Characterization of novel dual tandem CD19/BCMA chimeric antigen receptor T cells to potentially treat multiple myeloma

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Research

Abstract

Background: Chimeric antigen receptor (CAR) engineered T cells directed B cell maturation antigen (BCMA) showed transient recovery to multiple myeloma (MM). However, the expression of CD19 on immature plasma cell may escape the recognition by BCMA-CAR T, which restrict the efficacy and facilitate to relapse. The purpose of this study is to characterize a novel CAR structure with a tandem orientation of scFv-BCMA and scFv-CD19, tandem CAR (tan-CAR), to provide an effective solution for killing both BCMA and/or CD19 expression MM cells.

Method: Single-chain variable fragment (scFv) sequences from the anti-CD19 antibody FMC63 and the anti-BCMA antibody C11D5.3 were ligated in tandem with transmembrane and T cell signaling domains to achieve tan-CAR construct. The therapeutic specificity and efficiency were analyzed for tan-CAR T cells activation, proliferation, cytokine release and cytolytic toxicity *in vitro*. Also, *in vivo* efficacy evaluation conducted in xenograft mouse models with the combination of two corresponding target tumor cells, in comparison with conventional CAR.

Results: The *in vitro* studies demonstrated specific activation of tan-CAR T cells to the K562 tumor cell overexpressing CD19, BCMA, or both. Besides, it also elicits the comparable immunoreactivities, in terms of proliferation, cytokine release and cytolytic activity compared to single scFv modified CAR T cells. Importantly, the *in vivo* studies of tan-CAR-transduced T cells results specific inhibition of tumor growth in xenograft model that express combined tumor antigen i.e. CD19 and BCMA. Moreover, systemic administration of tan-CAR resulted in complete tumor remission, whilst neither BCMA-CAR T nor CD19-CAR-T could.

Conclusion: A novel tan-CAR T was successfully designed and showed the significant antitumor efficacy for combined antigen-positive tumor cells *in vitro* and *in vivo*. However, the single CAR T cells with targeting one antigen didn't achieve similar potency. The data from this study suggest a novel strategy to help reduce relapse due to existing CD19-expressing multiple myeloma cells or downregulation of the BCMA antigen after CAR-based treatment of multiple myeloma.

Introduction

Multiple Myeloma (MM) is a malignant neoplasm state of plasma cells where uncontrolled expansion and proliferation of clonal plasma cells lead to destruction of bone marrow failure end with organ damage [1]. Concurrently, several new drugs and regimen are approaching to treat MM. Though these regimens provide safer and prolonged standard, limited number patients show response to typical strategies treatment[2-4]. Therefore, an atypical innovative strategy being a prerequisite for significant therapeutic effect.

Concomitantly, adoptive cell therapy or cellular immunotherapy is now being an evolving treatment against cancer through redirect T cell by cellular engineering. Therefore, adoptively transferred chimeric antigen receptor (CAR) engineered autologous T cells reflected with unprecedented success in treatment

of hematological malignancy [5-7]. In parallel, diverse immunotherapeutic approaches are broadly under investigation to reap their effects with target antigen specificity and activation [8]. In recent years, CAR-T for MM treatment has shown considerable promising effect with manageable toxicities. In particular, B-cell maturation antigen (BCMA) reckoned as standard target due to its preferential expression on plasma cells [9-11]. To date, early phase clinical trial studies of anti-BCMA single-chain fragment variable (scFv) modified CAR T has shown undeniably high response rates. However, transient response with frequent relapse has been observed due to downregulation or lack of BCMA on tumor surface following CAR-T infusion [12]. Since, soluble BCMA drifted from tumor to neighboring area or circulation is potentially detrimental that disguise and block the recognition of BCMA+ MM cells by BCMA-CAR-T cells[13]. Furthermore, a study by R. Hajek *et al*. demonstrated that a group of CD19+ and BCMA+ residual malignant plasma cells exists in the tumor. These cells can drive self-renewal, myeloma propagation and chemo-resistance, which were considered as cancer stem cell like MM cells [14]. Moreover, sustained remission was observed in one patient with advanced multiple myeloma who received anti-CD19 chimeric antigen receptor T cells in conjunction with autologous stem cell transplantation [15]. Thus, this quest has fostered us to promote a combination of CD19 and BCMA for treating multiple myeloma to reduce the risk of relapse.

Herein, we demonstrate, for the first time to our knowledge, a novel CAR construct with tandem alignment contains dual scFv (tan-CAR) targeting CD19 and BCMA antigen. As a result, targeting alone or both antigens exert the equivalent notable significant cytotoxicity as conventional single scFv modified CAR-T in *in vitro* study. Apparently, *in vivo* studies depicted that T cells conferred with tan-CAR could not only characterize tumor cell with distinct antigen, but also promote effector cells to eradicate the mixture of the malignant cells expressing CD19 and BCMA with complete remission attainment. Finally, this duo tumor antigen targeting strategy may assist to reduce the relapse due to potential detrimental phase of BCMA antigen after CAR based treatment of multiple myeloma.

In this study, a novel dual tandem CD19/BCMA CAR lentiviral vector was constructed, and subsequently transduced into T cells to obtain the tandem CAR T cells. Tan-CAR T cells demonstrated a specific and efficient cytotoxicity against tumor cells overexpressing either CD19 or BCMA both *in vitro* and *in vivo*. This dual tumor antigen targeting strategy may also assist to provide a new strategy for treating MM patients and reducing the relapse due to potential detrimental phase of BCMA antigen or MM residues with CD19 expression.

