Genetic Modification of T Cells Redirected towards CS1

Enhances Eradication of Myeloma Cells

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Running title: CS1-Specific CAR T Cells for Myeloma Immunotherapy

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Translational Relevance

Multiple myeloma (MM) remains incurable despite the application of multiple current therapies, and thus development of novel and effective interventions is urgently needed. CD19 chimeric antigen receptor (CAR) T cells have been shown to be effective for treatment of chronic lymphocytic leukemia and acute lymphoblastic leukemia. However, MM cells do not express CD19, while universally expressing CS1, which has restricted expression on normal cells. In this study, we engineered T cells with a second generation CD28-CD3ζ CS1-CAR construct that we generated. Our results showed that these modified T cells efficiently destroyed human MM cells in vitro, ex vivo, and in vivo. Our preclinical study suggests that adoptive transfer of T cells expressing a CAR targeting CS1 presents a promising therapeutic strategy to treat MM. Future clinical trials on this novel strategy are warranted.
Abstract

Purpose: Our goal is to test if CS1 could be targeted by CAR T cells to treat MM.

Experimental Design: We generated a retroviral construct of a CS1-specific CAR and engineered primary human T cells expressing the CAR. We then tested the capacity of CS1-CAR T cells to eradicate human multiple myeloma tumor cells in vitro, ex vivo and in vivo using orthotopic MM xenograft mouse models.

Results: In vitro, compared to mock-transduced T cells, upon recognizing CS1 positive MM cells, CS1-CAR-tranduced T cells secreted more IFN-γ as well as IL-2, expressed higher levels of the activation marker CD69, showed higher capacity for degranulation, and displayed enhanced cytotoxicity. Ectopically forced expression of CS1 in MM cells with low CS1 expression enhanced recognition and killing by CAR T cells. Ex vivo, CS1-CAR T cells also showed similarly enhanced activities when responding to primary MM cells. More importantly, in orthotopic MM xenograft mouse models, adoptive transfer of human primary T cells expressing CS1-CAR efficiently suppressed the growth of human MM.1S and IM9 myeloma cells and significantly prolonged mouse survival.

Conclusions: CS1 is a promising antigen that can be targeted by CAR-expressing T cells for treatment of MM.
Introduction

Multiple myeloma (MM) is a B cell malignancy characterized by the aberrant clonal expansion of plasma cells (PCs) within the bone marrow (BM). In 2013, it has been estimated that 22,350 individuals will be newly diagnosed with MM in the U.S. and 10,710 people will die from it, accounting for 20% of the deaths from all hematologic malignancies (1,2). MM remains incurable despite the application of therapies including proteasome inhibitors, immunomodulatory agents, and stem cell transplantation (3,4). Therefore, development of novel and effective interventions are urgently needed.

Adoptive transfer of T cells engineered to express chimeric antigen receptors (CAR) can specifically recognize tumor-associated antigens, combining the advantages of non-major histocompatibility complex (MHC)-restricted recognition with efficient T cell activation and expansion (5-8). CARs generally incorporate an antigen recognition domain from the single-chain variable fragments (scFv) of a monoclonal antibody (mAb) with transmembrane signaling motifs involved in lymphocyte activation(9). T cells harboring first-generation CARs with the CD3ζ intracellular signaling domain alone often fail to persist or become anergic due to suboptimal activation, and thus only exhibit low efficiency (10-13). To address this limitation, new generations of CARs incorporating the intracellular domains of costimulatory molecules such as CD28, 4-1BB and OX40 to provide additional activating signals...
have been developed, and T cells grafted with these CARs have demonstrated improved expansion, activation, persistence and tumor-eradicating efficiency independent of costimulatory receptor/ligand interaction (13,14). Pilot clinical trials using T cells equipped with CD19-specific second generation CARs, incorporating either CD28 or 4-1BB costimulatory signal, to treat patients suffering from relapsed B cell malignancies have yielded exciting results (15-20). CD19-redirected CAR T cells cannot be applied to treat MM, given that CD19 is detectable on tumor cells in less than 5% of MM patients (21).

The cell surface glycoprotein CS1 is an attractive target antigen, as CS1 is highly and ubiquitously expressed on the surface of myeloma cells (22). CS1 is expressed at very low levels in the majority of immune cells including natural killer (NK) cells, some subsets of T cells, and normal B cells and is almost undetectable on myeloid cells (22). Notably, CS1 is negligibly expressed in human hematopoietic stem cells (22), which can be utilized for stem cell transplantation to treat hematological malignancies including MM. The functions of CS1 in MM remain incompletely understood, and it has been documented that CS1 may play a role in myeloma cell adhesion, clonogenic growth, and tumorigenicity (23,24). Targeting CS1 with the humanized mAb elotuzumab has been demonstrated to be safe in the clinic (23,25). Preclinical studies show that this antibody inhibits myeloma cell adhesion to bone marrow stromal cells (BMSC), induces NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC), and eradicates the xenograft tumors initiated by human myeloma cells in
immunodeficient mice (23,25,26). In addition, it has been reported that the CS1239-247-specific immunological HLA-A2 peptide presented by antigen-presenting cells can redirect cytotoxic T cells to specifically recognize and eradicate HLA-A2+/CS1+ myeloma cells(27). Based on the above evidence, we postulate that CS1 may be a suitable target for CAR-expressing T cells for treatment of MM.

