A compound chimeric antigen receptor strategy for targeting multiple myeloma

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Current clinical outcomes using chimeric-antigen receptors (CARs) against multiple myeloma show promise in the eradication of bulk disease. However, these anti-BCMA (CD269) CARs observe relapse as a common phenomenon after treatment due to the reemergence of either antigen-positive or -negative cells. Hence, the development of improvements in CAR design to target antigen loss and increase effector cell persistency represents a critical need. Here, we report on the anti-tumor activity of a CAR T-cell possessing two bioengineered and independent CAR receptors against the multiple myeloma antigens BCMA and CS1. We determined that the resulting compound CAR (cCAR) T-cell possesses consistent, potent and directed cytototoxicity against each target antigen population. Using multiple mouse models of myeloma and mixed cell populations, we are further able to show superior in vivo survival by directed cytototoxicity against multiple populations compared to a single-expressing CAR T-cell. These findings indicate that compound targeting of BCMA and CS1 on myeloma cells can potentially be an effective strategy for augmenting the response against myeloma bulk disease and for initiation of broader coverage CAR therapy.

INTRODUCTION

Multiple myeloma (MM), characterized as the abnormal accumulation of immunoglobulin producing plasma cells in the bone marrow, has seen treatment advances over the years with breakthroughs in both conventional and novel treatments. While proteasome inhibitors such as bortezomib have achieved improved outcomes when combined with traditional regimens such as lenalidomide, refractory and bortezomib-resistant MM still present a significant problem for patient prognosis.1–3

Recent clinical work utilizing chimeric-antigen receptor (CAR) T-cells, especially for the treatment of CD19+ B-cell acute lymphoblastic leukemia,4,5 represents a breakthrough in cell-mediated immunotherapy with response rates as high as 93%, prompting focus on its applicability to MM. To this point, several reports have demonstrated the potential of a BCMA-directed CAR T-cell in vitro and in vivo, with promising phase I clinical outcomes.6–8 BCMA (CD269, B-cell acute lymphoblastic leukemia maturation antigen) is highly expressed on both plasma cells and MM cells as a mediator of pro-survival signals, and is not normally expressed in other tissues.6,8 When compared to CD19 CAR results in clinical phase I data, the first BCMA-CARs have shown promising myeloma remission in a majority of the small trial patient population.7,9 However, similar to CD19 CAR trials with B-cell acute lymphoblastic leukemia relapse, BCMA-CAR therapy has observed MM relapse with recurrence of BCMA+ and BCMA– malignant cells.7 The former suggests a lack of potency and persistency, and the latter implicates antigen escape by selective pressure of the BCMA-CAR.

Several ideas have been introduced for the initiation of wider coverage CAR therapy, with the notion that eliminating a broader array of plasma cell lineages, or the construction of a more robust CAR, may allow for a more persistent patient response.10 Concurrent with the chemotherapy paradigm where a combination of multiple targets leads to improved outcomes versus the refractory outcomes of a single agent, more comprehensive coverage in MM cases can allow for more efficacious depletion of malignant cells. We present the idea of a ‘compound CAR’ whereby a single T-cell encoding two discrete CAR units can more broadly target and eradicate cell types of MM that are advantaged by single CAR selection.

We propose to augment BCMA targeting with CS1 (CD319, SLAMF7). CS1 is highly expressed in normal plasma cells and in over 95% of MM patient cells, with low levels of expression in lymphocyte subsets.11 CS1-directed monoclonal antibody (elotuzumab) therapy has been approved, and pre-clinical studies for a CS1-CAR have shown efficacy.12–14 We engineered a compound CAR (cCAR) comprising of a complete BCMA-CAR linked to a complete CS1-CAR via a self-cleaving P2A peptide15 thus expressing both functional CAR molecules on the T-cell surface. We demonstrate that this BCMA-CS1 cCAR (BC1cCAR) T-cell exhibits potent and specific anti-tumor activity against both antigens, independently and combined, in vitro, and in vivo, with implications for more comprehensive clinical outcomes.