Materials And Methods

Plasmid construction and lentiviral vector production

The tandem -CAR vector is a second-generation vector consisting of the following components in-frame from the 5' end to the 3' end. These includes CD8 signaling peptide sequences, anti-BCMA scFv (C11D5.3)[16], anti-CD19 scFv (FMC63AA 1-267, GenBank ID: HM852952.1), the hinge and transmembrane regions of the CD8α molecule, the cytoplasmic domain of CD28, and the CD3 zeta

signaling domain. The sequence was synthesized by Tsingke Biological Technology (Shanghai, China) and cloned into the plasmid of pUT-Backbone (Unicar-Therapy Biomedicine Technology Co., Ltd., Shanghai, China). The newly constructed lentiviral vector named tan-CAR. CD19-CAR and BCMA-CAR with the same cytoplasmic domain of CD28, and the CD3 zeta signaling domain were constructed in the same way. All lentivirus were generated by transient transfection of HEK 293T cells.

Cell lines

The chronic myelogenous leukemia cell line K562 was purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT). K562 cells were stably transduced with the lentiviral constructs encoding CD19 or BCMA, and the luciferase gene. Following transduction, single-cell luciferase-positive clones were selected for expansion, the stable cell lines named K562-CD19-luc or K562-BCMA-luc.

CAR-T cells preparation

Healthy donor-derived peripheral blood mononuclear cells were purified from the blood by gradient centrifugation using Lymphoprep™ (Oriental Hua Hui, Beijing, China), and then, human CD3+T cell were enriched by positive selection using magnetic bead separation (Miltenyi Biotec, Bergisch Gladbach, Germany). CD3+T cells were cultured and activated *in vitro* using anti-CD3/CD28 monoclonal antibodies (Miltenyi Biotec) in a 5% CO_2 atmosphere at 37°C for 18−24 h. The activated T cells were then transduced with lentivirus for 48 h. After transduction, the CAR T cells were cultured and expanded in a 5% CO_2 atmosphere at 37°C for 14 days with the T cell medium AIM-V (Gibco, Grand Island, NY, USA), which contained 100 IU/ml recombinant human interleukin-2 (IL-2; Peprotech, Rocky Hill, NJ, USA), 5 ng/ml recombinant human interleukin-7 (IL-7; Peprotech), 5 ng/ml recombinant human interleukin-15 (IL-15; Peprotech) and 10% autologous plasma[17].

Flow cytometry

For the flow cytometry assays, the cells were harvested and washed twice with 1 ml of phosphate-buffered saline containing 2% fetal bovine serum (FBS; Gibco). For the transduction efficiency and CD4/CD8 ratio analysis, the CAR T cells were labeled with protein L-FITC (ACRO Biosystems, Beijing, China), CD4-PE-Cy7 (eBioscience, San Diego, CA), and CD8-APC (eBioscience) for 45 min at 4°C in the dark. For detection of the CD19 CAR-expressing T cells, the CAR T cells were incubated with human CD19 protein-FITC (ACRO) for 45 min at 4°C in the dark. For detection of the BCMA-CAR T cells, the CAR T cells were labeled with human BCMA protein-FITC (ACRO) for 45 min at 4°C in the dark. The cells were washed twice before analysis by Attune NxT flow cytometer (Thermo Fisher, Waltham, USA).

T cell activation ability assay

T cell activation ability was confirmed by measuring the CD69 expression of the CAR T cells after cocultured with target cells for 24 h. Untransduced (NC) T cells were used as negative controls, and the T

cells transduced with CD19-CAR or BCMA-CAR served as positive controls. After cocultured, the cells were harvested and washed twice with 1 ml of phosphate-buffered saline containing 2% FBS, and then were labeled with CD69-PE (BiolegendCalifornia, USA), CD3-FITC (Biolegend) and protein L-FITC (ACRO) for 20 min at room temperature in the dark. CD69 expression in CAR+ T cells were detected by flow cytometry represented the CAR T cell activation ability.

Quantitation of T cell proliferation

Cell proliferation assays were performed using a Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) assay kit (Abcam) following the manufacturer's directions. In brief, the CAR T cells were labeled with 2.5 µM CFSE and then cocultured with inactivated Raji cells at a stimulator to responder (S:R) ratio of 2:1 (1 x 10^6 CAR T cells/ml) for 5 days in 96-well plates with a total of 200 µl of serum-free AIM-V (Gibco)medium per well. Flow cytometry was performed using an Attune NxT flow cytometer (Thermo Fisher) to detect the CFSE intensity. FlowJo V10 software (TreeStar, San Carlos, USA) was used for data analysis.

Cytotoxicity assays

Cytotoxicity assays were conducted by measuring lactate dehydrogenase activity in the supernatants of the effect and target cells coculture system using the Cytotoxicity Detection Kit (Promega, Madison, USA) following the manufacturer's protocol. All the transduced forms of CAR T cells (effector, E) cocultured with the K562 cell lines overexpressing CD19, BCMA or both antigens (target, T) at E:T ratios of 5:1, 2.5:1 and 1:1. Target cells and corresponding proportion of effector cells were seeded in 96-well plates in a total volume of 100 µl of serum-free RPMI 1640 media (Gibco) at 37°C for 6 h. After the coculture, 50 µl of supernatant from each well was transferred to a new 96-well plates and mixed with equal volume of lactic acid dehydrogenase substrate mixture for 20min at room temperature in the dark. The absorbance was recorded at 492 nm using a full wavelength reader Multiskan GO (Thermo Scientific). The percentage of tumor lysis was calculated with the following formula: %tumor lysis = (experimental LDH release - spontaneous LDH release)/ (maximal LDH release - spontaneous LDH release) × 100.