In the present study, we manipulated T cells to express a second generation CS1-specific CAR incorporating CD28-CD3ζ signaling moieties, and demonstrated that CS1-specific CAR T cells mediated enhanced cytokine release and cytotoxicity in response to CS1-expressing myeloma cells, which occurred in a CS1-dependent manner. Moreover, in orthotopic MM xenograft mouse models, CS1-directed T cells efficiently eradicated human myeloma cells and significantly prolonged mouse survival. Together, our data suggest that adoptive therapy with T cells armed with a CS1-specific CAR represent a promising strategy against relapsed MM.
Materials and Methods

Cell culture

Please see Supplemental Information. All cell lines were purchased from The American Type Culture Collection (ATCC) and have been authenticated with DNA profiling.

Mice

Six- to eight-week-old male NOD-scid IL2Rgamma<sup>null</sup> (NSG) mice were obtained from Jackson Laboratories. Mice were monitored frequently for MM disease progression, and sacrificed when they became moribund with the symptoms of hind limb paralysis, lethargy or obvious weight loss. All animal work was approved by The Ohio State University Animal Care and Use Committee.

Generation of CS1 specific CAR retroviral construct

The anti-CS1 single chain variable fragment (scFv) was derived from the hybridoma cell line Luc90. The coding domain sequences for variable regions of heavy (V<sub>H</sub>) and light (V<sub>L</sub>) chains were amplified separately and recombined using a linker by overlapping PCR reaction. The V<sub>H</sub>-linker-V<sub>L</sub> fragment was incorporated in frame with...
CD28-CD3ζ portion. The entire anti-CS1-scFv-CD28-CD3ζ fragment was then ligated into a retroviral vector designated Pinco (28,29) to generate a Pinco-CS1-CAR construct.

**Retroviral transduction of T lymphocytes**

The retroviral transfection and infection protocol was modified from our previous report (28,29) and has been detailed in Supplemental Information.

**Flow cytometry analysis**

For detection of CS1-CAR expression on the cell surface, transduced T cells were washed with phosphate-buffered saline (PBS) containing 4% bovine serum albumin (BSA), and incubated with biotin-labeled goat anti-mouse (Fab)2 polyclonal antibody or normal polyclonal goat IgG antibody (Jackson Immunoresearch) as an isotype control. Then cells were stained with Allophycocyanin (APC)-conjugated streptavidin (Jackson Immunoresearch) and anti-CD3 antibody conjugated to V450 (BD Biosciences). To determine the expression of CS1 on the surface of myeloma cells, the cells were stained with PE-conjugated mouse anti-CS1 mAb (eBiosciences) and APC-conjugated mouse anti-CD138 mAb (Miltenyi Biotec). Antibody staining was monitored with a BD LSRII flow cytometer. Data analysis was carried out using FlowJo software (Tree Star Inc.)
Immunoblotting

Cells were lysed in laemmli buffer. Lysates were separated by SDS-PAGE gel and transferred to nitrocellulose membrane (Millipore). The membrane was probed with mouse anti-human CD3ζ mAb (BD Pharmingen) and then with an HRP-conjugated goat anti-mouse IgG antibody. Antibody binding was revealed by using an enhanced chemiluminescence reagent (ECL, GE Healthcare Biosciences).

Generation of RPMI-8226 cells stably expressing CSI

The lentiviral transfection and infection protocol was modified from our previous report (28,29) and has been detailed in Supplemental Information.

Cytotoxicity assay

A standard 4-h $^{51}$Cr release assay was performed as described previously (30). Briefly, target cells were labeled with $^{51}$Cr and co-cultured with T cells at various effector/target ratios (E/T) in the wells of 96-well V-bottom plate at 37 °C for 4 h. Supernatants were harvested and transferred into scintillation vials containing a liquid scintillation cocktail (Fisher Scientific), and the release of $^{51}$Cr was measured on TopCount counter (Canberra Packard, Meriden, CT, USA). Target cells incubated in
complete medium or 1% SDS were used to determine spontaneous or maximal $^{51}$Cr release. Percentage of specific lysis was calculated using the standard formula: 100 x (cpm experimental release – cpm spontaneous release) / (cpm maximal release – cpm spontaneous release).

**Cytokine release assays**

Target cells were co-cultured with an equal number of effector cells in 96-well V-bottom plates at 37 °C for 24 h. Cell-free supernatants were harvested and assessed for IFN-γ and IL-2 secretion by enzyme-linked immunosorbent assay (ELISA) using corresponding ELISA kits from R&D system according to the manufacturer’s protocol.