MATERIALS AND METHODS

Construction of xenogeneic mouse models

Three mice models were used to analyze anti-myeloma effect and compound antigen targeting: (1) MM1S MM tumor model N=10, (2) BCMA-K562 and CS1-K562 mixed population tumor model N=15 and

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(3) BCMA-K562 and CS1-K562 separate tumor models N=19. Male 12-week-old NOD scid gamma mice (NOD.Cg-Prkdcscid Ii2qtm1Wjl/Szl) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and used under a Stony Brook University IACUC-approved protocol. NOD scid gamma mice were irradiated with a sublethal (2.0 Gy) dose of gamma. Next day (day 1), mice were intravenously (i.v.) injected with (1) 1.0 × 10⁷ MM1S cells per mouse, or (2) 0.5 × 10⁷ total BCMA-K562 mixed with CS1-K562 in a 4:1 ratio, or (3) 1 × 10⁷ BCMA-K562 or CS1-K562 cells per mouse. Increased number of injected K562 tumor cells in model 3 was used to investigate CAR T efficacy under higher tumor burdens. Three days (day 4) following tumor cell injection, mice were intravenously injected via tail vein with a one course dose consisting of (1) 7.5 × 10⁶ BC1cCAR or control T cells, (2) 7.5 × 10⁶ BC1cCAR, BCMA-CAR, or control T-cells, (3) 7.5 × 10⁶ BC1cCAR or control T-cells.

Note: For all mice studies, one dose is defined as total cells injected; actual dose of CAR+ T-cells is lower due to efficiencies of gene transfer ~20%.

Luciferin injection and IVIS imaging was accomplished as previously described. Additional methods (including in vitro assays and flow cytometry methods) are described in supplementary Materials.

RESULTS

Generation of BCMA-CS1 cCAR (BC1cCAR) T-cells

The BC1cCAR construct is a two-unit CAR composed of a complete BCMA-CAR fused to a complete CS1-CAR by a self-cleaving P2A peptide, enabling independent expression of both CAR receptors separately on the T-cell surface. Expression was measured by FACS against control T-cells. Flow cytometry analysis of the MM10-G sample revealed a mixed spectrum of target antigen expression (Supplementary Figure 1). We established a model that allowed us to test the BC1cCAR scFv functionality independently. A CML cell line, K562, negative for myeloma markers was induced to express either CS1 (CS1-K562) or BCMA (BCMA-K562). After confirming independent antigen expression in each cell line (Figure 4a), we determined BC1cCAR T-cell targeting functionality through co-culture experiments.

In short-term cultures (overnight), BC1cCAR T-cells exhibited cytotoxic activity against BCMA-K562 cells. There were no off-target effects against wild-type K562 cells negative for either antigen (Figure 4b). Short-term cultures against CS1-K562 cells also showed similar responses against CS1-expressing target cells.

To further evaluate the BC1cCAR’s ability to kill diverse primary myeloma cell types, primary samples were chosen to exhibit a spectrum of target antigen expression (Supplementary Figure 1). Flow cytometry analysis of the MM10-G sample revealed a mixed tumor with double positive BCMA+ CS1+ as well as CS1+ only population subsets. MM7-G sample showed a complete BCMA+ CS1+ phenotype while bone marrow aspirate MM11-G exhibited a noisy BCMA+dim CS1+dim phenotype. BC1cCAR T-cells showed robust (>80%) dose-dependent ablation of the MM7-G primary patient sample (Figure 3a).

BC1cCAR also showed targeted and specific lysis ability, by significantly ablating both BCMA+ CS1+ and BCMA+ CS1+ population subsets in MM10-G co-cultures. At an E:T ratio of 2:1, BC1cCAR T-cells ablated over 60% of the BCMA+ CS1+ population, and 70% of the CS1+ only population with slight dose-dependent increases (Figure 3b). BC1cCAR T-cells were also able to demonstrate dose-dependent cytotoxic activity against the MM11-G cells (Figure 3c). Across the cytotoxicity screening, BC1cCAR T-cells exhibited robust anti-tumor activity against both myeloma cell lines and primary tumor cells expressing different combinations of BCMA and CS1 (Figure 3d).