CD107a degranulation assay

For the CD107a degranulation assay, 1 x 10^6 CAR T cells were cocultured with target cells at a 5:1 ratio in 96-well plates with a total of 200 µl of medium per well for 6 h. The Golgi inhibitor monensin (Invitrogen, Carlsbad, US) was added before the incubation. Cocktails (Invitrogen, Carlsbad, US) were added to the positive control group before the coculture. After the 6 h incubation, cells were labeled with CD107a-APC, CD3-FITC and CD8-PE. All the antibodies purchased from Becton, Dickinson and Company Co., Ltd (New Jersey, USA). Before flow cytometry analysis, the cells were collected and washed twice. Flow cytometry was performed on an Attune NxT flow cytometer (Thermo Fisher), and the results were analyzed by FlowJo V10 software (TreeStar).

Cytokine secretion analysis

The cytokines were measured using a Th1/Th2 Cytometric Bead Array (CBA) Kit II (BD Bioscience) according to the manufacturer's instructions. Briefly, different CAR-transduced T cells cocultured with different type of K562 cells at an E:T ratio of 5:1 in a 96-well plate with total 200µl volume of RPMI 1640 medium (Gibco). After 24 h co-incubated, supernatants were harvested and the cytokine secretion levels were measured. The capture microspheres for the seven cytokines (IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-a, and IL-17A) were first mixed and then incubated with the sample and fluorescent antibody for 3 h. The mixture was washed and measured by flow cytometry (Thermo Fisher). The concentration of each cytokine was calculated from standard curves.

Xenograft animal model

Mouse experiments were performed following approval from the Institutional Animal Care and Use Committee of East China Normal University. Four to six-week-old male NOD/scid/γc-/- (NSG) mice were purchased from Biocytogen Co., Ltd (Beijing, China). To verify the tan-CAR antitumor effects, animal models established via injection of K562-CD19-luc or K562-BCMA-luc or a combination of the two kinds of targeting cells. Total $7×10^6$ cells of two kinds of tumor cells at a ratio of 1:1 of in 200 µl phosphate buffered saline (PBS) were injected via the tail vein into mice on day 0. The mice were then randomly divided into four groups and treated with either $1x10^7$ CD19-CAR T cells (n=3), $1x10^7$ BCMA-CAR T cells (n=3), $1x10^7$ tan-CAR T cells or same number of un-transduced T cells (n=3, negative treatment control)on day 8 and day 10. For the mice received only K562-CD19-luc or K562-BCMA-luc, received CD19-CAR T cells or BCMA-CAR T cells for the treatment. Tumor progression was monitored by bioluminescent imaging every four days beginning on day 7. The mice were sacrificed when moribund or upon the development of hind-limb paralysis. For *in vivo* imaging, the mice were injected intraperitoneally with 150 mg/kg D-luciferin (Yeasen, Shanghai, China) and imaged under isoflurane anesthesia using the Xenogen-IVIS system. Fluorescence was quantified using Living Image software (IVIS Lumina Series, PerkinElmer, Massachusetts, USA).

Statistical analysis

Statistical analyses were carried out using GraphPad 8.0. Biological replicates of *in vitro* (n = 3) and *in vivo* data (n = 3) are presented as the mean ± SD. Statistical analysis was performed differences between individual treatment groups and the untransduced control group were analyzed for significance using one-way ANOVA. Statistically significant findings were defined as $*p < 0.05$.

Results

Generation and characterization of the tan-CAR-transduced T cells

To authenticate and develop a CAR strategy that can simultaneously target CD19 and BCMA both respectively, we designed and generated a novel CAR construct in tandem orientation of CD19-scFv and BCMA-scFv domains. Consequently, this tandem-CAR comprise of bi-specifically fused two of those domains with a CD28 costimulatory domain and a CD3ζ-mediated activation signal domain. In parallel,

to assessed the specificity of T cell activation, we constructed two positive control CARs including single-receptor CARs against CD19 (CD19-CAR) and single-receptor CARs against BCMA (BCMA-CAR) following identical co-stimulatory domain and activation domain as in tan-CAR (Fig 1A). In addition, our data demonstrated that tan-CAR not only induced activation of T cells but also effectively instruct to kill target cells that expressed either CD19 or BCMA compared to conventional CAR. Afterwards, transduction efficiency of these artificial constructs evaluated by lentivirus vector that contain tan-CAR, CD19-CAR, BCMA-CAR transduced to primary T cells. The surface expression of antigen receptors of corresponding modified T cells typically yielded from 46 to 55 % individually that verified through flow cytometry using L-protein-FITC (Fig. 1B). Similarly, for validation of co-expression of both CD19 and BCMA on transduced T-cells, we introduced tan-CAR effector cells to human CD19 protein or human BCMA protein to define specific CAR T cell detection (for details, see Methods). Subsequently, tan-CAR transduced T cells demonstrated equivalent levels of double scFv molecules i.e 59% BCMA-scFv and 53% CD19-scFv respectively compared to typical single scFv CAR and more comparable to detect scFv expression by L protein (Fig. 1B). These results suggested that tandem fusion of two respective scFv protein could successfully developed with an optimal equivalent expression of tan-CAR on primary T cells. Later, we also examined the effect of the tan-CAR construct on the CD4 to CD8 ratio in the subsequent transduced tan-CAR T cells which demonstrated in figure 1C. As depicted, tan-CAR modified T cells produced equal quantity of CD4 to CD8 transduced T cell ratios compared with two distinct corresponding CAR T cells of CD19 and BCMA which may translate with similar efficacy of the CAR from these three following groups of CAR T cells.