**CD107a degranulation assay**

CD107a assay was performed as described previously with some modification(31). Briefly, MM target cells (2.5 x 10^5) were co-cultured with an equal number of effector cells in 0.2 mL/well in 96-well V-bottom plates. Control cells are either mock- or CS1-CAR-transduced T cells incubated without target cells. Anti-CD107a or IgG1 isotype antibody conjugated to APC (BD Biosciences) together with 1 µl monensin (BD Biosciences) was added and incubated at 37 °C for 4 h. Cells were further stained with PE-conjugated CD69 and V450-conjugated CD3 antibodies, and
analyzed using a LSRII flow cytometer (BD Biosciences).

**Intracellular staining of granzyme B and perforin**

Mock- or CS1-CAR-transduced T cells were washed and stained with V450-conjugated anti-human CD3 mAb. Subsequently, cells were fixed and permeabilized using Cytofix/Cytoperm kit (BD Biosciences), labeled with APC-conjugated anti-granzyme B (Invitrogen), APC-conjugated anti-perforin antibody (eBiosciences) or a mouse APC-conjugated isotype antibody, and then analyzed on a BD LSRII flow cytometer (BD Biosciences).

**In vivo treatment of MM-bearing mice and bioluminescence imaging**

MM.1S and IM9 myeloma cells were retrovirally transduced with Pinco-pGL3-luc/GFP virus expressing firefly luciferase (FFL), and GFP positive cells were sorted using the aforementioned method, yielding MM.1S-GL3 and IM9-GL3 cells, respectively. Male NSG mice were intravenously (i.v.) injected with $8 \times 10^6$ MM.1S-GL3 cells or $5 \times 10^5$ IM9-GL3 cells in 400 $\mu$L of PBS via tail vein on day 0 in order to establish a xenograft orthotopic MM model. On day 7 and day 14 (MM.1S) or day 21 (IM-9), the mice were intravenously (i.v.) administered with $10 \times 10^6$ effector cells, CS1-CAR-transduced T cells or mock-transduced control cells, in 400 $\mu$L of PBS via tail vein. Five weeks after inoculation with MM cells, the mice were
intraperitoneally (i.p.) infused with D-luciferin (150 mg/kg body weight; Gold Biotechnology), anesthetized with isoflurane, and imaged using In Vivo Imaging System (IVIS) with Living Image software (PerkinElmer).

**Statistical analysis**

Unpaired Student’s *t* test was utilized to compare two independent groups for continuous endpoints if normally distributed. One-way ANOVA was used when three or more independent groups were compared. For survival data, Kaplan-Meier curves were plotted and compared using a log-rank test. All tests were two-sided. *P* values were adjusted for multiple comparisons using Bonferroni method. A *P* value less than 0.05 is considered statistically significant.
Results

Generation of primary T cells expressing CS1-specific CAR

We constructed a Pinco retroviral vector encoding a second generation CS1-specific CAR (Pinco-CS1-CAR), which consisted of anti-CS1 scFv, the hinge and transmembrane regions of the CD8 molecule, the CD28 costimulatory signaling moiety, and the cytoplasmic component of CD3ζ molecule (Fig. 1A). Anti-CD3/CD28 antibody-activated primary T cells from a healthy donor were transduced with retroviral particles encoding CS1-CAR or empty vector (mock) and sorted for expression of GFP, which was encoded by the retroviral construct. To determine whether CS1-CAR was successfully transferred, the sorted cells were lysed and subjected to immunoblotting with an anti-CD3ζ mAb. As shown in Fig. 1B, in contrast to the mock-transduced T cells, which only expressed endogenous CD3ζ protein, CS1-CAR-transduced T cells obviously expressed the chimeric CS1-scFv-CD28-CD3ζ fusion protein at the predicted size in addition to native CD3ζ. Expression of CS1-CAR on the cell surface was demonstrated by staining transduced T cells with a goat anti-mouse Fab antibody that recognized the scFv portion of anti-CS1, which detected expression of the scFv on 70.3% of CS1-CAR-transduced T cells, while expression remained almost undetectable on mock-transduced T cells (Fig. 1C).
Recognition of CS1+ myeloma cell lines by CS1-specific CAR T cells