Functional evaluation of BC1cCAR antigen-specific activity

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Figure 1. CAR construction and expression. (a) Two discrete CAR units: an anti-BCMA CAR comprised of: a CD8-derived hinge (H) and transmembrane (TM) regions, and 4-1BB co-activation domains linked to the CD3ζ signaling domain is fused to a complete anti-CS1 CAR by a self-cleaving P2A peptide. A strong spleen focus forming virus promoter (SFFV) and a CD8 leader sequence were used for efficient expression of the BC1cCAR molecule on the T-cell surface. (b) Expression was measured by FACS against control T-cells.
In addition, BC1cCAR T-cells appeared to have a stronger cytotoxic effect than a CS1-specific CAR against CS1-K562 cells (Figure 4b).

Residual tumor populations possessing a non-target antigen may lead to relapse in patients who have undergone treatment using a single-antigen CAR. Thus, to model more clinically relevant mixed antigen-expressing cell populations, we conducted combined co-culture experiments. BCMA-K562 and CS1-K562 cells were mixed in 1:1 ratios in a sustained (48 h) culture to assay for residual antigen-positive populations. Next, histograms were constructed that represented populations of T-cells and target tumor cells with residual gated target tumor populations marked (Figure 4c). We found that compared to control T-cells, BCMA-specific CAR and CS1-specific CAR T-cells had profound cytotoxic effects against their respective target populations. However, CS1-CAR left a significant residual BCMA+ population, whereas BCMA-CAR achieved a high degree of cytotoxicity but left a small CS1+ population. In contrast, the BC1cCAR T-cells effectively depleted both target populations (Figure 4c).

Tumor re-challenge demonstrates sequential killing ability of BC1cCAR T-cells

We next investigated the ability of BC1cCAR T-cells to kill tumor cells in a sequential manner under unfavorable microenvironments caused by cell lysis, debris and tumor re-challenge. Using the scheme in Figure 5a, we conducted long-term co-cultures using MM1S cells as a model myeloma tumor and periodically re-challenged BC1cCAR and single BCMA-CAR and CS1-CAR T-cells with fresh MM1S cells to simulate tumor expansion or relapse. Even without exogenous cytokines, we found that all CAR treatments depleted target antigens after 48 h, with significant clustering and T-cell proliferation (Figure 5b). In contrast, control T-cells showed no response or proliferation, and yielded a tumor cell population twice its initial size. After re-challenging all treatment wells with fresh MM1S cells we found that all CARs still retained a high degree of cytotoxicity. By 108 h, new MM1S cells were virtually depleted by both BCMA-CAR and the BC1cCAR,
while the CS1-CAR displayed incomplete killing of the new MM1S cells (Figure 5c). All CAR-mediated tumor lysis and cytotoxicity stopped after 168 h; however, BCMA-CAR and BC1cCAR still showed detectable minority T-cell populations while control T-cells and CS1-CAR T-cells were virtually undetectable (data not shown).

In order to evaluate the in vivo activity of BC1cCAR T-cells, we developed a myeloma mouse model with luciferase-expressing MM1S cells to induce fluorescence visible tumor formation. The BC1cCAR T-cells significantly reduced tumor burden and...
prolonged survival in MM1S-injected mice when compared to control T-cells. Mice were given a single dose of BC1cCAR or control T-cells and tumor burden assayed by IVIS imaging (Figure 6a). There were highly significant differences ($P < 0.0003$) in tumor burden between the control group and the BC1cCAR treatment group from day 6 onwards (Figure 6b). CAR-injected mice also had significantly more favorable survival outcomes (Figure 6c).

Mixed antigen population mouse models demonstrate superior tumor burden control by cCAR expressing cells versus single CAR expressing cells.

To model heterogeneous cell populations and potential antigen escape, we injected mice with a 4:1 mix of BCMA:CS1-expressing K562 cells and treated on day 3 with $7.5 \times 10^6$ of either control, BCMA-CAR or BC1cCAR T-cells. CS1-CAR T-cells were excluded on the basis of inferior in vitro efficacy. On day 3, two control mice
Scheme for rechallenging CAR cells with MM1S after initial cytotoxicity for sequential killing assay