tan-CAR transduced T cells activation via CD19 or BCMA

CD69 is one of the most common markers for T cell activation [18, 19]. To demonstrate specific activation of tan-CAR transduced T cells which triggered by corresponding target cells, a human leukaemia cell line K562. Therefore, K562 cells were utilized and modified by lentivirus encoding CD19, BCMA or both antigens to develop the corresponding target cells of K562-CD19, K562-BCMA or K562-CD19+BCMA. Consequently, T cell activation to diverse categories of K562 cells were analyzed by expression of CD69 marker on T cell surface through flow cytometry and adequately expressed for activation with specificity (Fig. 2A). In particular, the tan-CAR modified T cells exhibited substantially high CD69 expression level that was equivalent to single receptor modified T cells CD19-CAR, BCMA-CAR. This data showed that tandemly modified CAR transduced T cells express considerable equivalent activation to distinctly antigen positive target cells for CD19, BCMA or both antigens compared to conventional single CAR modified T cells.

Tumor cells-induced T cell proliferation using CD19 or BCMA antigen

CAR-T cell proliferation due to endorsement of tumor cell is a fundamental requirement and crucial factor for augmenting anti-tumor efficacy of T cells [20]. Therefore, our next investigation was to determine whether the proliferation of the tan-CAR T cells was dependent on tumor cells expressing either CD19 or BCMA.

Different scFv modified T cells were labeled with CFSE, followed by cocultured with corresponding target cells to evaluate T cell proliferation activity by detecting a decrease fluorescent dye in the CFSE. In this study, the tan-CAR T cells demonstrated high proliferation towards the target cells of overexpressing CD19 and BCMA or both, along with the combination of K562-CD19 and K562-BCMA at a ratio of 1:1. Expectedly, the propagation level of tan-CAR was similar to its corresponding single-scFv CAR T positive control groups of CD19-CAR T cells and BCMA-CAR T cells, respectively. In contrast, the NC T cells showed limited proliferation after incubation with the target tumor cells as above (Fig. 2B). Together, these results showed that tan-CAR T cells as well as their corresponding single scFv-CAR T cells have similar significant proliferative activities with antigen-stimulation specificity.

Determination of cytotoxic efficacy of tan-CAR T cells

To evaluate cytotoxic specificity of tan-CAR T cells, we first quantified the level of LDH in the supernatant that was released from apoptotic target cells of each subsequent group. This study was systemically designed with distinct effector T cells including NC T, CD19-CAR, BCMA-CAR and tan-CAR T cell group. Corresponding effector T cells were co-cultured with different types of target cells of K562-CD19, K562-BCMA, K562-CD19+BCMA and wild type K562 as the negative control. After a 24 hour of incubation, a significant cytotoxicity was observed in tan-CAR T cells with all three target cell groups. However, among them conventional single CAR T cell only showed the cytotoxicity with its corresponding target cells. Expectedly, the negative control effector T cell (NC T) group didn't show significant cytotoxicity to any targeting groups. (Fig. 2C).

Afterwards, a degranulation assay was proceeded to validate the cytolytic function of T cells through quantify the CD107a expression on cell surface by flow cytometry. After 6-hour incubation, a significant high expression of CD107 was observed in tan-CAR modified T cells compared to NC T cells. In parallel, similar upregulation was observed in corresponding single conventional CAR construct. Thus, the CAR T cells expressing a single scFv were only responsive to their own corresponding antigens (Fig. 2D).

Lastly, the proinflammatory cytokine release assay was carried out to further confirm the CAR T cell cytotoxic activities. In our experiment, we measured the cytokine levels of effect T cells including tan-CAR T cells, CD19-CART, BCMA-CART and the NC T cells after cocultured with corresponding targeting cells, as well as a K562 negative control cell, using the Th1/Th2 Cytometric Bead Array (CBA)Kit II. As we expected, the tan-CAR T cells were observed with significant comparable cytokine production level compared to CD19-CAR T and BCMA-CAR T cells. Also, the NC T cells released limited level of cytokines when encountering the target cells (Fig. 2E). Collectively, the above experiments demonstrated that the cytotoxicity level of tan-CAR engineered T cell is equivalent to the conventional CAR-T cells. Following cytotoxicity demonstrated that this tan-CAR construct exerts therapeutic intervention with significant proliferation and antigen specificity.