We evaluated the surface expression of CS1 in four commonly used myeloma cell lines NCI-H929, IM9, MM.1S and RPMI-8226 by flow cytometry, and revealed that CS1 protein was variably expressed in these cell lines with much higher expression in NCI-H929, IM9 and MM.1S cells than RPMI-8226 cells with minimal CS1 expression (Fig. 2A). As a negative control, the transformed human kidney cell line, 293T, did not express CS1 on its surface (Supplemental Fig. 1A). To determine the capacity of CS1-CAR T cells for recognition of myeloma cells with endogenously expressing CS1, IFN-γ and IL-2 secretion was measured via ELISA in supernatants from mock-transduced T cells or CS1-CAR-transduced T cells in the presence or absence of each myeloma cell line. Mock-transduced T cells and CS1-CAR-transduced T cells each alone produced negligible levels of IFN-γ and IL-2 (Fig. 2B and C); however, after exposure to NCI-H929 and IM9 cells expressing high levels of CS1, significantly greater amounts of IFN-γ and IL-2 proteins were secreted by CS1-CAR T cells but not by mock T cells. In response to MM.1S cells with high levels of CS1 expression, CS1-CAR-transduced T cells also produced a higher amount of IFN-γ than mock-transduced T cells (Fig. 2B) while, for unknown reasons, CS1-CAR-transduced T cells could not be triggered by this cell line to secrete higher levels of IL-2 than mock-transduced T cells (Fig. 2C). In addition, compared to corresponding mock-transduced subsets of T cells, both CD4+ (CD8-) and CD8+ CS1-CAR T cells displayed increased IFN-γ secretion in response to NCI-H929 or
MM.1S cells (Supplemental Fig. 2A). For RPMI-8226 cells with very low levels of CS1 expression, both mock-transduced T cells and CS1-CAR-transduced T cells produced low levels of IFN-γ and IL-2 that were comparable to background (Fig. 2B and C). These findings suggest that, compared to mock-transduced T cells, CS1-CAR-transduced T cells can more specifically recognize MM cells with high levels of endogenous CS1 expression, and become more activated after the recognition of these MM cells.

In vitro cytolytic potency against myeloma cells triggered by CS1-specific CAR

To determine whether enhanced recognition of CS1+ myeloma cells by CS1-CAR T cells could lead to more efficient tumor cell lysis, a standard 4-h chromium-51 release assay was performed. NCI-H929, IM9 and MM.1S cells, which express high levels of CS1, were resistant to mock-transduced T cell-mediated killing, even at E/T ratios as high as 20:1; however, these cells were efficiently lysed by CS1-CAR T cells at all E:T ratios tested (Fig. 3A, left three panels). However, compared to mock-transduced T cells, the cytolytic activity of RPMI-8226 cells expressing low levels of CS1 could only be slightly augmented upon co-incubation with CS1-CAR-transduced T cells (Fig. 3A, right one panel). We further characterized degranulation and activation of T cells by assessing expression of CD107a and CD69 in mock-transduced T cells and CS1-CAR-transduced T cells following incubation with or without NCI-H929 myeloma cells which, as mentioned above, triggered a strong response in CS1-CAR T
cells with respect to cytokine release and cytolytic activity. Consistent with the
disclosed data regarding cytokine release and cytolytic activity, degranulation
and activation occurred to a greater extent in CS1-CAR T cells than in mock T cells in
response to NCI-H929 cells, as evidenced by upregulation of surface co-expression of
mobilized CD107a and the activation marker, CD69 (Fig. 3B). Moreover, compared
to corresponding mock-transduced subsets of T cells, we observed that both CD4
(CD8) and CD8+ CS1-CAR T cells exhibited increased levels of degranulation when
stimulated by NCI-H929 or MM.1S cells (Supplemental Fig. 2B). In addition, using
an intracellular staining approach, we demonstrated that, compared to
mock-transduced T cells, CS1-CAR-transduced T cells expressed significantly higher
levels of granzyme B, but not perforin, even in the absence of target cells (Fig. 3C
and 3D), suggesting that granzyme B may be predominantly involved in mediating
the cytolytic activity of CS1-redirected T cells. This finding is in line with a previous
report showing T cells grafted with a carcinoembryonic antigen (CEA)-specific CAR
incorporating a combined CD28-CD3ζ signaling moiety harbored elevated levels of
granzyme B compared to unmodified T cells (32).