Day 1 - Culture setup with initial MM1S tumor
Day 3 - Label and rechallenge with MM1S tumor cells
Day 5-6 - Label and rechallenge with MM1S tumor cells
Day 7-8 - Label and collect

b) CAR cell proliferation and antigen depletion after 48 hours of culture

Compound CAR T-cells target BCMA and CS1 myeloma antigens

Figure 5. Long-term sequential killing assay and tumor re-challenge. (a) Assay was conducted over a period of 168 h without exogenous cytokines and initial culture was performed using a 1:1 E:T ratio of CAR cells or control cells mixed with BCMA-CS1+ MM1S cells. After 48 h, flow cytometry analysis was acquired for a small sample collection and MM1S cells were re-introduced into each treatment well. Repeated through the 168 h time-point. (b) T-cell proliferation and response after 48 h. Images were taken on the day of flow cytometry acquisition and cells were stained with anti-BCMA, anti-CS1 and anti-CD3 antibodies, MM1S cells (circled, blue). (c) Similar image acquisition and FACS analysis was performed at the 108 h time mark.
died as a result of the injection procedure and were excluded from analysis. Tumor burden was visualized by fluorescence (Figure 7a).

At day 10, both CARs exhibited over 50% tumor reduction compared to GFP control, increasing to over 60% by day 12 (Figure 7a, right). By day 10, BC1cCAR outpaced BCMA-CAR in tumor suppression by 6% and this spread increased to 17% by day 12, potentially modeling the inability of BCMA-CAR to lyse residual CS1-K562 cells (20% of tumor injected). Survival outcomes for all CAR T-cell-treated mice were significantly improved over the control group. There was also a significant improvement ($P < 0.05$) in survival for the BC1cCAR group versus the BCMA-CAR group (Figure 7b). While both CARs were efficacious in controlling tumor growth, the BC1cCAR demonstrates more robust control compared to a single target option.

Enhanced T-cell persistency and maintenance of tumor depletion by compound CAR T-cells in independent antigen mouse models

To assay specific BCMA and CS1 antigen-expressing cell depletion and verify compound scFv efficacy, a third mouse model was constructed in which four groups consisting of five mice each were injected with either BCMA-K562 or CS1-K562 cells, with control and BC1cCAR T-cells administered to each tumor group ($n = 19$ as a result of an early spontaneous mouse death). At time of killing (various: day 30–80+), mice whole blood and liver tissues were screened for T-cell and tumor populations. Both hematological tissue types show consistent tumor presence in control groups when compared to cCAR groups (Figures 8a and b, Supplementary Figures 2). Aggregate tissue analysis of averaged tumor cell populations in both tissues show consistent trends of depleted tumor burden in cCAR-treated mice groups (Figure 8b). In both the blood and liver, control T-cells were unable to persist beyond the 30-day mark and exhibited significant tumor burden in both tissue types (Figures 8b and c). In contrast, cCAR-treated mice showed significant T-cell expansion and persistency compared to control T-cells across all mice even at day 30+ (Figure 8c), correlating with observed increased anti-tumor activity and supporting overall improved survival.

**DISCUSSION**

Several approaches exist for coverage of multiple targets, including bispecific CARs and dual CAR combination therapy. However, bispecific antibodies for CAR applications remain difficult to optimize and co-administration of two CAR T-cell populations result in increased observations of tonic signaling and reduced efficacy. Co-cultures of two CAR T-cell populations have resulted in a disproportionate expansion of one population over another, creating a dominant CAR T-cell population, potentially reducing clinical impact. Compound CAR therapy may sidestep these limitations by expressing...
independent CAR molecules in roughly equal ratios on a single T-cell. Limited available data on patient outcomes from ongoing BCMA-CAR trials show that the majority of myeloma relapse is BCMA+, with one patient developing BCMA- relapse. Patients with the strongest treatment response had the highest BCMA-CAR T-cell count, suggesting that robust in vivo CAR T-cell proliferation and persistency lead to better treatment outcomes. We propose that targeting CS1 can potentially augment the anti-tumor response of a compound CAR when compared to a single BCMA or CS1-CAR, and, furthermore, can eliminate any BCMA- CS1+ myeloma cells that may be a source of potential relapse. To study the efficacy of the compound CAR we identified three main criteria for its viability as an enhanced CAR: (1) basic cytotoxic functionality, (2) independent antigen targeting and (3) relative persistency and activity in vitro and in vivo.
We confirmed the basic cytotoxicity properties of the BC1cCAR against both myeloma cell lines and primary myeloma samples including both dual positive phenotypes (BCMA⁺CS1⁺), as well as singular positives (BCMA⁺CS1⁻ or BCMA⁻CS1⁺). Long-term cultures with the BC1cCAR T-cells were comparable to each of its single-unit CARs when cultured with repeated tumor re-challenge experiments, suggesting that compound CAR technology is not likely to inhibit T-cell activity and proliferation compared to current design paradigms.