tan-CAR-transduced T cells effectively clear tumors *in vivo*

After indorsing the antigen specificity and cytotoxicity of tan-CAR T cells *in vitro*, we performed *in vivo* study to evaluate tumor regression in a xenograft model. In this model, mice were treated with the target cells of K562-CD19-luc, K562-BCMA-luc or the mixture of the two target cells at 1:1 ratio. Afterwards, modified T cells including tan-CAR T cells, two types of corresponding conventional single-scFv CAR T cells, as well as the NC T cells were intravenously injected at day 8th or day 10th (Fig. 3A). Therefore, the tumor growth was monitored by IVIS imaging, and representative images showed the progression or inhibition of disease of each group (Fig. 3B). Single tumor antigen established mouse model, whether K562-CD19-luc or K562-BCMA-luc, treated with conventional single CAR-modified T cells targeting CD19 or BCMA showed significant decrease of tumor burden based on bioluminescence imaging analysis. However, the conventional single scFv CAR T cells of anti-CD19 or anti-BCMA could not inhibit tumor cell growth in the combined tumor antigen targeting xenograft model. Consequently, it is found that only tan-CAR T cells showed an efficient tumor inhibitory response including exhibited tumor remission with antigen specificity. The signal intensity was plotted over time, as shown in Fig. 3C. This result demonstrated that the conventional single CAR-modified T cells could only specifically target the corresponding antigen overexpressing tumor cells but had no antitumor effect on tumor cells with other antigen. Most notably, the tan-CAR T cells had a remarkable and specific antitumor effect *in vivo* towards tumor cells expressing either CD19 or BCMA (p < 0.05). Taken together, with the harmony of our in-vitro results, tan-CAR T cells can successfully eliminate both CD19 and BCMA expression tumor cells with high antigen specificity and as well as comparable efficacy to conventional single scFv CAR-T cells *in vivo*.

Discussion

Adoptive transfer of engineered T cell in treatment of cancer is a promising approach, however relapse is still a potential challenge need more optimized way to improve [12]. For instance, previously studies demonstrated that adoptive transfer of anti-BCMA CAR T cells to refractory and relapsed multiple myeloma patients initially showed positive therapeutic response but relapse could occur in a short time after CAR T-cell therapy [12, 22]. Intriguingly, downregulation or released off target antigen ability of tumor may be feasible reason to relapse after a conventional single-scFv CAR-T treatment [12]. And existing of CD19 expression on the plasma cell with a cancer stem cell like property act as a poor prognostic indicator [23]. Sequential delivery of anti-BCMA and anti-CD19 obtained good therapeutic effect and their preliminary data suggest that amplification of anti-CD19 CAR T cells might be associated with response and minimal residual disease negative status [24]. However, patients with lymphocytopenia i.e. having insufficient T cells for producing two CAR-T products as well as high manufacturing cost are important limitation to be considered, and sequential delivery of two kind of CAR-T products might resulted with lack of efficiency of second CAR infusion[25].

The linkage of dual specific antigens targeting into a single tandem CAR had already proposed in theory and it is conceivable to generate of bi-specific chimeric receptor [26]. Tan CAR enables T cells to recognize two target antigens and initiate specific killing the tumor cells expression both antigens or either antigen. The most important thing for tan CAR design is the comparable cytotoxic efficacy to the

conventional single CAR. In particular, the rigorous application tan-CAR depicted equivalent specificity and effective compared to single disease model and higher disease burden setting [27]. In a murine glioblastoma model Tan CAR T cells show therapeutic potential to improve glioblastoma control by coengaging HER2 and IL13Rα2 in an augmented, bivalent immune synapse that enhances T cell functionality and reduces antigen escape [28]. To ameliorate relapse condition after BCMA targeting CAR-T therapy in MM treatment we align two scFvs of CD 19 and BCMA in a single targeting domain of CAR molecule to generate tandem CAR (tan-CAR). This tan-CAR construct was characterized by successfully transduced lentiviral vector to primary human T cells (tan-CAR T) with expressing the both scFvs of anti-CD19 and anti-BCMA as a single domain. Accordingly, this duo-scFv CAR construct represents activation capability via binding of either BCMA or CD19. Moreover, it also showed efficacy of tan-CAR T both *in vitro* and *in vivo* against the corresponding antigen expressing tumor cell lines. Most notably, the tan-CAR-engineered T cells demonstrated *in vitro* equivalent efficacy of activation, proliferation, cytokine release and cytotoxicity compared to its corresponding single-scFv CAR-T based therapy of CD19-scFv or BCMA-scFv. The intrinsic enhancement of tan-CAR T cell was also investigated using immunodeficient mouse model bearing combined target tumor cells of K562-CD19-luc and K562-BCMA-luc. Moreover, the tan-CAR T specificity and efficacy evaluated with comparison of its therapeutic control groups with only single-scFv CAR T cells. Notably, tan-CAR enabled T cells to recognize single or dual positive antigens and significantly prohibited tumor progression in combined targeting tumor mice model, while tumor burden increased rapidly in single-scFv CAR-T cells treated mice. Therefore, bivalent nature of tan-CAR incorporates specific inhibition of the progression of the tumor with either CD19 or/and BCMA antigen expression, where the single scFv CAR T cells could only show anti-tumor effects towards the corresponding single antigen positive tumor cells, but not both.