**Forced overexpression of CS1 in target cells enhances recognition and killing by
CS1-specific CAR T cells**

The considerably stronger response in CS1-CAR T cells in terms of cytokine release
and cytotoxicity when stimulated by myeloma cells expressing high levels of CS1
prompted us to investigate whether ectopic expression of CS1 in myeloma cells with endogenously low levels of CS1 expression could elicit an increase in cytokine release and cytolysis. To this end, RPMI-8226 myeloma cells with low levels of endogenous CS1 expression were transduced with lentiviruses encoding human CS1 or PCDH empty vector as a mock-transduced control. The transduction efficiency was monitored by detection of GFP protein encoded by the lentiviruses, and the percentage of GFP positive cells was over 90% by flow cytometric analysis (data not shown). Overexpression of CS1 was confirmed by staining the surface of the transduced cells with a PE-conjugated anti-CS1 antibody (Fig. 4A). Chromium-51 release assay indicated that forced CS1 expression resulted in a discernible increase in the susceptibility of RPMI-8226 cells to lysis by CS1-CAR-transduced T cells as opposed to mock-transduced T cells (Fig. 4B). Then we assessed IFN-γ and IL-2 production via ELISA and observed that, compared to mock-transduced T cells, CS1-CAR-transduced T cells produced significantly higher amounts of IFN-γ and IL-2 in response to RPMI-8226 cells overexpressing CS1; meanwhile, there was only a moderate increase in IFN-γ secretion and no change in IL-2 secretion when CS1-CAR T cells were co-cultured with empty vector-modified RPMI-8226 cells (Fig. 4C and 4D). Likewise, overexpression of CS1 in CS1-293T, a transformed cell line, also triggered enhanced cytokine release and cytolysis by CS1-CAR T cells (Supplemental Fig. 1B-D). This was consistent with other previous reports on CAR T cells targeting other tumor antigens (33,34). These findings corroborated that increased recognition and killing of target cells by CS1-CAR T cells occurred in
CS1-dependent manner.

**Improved recognition and killing of primary myeloma cells by autologous CS1-specific CAR T cells**

To study the effects of CS1-specific CAR T cells in a more clinically relevant context, we investigated whether CS1-CAR-transduced autologous T cells could efficiently recognize and kill tumor cells freshly isolated from myeloma patients. Like T cells from healthy donors, T cells from relapsed myeloma patients were successfully expanded and manipulated to express CS1-CAR by retroviral infection, as manifested by 60.7% of T cells staining positively with both anti-mouse Fab and anti-human CD3 antibodies determined by flow cytometry (Fig. 5A). Primary CD138+ myeloma cells from patients were isolated using positive magnetic selection, and primary myeloma cells were observed to be uniformly positive for surface expression of CS1 using flow cytometry (Fig. 5B). By chromium-51 release assay, we observed that myeloma cells from patients were highly resistant to lysis by autologous mock-transduced T cells, but became susceptible to autologous CS1-CAR-transduced T cells even at a low (2.5:1) Effector/Target ratio (Fig. 5C). In agreement with these cytotoxicity results, autologous CS1-CAR T cells produced significantly higher amounts of IFN-γ in response to myeloma cells compared with autologous mock-transduced T cells (Fig. 5D). These findings demonstrate that CS1-CAR-equipped T cells can efficiently recognize and eradicate myeloma cells in the autologous setting ex vivo.
CS1-directed T cells suppress in vivo tumor growth and prolong survival of tumor-bearing mice in orthotopic xenograft myeloma models

The therapeutic potential of CS1-CAR T cells was evaluated in an MM.1S-grafted NSG mouse model. Intravenous injection of MM.1S cells has been widely used to establish a mouse xenograft model of MM, since this can lead to the engraftment in bone marrow and bone, as well as consistent establishment of multifocal bone lesions, which closely recapitulates human MM35,36. To facilitate monitoring of tumor growth, we engineered MM.1S cells to express both GFP and firefly luciferase by retroviral infection, and GFP+ cells were sorted and intravenously grafted into NSG mice to initiate tumor growth. These mice were then intravenously infused with mock-transduced T cells, CS1-CAR-transduced T cells or PBS. In agreement with the previous reports (35,36), bioluminescence imaging using IVIS showed that MM.1S-bearing NSG mice in the PBS-treated group developed disseminated tumor lesions in skulls, vertebrae, pelvis and femurs (Fig. 6A), and the majority of the mice displayed hind leg paralyses five weeks after inoculation of tumor cells (data not shown). Infusion of CS1-CAR T cells remarkably reduced tumor burden as determined by bioluminescence imaging as well as prolonged the overall survival of MM.1S-bearing NSG mice, whereas infusion of mock-infected T cells failed to result in efficient tumor eradication and improved survival of mice (Figs. 6A and 6B).
To further validate the in vivo anti-MM capacity of CS1-CAR T cells, we evaluated the impact of CS1-CAR T cells using an IM9-grafted NSG mouse model. Similar data to those shown using MM.1S were observed. Bioluminescence imaging indicated that infusion of CS1-CAR-transduced T cells could efficiently eradicate tumors established in IM9-bearing mice, while infusion of mock-transduced T cells failed to reduce tumor burden (Supplemental Fig. 3A). Forty-four days after the initial treatment, a 100% survival rate was observed for IM9-bearing mice receiving CS1-CAR T cell infusion, while the survival rate was only 28.6% and 16.7% for control mice receiving mock T cells and PBS, respectively (Supplemental Fig. 3B).
Discussion

