicillin-Streptomycin.

**Primary human T cell culture and lentiviral transduction**

All experiments were performed on PBMC isolated from de-identified human Buffy Coat samples purchased from the Pittsburgh Central Blood Bank fulfilling the basic exempt criteria 45 CFR 46.101(b)(4) in accordance with the University of Pittsburgh IRB guidelines. Human T cells were cultured in supplemented RPMI media as described for cell lines above, however, 10% Human AB serum (Gemini Bio Products) was used instead of FBS, and the media was further supplemented with...
100 U/ml human IL-2 IS and 1 ng/ml IL-15 (Miltenyi Biotec). PBMC were isolated from a Buffy Coat from healthy volunteer donors using Ficoll centrifugation and total human T cells were isolated using the Human Pan T cell isolation kit (Miltenyi Biotec). T cells were stimulated and expanded using TransAct Human T cell Activation Reagent (Miltenyi Biotec). For transduction, two days after addition of TransAct, lentivirus was added to cells at an MOI of 10–50 in the presence of 6 μg/ml of DEAE-dextran (Sigma Aldrich). After 18 hours, cells were washed and resuspended in fresh T cell media containing 100 U/ml IL-2 IS and 1 ng/ml IL-15. For additional 12 days of stimulation and expansion, CAR+ cells were flow-sorted by TagBFP expression. To obtain sufficient numbers of cells for experiments, sorted CAR+ cells then underwent an additional TransAct stimulation cycle prior to being assayed.

Flow cytometry staining

Cells were stained using the indicated antibodies and diluted in flow cytometry buffer (PBS + 2% FBS), for 30 minutes at 4 °C followed by two washes with flow cytometry buffer. Live cells were gated based on forward and side scatter and CAR+ cells were gated on TagBFP expression. 50,000 total events were recorded per sample.

Plate-immobilized biotin stimulation assay

High protein-binding 96 well flat-bottom plates (Corning) were coated with 10 μg/ml of biotinylated antibody in PBS or with PBS alone for 2 hours at 37°C and washed 2 times with PBS. 100,000 CAR T cells were incubated on the plate for 18 hours. After incubation, cells were stained with antibodies against T cell activation markers CD69-PE (BD Biosciences), CD62L-FITC (BD Biosciences) and CD107a-APC (BD Biosciences) and evaluated for marker expression by flow cytometry.

CAR T cell and target cell antibody-mediated activation co-incubation assay

100,000 primary T cells were co-cultured with 10,000 target cells and the indicated amounts of biotinylated antibodies for 18 hours. After incubation, cells were stained with antibodies against T cell activation markers CD69-PE (BD Biosciences) and CD62L-FITC (BD Biosciences) and evaluated for activation marker expression by flow cytometry. Supernatants from these co-cultures were also collected and analyzed for the presence of IFNγ by ELISA (BioLegend). Assays were performed in triplicate and average IFNγ production was plotted with standard deviation.

Target cell lysis assay

Target cells were stained with Cell Trace Yellow following manufacturer’s recommendation (ThermoFisher), re-suspended in DMEM-media and plated at 10,000 cells per well in 50 μl in a 96 well V-bottom plate. 50 μL of CAR T cells were added at E:T ratio of 10:1 (100,000 effector cells). Plates underwent a quick spin to collect cells at the bottom of the wells and were incubated at 37°C for 18 hours. To identify lysed cells, samples were stained with Ghost Dye Red Viability Dye (Tonbo Biosciences) and analyzed by flow cytometry. Target cells were identified by Cell Trace Yellow and lysed target cells were identified by positive Ghost Dye staining. Specific cytotoxicity was calculated by the equation: 100 * (% experimental lysis – % target-only lysis) / (100 – % target-only lysis).

Disclosure statement

The authors declare no conflicts of interest.

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