In summary, the present study described the first report of intensive pre-clinical characterization of novel dual tandem CD19/BCMA scFv chimeric antigen receptor T cells to potentially treat multiple myeloma. Our study demonstrated that the tandem CAR with the linkage of two specific antigen of CD19 and BCMA scFvs enable CAR-T cells to target tumor cells in a bivalent manner. On the basis of corroborated analysis of our data depicted that tan-CAR transduced T cells not only effectively kill malignant BCMA-expressing cancer cells, but also eradicate CD19 expressing cancer cells. Since, BCMA antigen downregulation or existing of CD19 residues might be the two main obstacles of conventional single-scFv BCMA-CAR based therapy. Therefore, targeting two antigens at the same time might be a considerable and promising approach to address this limitation and reduce relapse. Besides, this novel construct might be a reasonable approach to reduce production cost against sequential CAR-T treatment for myeloma, as well a tan-CAR combination strategy could be applied for solid tumor which required dual target therapy.

Conclusion

The notable designed and well characterized tan-CAR can specifically recognize both CD19 and BCMA, and exert the significant antitumor efficacy to corresponding dual specific tumor cells *in vitro* and *in vivo.* This novel and effective tan-CAR construct may become an effective option for treatment of

refractory and relapsed multiple myeloma expressing both CD19 and BCMA or relapsing after the BCMA-CAR treatment.

Abbreviations

NC, untransduced T cell;

scFv, single-chain variable fragment;

tan-CAR, tandem-chimeric antigen receptor;

BCMA, B cell maturation antigen.

PC, positive control;

TCM, T cell medium;

CAR, chimeric antigen receptor;

BCMA, B cell maturation antigen;

IL-2, interleukin 2;

IL-4, interleukin 4;

IL-6, interleukin 6

IL-10, interleukin 10;

TNF-α, tumor necrosis factor-alpha;

IFN-γ, interferon gamma;

IL-17A, interleukin 17A;

NC T, negative control T cells

Declarations

Ethics approval and consent to participate

Not applicable

Protection of Animal subjects

The institutional and national guidelines for the care and use of laboratory animals were followed and overseen by the animal facility of the East China Normal University.

Consent for publication

Not applicable

Availability of data and material

The data-sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

LY, DPW, LQK contributed to the concept development and study design and wrote the manuscript. NX and MHL participated in the figure preparation and revised the paper. All authors read and approved the final manuscript.

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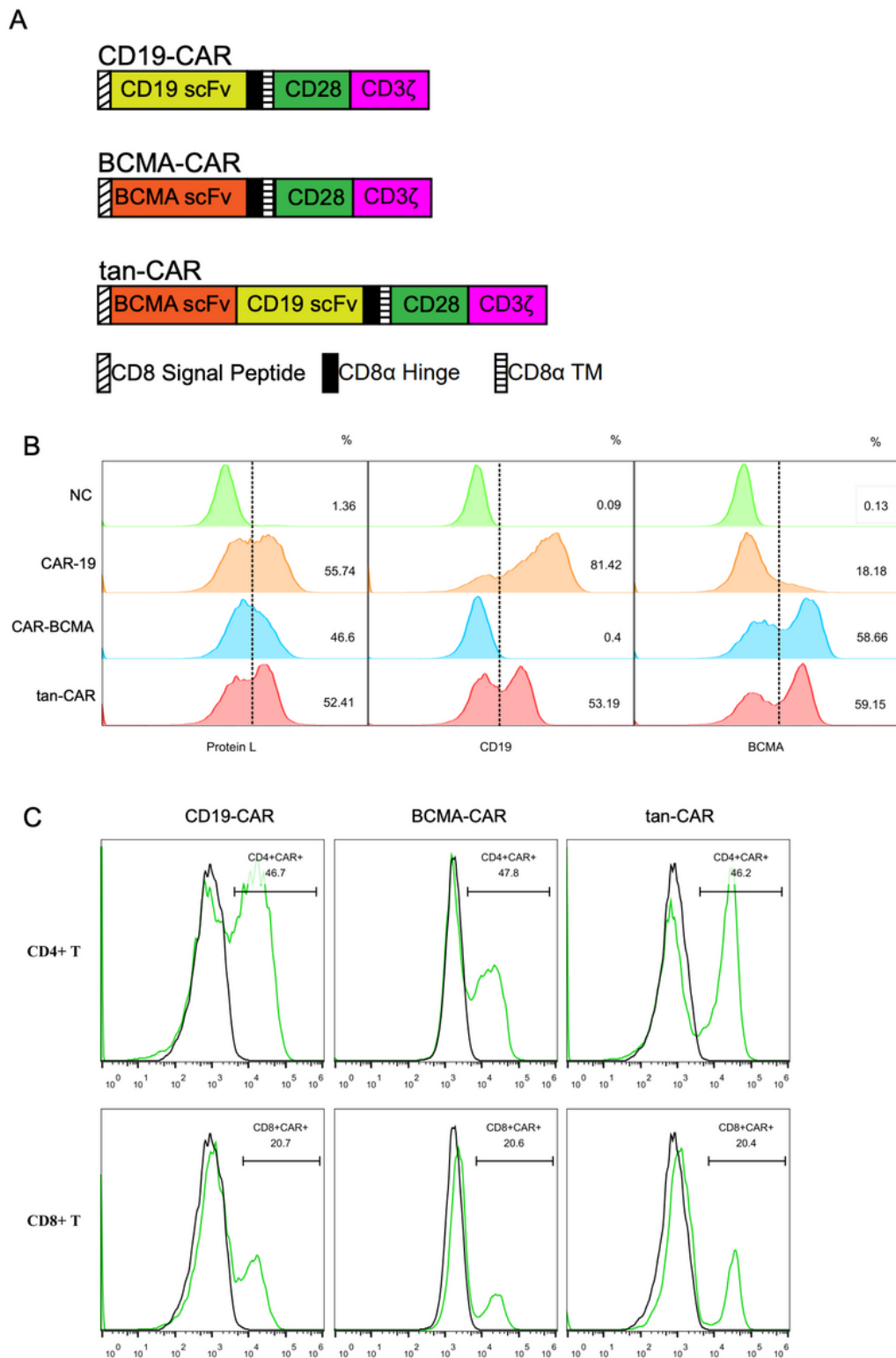
Figures