After being researched for over a decade, CD19 CAR T cells have been successfully applied to the clinic for treatment of refractory chronic lymphocytic leukemia (CLL) and acute lymphoblastic leukemia (ALL) patients, although normal B cells also express CD19 (15-18). These encouraging studies have fostered a great deal of interest in the application of CAR-based immunotherapy for other types of cancer. Unfortunately, tumor cells from most types of cancer, including MM, do not express CD19, suggesting that the identification and characterization of tumor-associated antigens (TAA) specific to certain types of cancer will be critical. In this study, we tested our hypothesis that CS1 is an excellent TAA to be targeted by CAR T cells for treatment of MM. CS1 is highly and uniformly expressed on MM cells from almost all patients, while having a restricted pattern of expression on normal cells and tissues (22). CS1 expression is maintained on MM cells from patients, even after disease relapse (22). Importantly, another immunotherapeutic approach for targeting CS1 using an antibody has been proven safe, and is under phase III clinical trial studies (25). We therefore reasoned that CS1 would be a unique antigen to be targeted by CAR T cells for the treatment of MM. Compared to mock T cells, CS1-CAR T cells show markedly enhanced cytotoxicity against CS1-expressing MM cell lines and primary tumor cells freshly isolated from MM patients. When co-cultured with MM cells, CS1-CAR T cells become more activated compared to the vector-transduced control cells, and both CD4+ and CD8+ CS1-CAR T cells exhibit more efficient
activation. Here, we present strong evidence that the effects of CS1-CAR T cells are CS1-dependent, as these cells do not respond well to RPMI-8266 cells, which only express low levels of CS1. Moreover, ectopic overexpression of CS1 in the RPMI-8266 target cells significantly enhances the response of CS1-CAR T cells. Importantly, CS1-CAR T cells prolong the survival of the mice bearing orthotopic MM.1S and IM9 tumors. All this evidence supports CS1 as a promising target for development of CAR T cells to treat MM.

In recent years, both antibody-mediated therapy and adoptive transfer of CAR T cells have emerged as attractive immunotherapeutic approaches against hematological malignancies. Compared to antibody-mediated therapy, CAR T cells bear the advantage in that they have the potential to replicate \textit{in vivo}, and thus may suppress tumor growth as well as prevent relapse for a prolonged period of time (15). Moreover, CAR T cells may persist with a memory phenotype, which enables them to respond more promptly and on a larger scale upon a second exposure to tumor cells (18). Additionally, considering that either anti-CS1 mAb treatment or CS1-specific CAR T cells can efficiently eradicate myeloma cells both \textit{in vitro} and \textit{in vivo}, it will be intriguing to unravel whether combinational treatment can lead to a synergistic anti-MM effect. A combination of CAR T cells with anti-PD-1 antibody has also been recently demonstrated as a good synergistic approach (37,38). In addition, it has been speculated that chemotherapy may potentiate the effect of CAR T cells in multiple ways, including enhancing their engraftment and migration towards tumor cells, as
well as potentiating the capability of CAR T cells to eradicate stressed tumor cells that would otherwise survive the chemotherapy (15,39,40). We therefore believe that infusion of CAR T cells after chemotherapy would be an effective approach to prevent MM relapse.

There are not many appropriate options for targeting TAAs with CAR T cells in the treatment of MM. Except for CS1, B cell maturation antigen (BCMA) and CD38 and were identified as targets for development of CAR T cells against MM (41)-(42). BCMA appears to be an excellent MM-associated antigen to be targeted (42), while CD38 has widespread and abundant expression on hematopoietic and non-hematopoietic tissues (43), as well as on common myeloid progenitors, increasing the potential for broad myelotoxicity (44), despite the relative safety of naked CD38 antibodies in phase 1 trials in relapsed myeloma (45). Compared to CD38, CS1 appears to have more restricted expression, considering it only has low levels of expression on NK and some T cell subsets (22). Of note, CS1-CAR T cells displayed much lower in vitro cytotoxicity against NK and T cells than against MM cells (Supplemental Fig. 4), an effect that is comparable to that of an anti-CS1 mAb that is being used in the clinic (22). As recently reported for CD44V6 CAR-T cells (46), future in vivo studies that incorporate the suicide gene iCaspase 9 will mitigate on-target but off-tumor potential cytotoxicity against normal T and NK cells by CS1-CAR T cells. In addition, since CS1 is not expressed on hematopoietic stem cells (22), we postulate that hematopoietic stem cells can continuously generate new NK and
T cells to compensate for any decrease in T cells caused by CS1-CAR T cells.

We believe a parallel comparison of the safety and anti-MM efficiency of CAR T cells targeting each of these above antigens would be ideal. Moreover, in a recent report addressing the application of CD19-specific CAR T cells in the treatment of patients with ALL, it has been demonstrated that one ALL patient relapsed with the emergence of CD19 negative blast cells after receiving infusion of the CAR T cells (16). This suggests that tumor cells may develop the ability to evade the immune system through downregulation or loss of target proteins after treatment with CAR T cells. One possible strategy to resolve this problem is to apply CAR T cells targeting an additional tumor antigen (16). Therefore, in a setting where MM patients treated with CD38- or BCMA-CAR T cells relapse with CD38 negative or BCMA negative tumor cells, CS1-CAR T cells could provide an opportunity to further treat these patients. However, the safety of this proposed strategy remains to be addressed.