Figure 8. Improved BC1cCAR T-cell persistency and maintenance of tumor suppression in separate antigen models. (a) Whole blood samples from mice injected with either BCMA-K562 or CS1-K562 tumor cells (n = 5 per group) were taken at the time of killing. Histogram population of BCMA or CS1-positive peaks represent tumor presence. (b) Aggregate tissue analysis of both whole blood and liver samples across killed mice are summarized. Mice tumor cell counts were established by FACS of antigen-positive cells per 250 000 cells collected per sample and averaged across all mice per treatment group. (c) Whole blood and liver tissues were also analyzed for T-cell persistency by CD3 expression at the time of killing summarized across all sacrificed mice (right).
Independent targeting of multiple antigens (BCMA, CS1) was verified through generation of single antigen-expressing CML lines. The BC1cCAR was able to ablate both the BCMA-expressing K562 (BCMA-K562) and the CS1-expressing K562 (CS1-K562) cells independently in co-culture assays. Further comparisons with single CARs encoding either anti-BCMA or anti-CS1 scFvs showed that while each single CAR ablated its own target antigens, both single CARs were unable to ablate a residual population of the other antigen, in contrast to successful dual ablation by the BC1cCAR.

Finally, we showed the BC1cCAR promoted sustained in vivo activity against the MM1S cell line, as well as superior murine survival in a mixed cell model. Analyses of both the mixed BCMA-CS1-K562 mouse model and the separate BCMA-K562 or CS1-K562 models showed that BC1cCAR T-cells achieved the same or better in vivo imaging results compared to a BCMA single CAR while promulgating superior survival outcomes. In comparison to control T-cells in the two mouse groups injected with BCMA- or CS1-expressing K562 cells, BC1cCAR T-cells were able to proliferate and persist in mouse circulatory tissues even by day 50+, at greater rate than single BCMA-CAR mice at the survival endpoints. All compound CAR-treated mouse hematological tissues were virtually free of tumor with large populations of expanded BC1cCAR T-cells at these endpoints (Figure 8c).

The BC1cCAR holds potential to be a more comprehensive option for myeloma therapy due to the high expression of both BCMA and CS1 on MM cells. BCMA signaling protects myeloma cells from apoptosis and has been utilized in CAR applications.8,9,23 CS1 signaling has roles in myeloma pathogenesis and specific immune cell activities.11,24–26 However, compound CAR therapy involving discrete CAR units in a single T-cell requires consideration of the safety of targeting each antigen, in addition to potentially additive or multiplicative therapeutic effects. BCMA has been well-qualified as a dominant myeloma plasma cell marker with little expression on hematopoietic or other healthy tissues,9 while CS1 has low levels of expression on small subsets of immune cells.11 anti-CS1 elotuzumab therapy has been successfully used in clinic for the treatment of myeloma without evidence of elotuzumab-mediated depletion of CS1+ lymphocytes.16,24,27 Still, close monitoring of such combination therapy will prove useful for analyzing its long-term effects.

The development of BCMA and CS1 CARs signal the beginning of practical cell therapy for myeloma. As the first trials show, the need for more comprehensive tumor coverage is a hallmark of cancer treatment. A multi-pronged approach is a logical extension of the cancer treatment paradigm, and we show that a compound CAR may be an effective method for augmenting current MM therapeutic efforts.

CONFLICT OF INTEREST

Yupo Ma is a co-founder of iCell Gene Therapeutics, LLC. The remaining authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Designed and performed the experiments, interpreted data and wrote the manuscript: KHC, YM; Performed experiments: MW, KGP, HL, Wrote the manuscript: LEY, JCP, XS, XC, HS, ELHL, XJ.

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