Figure 1

Generation and characterization of the tan-CAR-transduced T cells. A) Schematic representation of the plasmid design to construct single and tandem- CAR modified T cells. Tan-CAR is the second-generation CAR construct consisting of a CD19-scFv and BCMA-scFv in a tandemly fused domain, a CD28 costimulatory domain and a CD3 zeta-mediated activation signal domain. B) Transduction efficiency analysis of the CAR-modified T cells. CAR engineered T cells were successfully constructed by lentivirus

transduction. T cells were transduced with the lentiviral vectors CD19-CAR, BCMA-CAR, or tan-CAR. Transduction efficiency was measured by flow cytometry using protein L, the human CD19 protein or the human BCMA protein. C) CD4+ T cell to CD8+ T cell ratios in the CAR-engineered T cells. Quantifying fractions of the CAR-positive CD4+ T and the CAR-positive CD8+ T cells in different CAR groups of CD19-CAR, BCMA-CAR, and tan-CAR-transduced T cells. Data are representative of three donors.
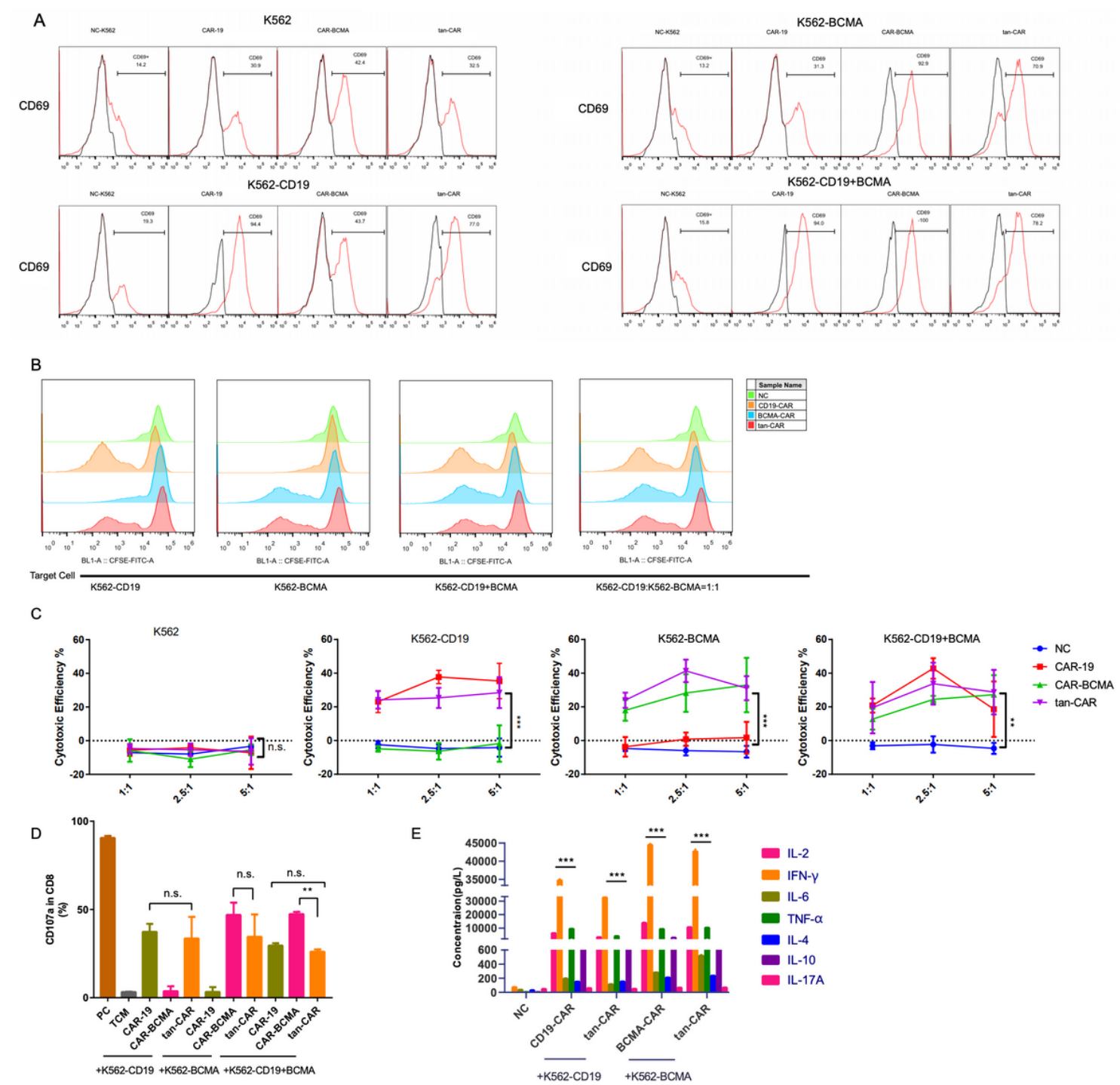