One limitation of T cell-based immunotherapy to treat MM is that the infusion of major histocompatibility complex (MHC) mismatched allogeneic T cells may cause graft-versus-host disease (GVHD). Therefore, current immunotherapy for MM focuses on modulation of autologous immune cells. Unfortunately, in MM patients, immune cells including T cells are anergic due to immunosuppression (47), which is evidenced by our data showing that patient MM cells are resistant killing by mock-transduced autologous T cells (Fig. 5C). For the same reason, allogeneic stem
Cell transplantation has been applied to treat MM, and has proven effective in a small subset of patients (48). These observations imply that allogeneic CAR T cells may have higher anti-MM activity than autologous CAR T cells, as the former can be from third-party healthy donors without immunosuppression. Interestingly, allogeneic CAR T cells have recently been shown to attenuate GVHD in an animal model, although this finding awaits further confirmation (49). Another approach to overcome the problem of autologous T cells is to utilize allogeneic CAR NK cells, as NK cells have been shown to suppress GVHD in a mouse model (50), although expansion of primary human NK cells is still challenging.

In summary, we demonstrate that CS1 is a promising antigen for targeting by CAR T cells in the treatment of MM. Our efforts to utilize CS1-CAR T cells for treatment of refractory MM patients are ongoing.

Authorship

Contribution: J.C. designed research, performed experiments, and wrote the paper; S.H, Y.D., Y.P., L.Y., T.H. performed experiments; Q.W., C-H.K., S.M.D., X.H., X.B. designed research and reviewed the manuscript; J.Z. analyzed data; C.C.H. devised the concept, designed research and edited the manuscript; J.Y. devised the concept, designed research, supervised the study, and wrote the paper.
References


Abstract 2012; 120.


Figure legends:

Figure 1. Generation and expression of CS1-specific second-generation CAR. A, Schematic diagram of the Pinco-CS1-CAR retroviral construct containing a single-chain variable fragment (scFv) against CS1 linked to CD28 and CD3ζ endodomains. LTR: long terminal repeat, SP: signal peptide, VH: variable H chain, L: linker, VL: variable L chain. B, PBMCs were activated with CD3 and CD28 beads and transduced with the Pinco-CS1-CAR or Pinco construct. GFP positive cells were sorted, and cell lysates were subjected to immunoblot analysis under reducing conditions with anti-human CD3ζ primary antibody. C, Mock- or CS1-CAR-transduced T cells from healthy donors were stained with biotin-labeled goat anti-mouse Fab specific or isotype-matched control antibody, followed by streptavidin and CD3 antibody staining.

Figure 2. CS1redirected T cells secrete more IFN-γ and IL-2 than mock T cells in response to CS1-expressing myeloma cell lines. A, Flow cytometric analysis of CS1 expression on the surface of myeloma cell lines. The four myeloma cell lines indicated were stained with PE-conjugated anti-CS1 mAb antibody (solid line) or isotype-matched control Ab (dotted line). B, Mock- or CS1-CAR-transduced healthy donor T cells (2 × 10^5) were cultured alone (No target) or stimulated with an equal
number of myeloma cells expressing different levels of CS1 for 24 h, and the
supernatants were harvested to measure IFN-γ secretion via ELISA. C, Cells were
treated as in (B), and IL-2 secretion in cell-free supernatants was determined via
ELISA.

**Figure 3. CS1-redirected T cells preferentially eradicate myeloma cells obviously expressing CS1 protein.** A, ¹⁷⁷Cr-labeled NCI-H929, IM9, MM.1S and RPMI-8226 myeloma cells (5 × 10³) were co-cultured with mock- or CS1-CAR-transduced T cells at the indicated Effector/Target (E/T) ratios for 4 h, and target lysis (¹⁷⁷Cr release) was measured. B, Expression of the degranulation marker CD107a and the T cell activation marker CD69 on mock- or CS1-CAR-transduced T cells were evaluated by flow cytometry following 4 h co-culture with NCI-H929 cells. Compared to mock-transduced T cells, CS1-CAR-transduced T cells displayed superior degranulation and higher T-cell activation in response to CS1-expressing NCI-H929 cells. C, Mock- and CS1-CAR-transduced T cells were permeabilized for intracellular staining with mAb specific for granzyme B and perforin, and analyzed by flow cytometry.

**Figure 4. Ectopic overexpression of CS1 in MM cells triggers enhanced cytotoxicity and cytokine secretion after recognition by CS1-CAR T cells.** A, Flow cytometric staining for CS1 protein or IgG isotype control (dotted line) on the surface of RPMI-8226 cells overexpressing CS1 (RPMI-8226-CS1, solid heavy line) or an
empty vector control (RPMI-8226-PCDH, solid light line). B, Cytotoxicity of mock- or CS1-CAR-transduced T cells against RPMI-8226-CS1 and RPMI-8226-PCDH cells. RPMI-8226-CS1 and RPMI-8226-PCDH cells were incubated with mock- or CS1-CAR-transduced T cells at indicated E/T ratios for 4 h, and specific lysis was determined using a standard $^{51}$Cr release assay. C, Mock- or CS1-CAR-transduced T cells ($1 \times 10^5$) were cultured alone or stimulated with an equal number of either RPMI-8226-CS1 or RPMI-8226-PCDH cells. Cell-free supernatants from these cultures were used to determine IFN-γ secretion via ELISA. D, Supernatants from cell cultures in (C) were assayed for IL-2 secretion via ELISA.

Figure 5. CS1-CAR T cells specifically recognize and eliminate CS1-expressing human primary myeloma cells ex vivo. A, PBMCs from MM patients were activated with anti-CD3 and anti-CD28 beads and transduced with Pinco-CS1-CAR or Pinco construct (mock) as described in Methods. Cells were stained with anti-mouse Fab and anti-human CD3 antibodies. Results from 1 of 4 patients with similar data are shown. B, Flow cytometric staining for CS1 protein in CD138$^+$ myeloma cells freshly isolated from MM patients. Results from 3 of 10 patients with similar data are shown. C, CD138$^+$ myeloma cells in (B) were co-cultured with autologous mock- or CS1-CAR-transduced T cells in (A) at indicated E/T ratios for 4 h, and specific lysis was determined using a standard $^{51}$Cr release assay. D, Cells were treated as in (C) except that the E/T ratio was 1:1 and the incubation time was extended to 24 h, and IFN-γ secretion was determined via ELISA.
Figure 6. CS1-redirected T cells inhibit tumor growth and prolong mouse survival in an orthotopic MM.1S xenograft mouse model. A, Dorsal and ventral bioluminescence imaging of five representative mice bearing MM.1S tumors from each indicated group. NSG mice were i.v. inoculated with $8 \times 10^6$ MM.1S cells expressing luciferase (day 0). On day 7 and day 14 after inoculation, each mouse received PBS (placebo control group), $10 \times 10^6$ mock T cells (mock control group) or CS1-CAR T cells (CAR treatment group). B, Kaplan-Meier survival curve of MM.1S-bearing mice treated with PBS, mock T cells or CS1-CAR T cells.
**Figure 1**

**A**

Anti-CS1 scFv

5’LTR  SP  VH  L  VL  Hinge  CD28  CD3ζ  3’LTR

**B**

Mock T cells

CS1-CAR T cells

Chimeric Receptor

Nonspecific

Native CD3ζ

β-actin

**C**

Mock T cells

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Mock T cells

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Mock T cells

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Mock T cells

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Figure 2

A

B

C
Figure 3

A

NCI-H929

IM9

MM.1S

RPMI-8226

Specific lysis (%) vs. E/T ratio

Mock T

CS1-CAR T

Specific lysis (%) vs. E/T ratio

Mock T

CS1-CAR T

Specific lysis (%) vs. E/T ratio

Mock T

CS1-CAR T

Specific lysis (%) vs. E/T ratio

Mock T

CS1-CAR T

B

Mock T

CS1-CAR T

 Alone + NCI-H929

 Alone + NCI-H929

CD69-PE

CD107a-APC

C

D

Granzyme B

Perforin

Count

Count

 IgG

Mock T

CAR T

 IgG

Mock T

CAR T

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Figure 4

A

B

C

D

- Isotype
- RPMI-8226-PCDH
- RPMI-8226-CS1

Specific lysis (%)

20:1 10:1 5:1

E/T ratio

- RPMI-8226-PCDH + Mock T
- RPMI-8226-PCDH + CS-CAR T
- RPMI-8226-CS1 + Mock T
- RPMI-8226-CS1 + CS-CAR T

- No target
- RPMI-8226-PCDH
- RPMI-8226-CS1

IFN-γ (pg/ml)

Mock T CS1-CAR T

IL-2 (pg/ml)

Mock T CS1-CAR T

0 1000 2000 3000

0 100 200 300

** *
Figure 5

A

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B

Patient 1

Patient 2

Patient 3

CS1-PE

C

Patient 1

Patient 2

Patient 3

Specific lysis (%) vs E/T ratio

D

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Figure 6

A

Dorsal images

Ventral images

PBS

Mock T

CS1-CAR T

B

Percent survival

Days

PBS (n=7)
Mock T cells (n=7)
CS1-CAR T cells (n=7)

P < 0.001, Mock T vs. CS1-CAR T cells
P < 0.001, PBS vs. CS1-CAR T cells
Genetic Modification of T Cells Redirected towards CS1 Enhances Eradication of Myeloma Cells

Jianhong Chu, Shun He, Youcai Deng, et al.

Clin Cancer Res  Published OnlineFirst March 27, 2014.

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