Figure 2

Tan-CAR transduced T cells responded in vitro to tumor cells overexpressing CD19, BCMA, or both. A) The CAR T cell activation ability evaluated by CD69 expression. T cell activation marker CD69 was

significantly expressed on tan-CAR T cells compared with that of other conventional CAR while cocultured with wild type K562 tumor cells or K562 cells overexpressing CD19, BCMA, or both. The expression levels of CD69, a marker of T cell activation, measured by flow cytometry (n = 3). B) Proliferative capacity analysis after targeting inactivated tumor cells. Different CAR-modified T cells were labeled with CFSE before coincubation with replication-incompetent target cells for 5 days, and T cell proliferation was detected using flow cytometry. C) The CAR T cell cytotoxic activity detected by quantification of the level of LDH in the supernatant of groups with different ratios (shown on the X-axis). Data has shown are the mean ± SD, *P < 0.05; **P < 0.01; ***P < 0.001 vs the untransduced T cell as control from the same donor. D) Degranulation of modified T cells were measured by the expression of CD107a on the transduced CD8+ T cells after coculture with different K562 tumor cells. E) The cytokine release of each sample including IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17A was measured in the cocultured supernatants of transduced T cell and tumor cell. Bars represent the mean ± SD of replicate samples. Data are representative of three independent experiments, each with CAR-transduced T cells from a different donor.
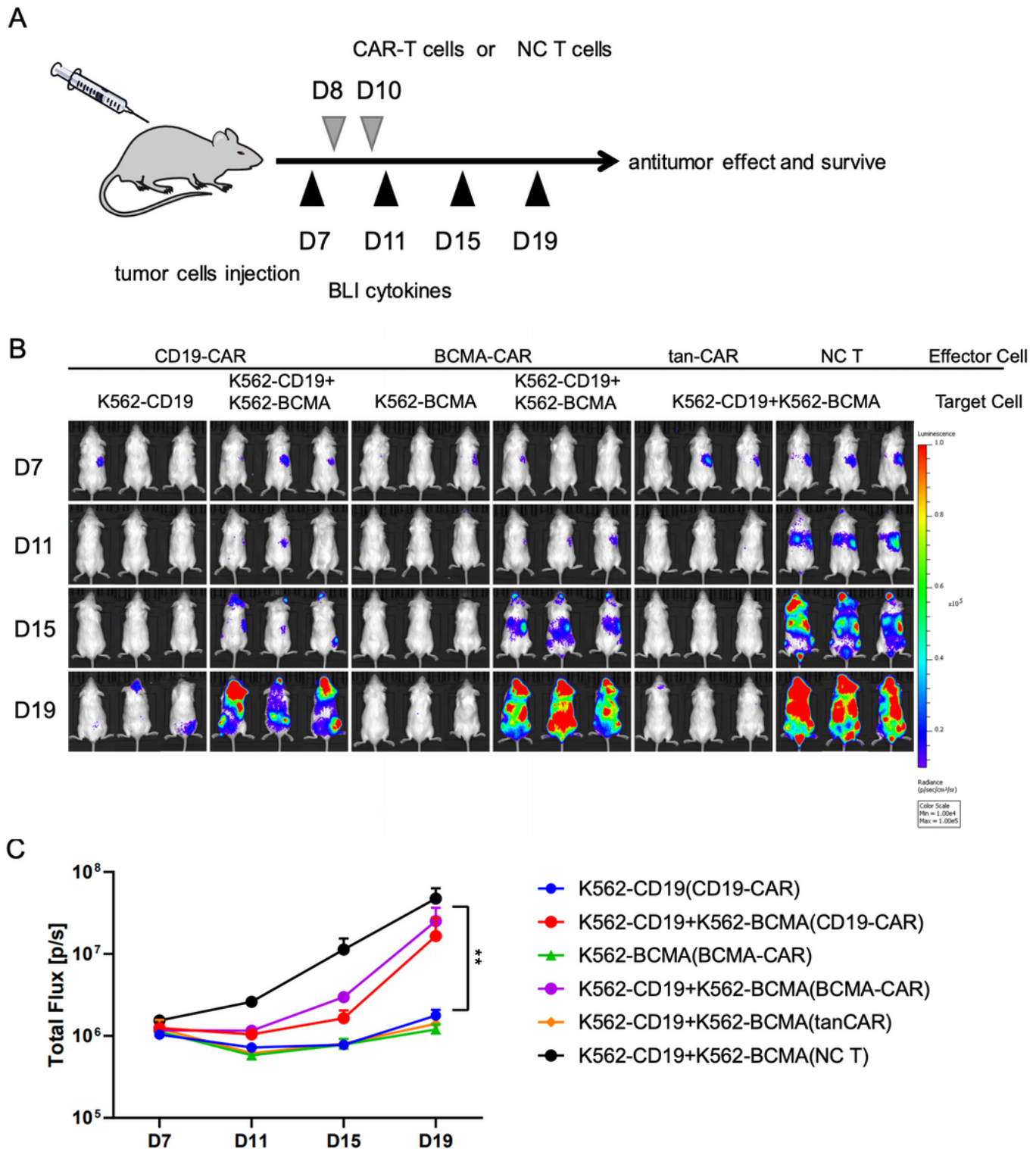
Figure 3

Tan-CAR-transduced T cells effectively clear tumors in vivo A) Schematic of the xenograft model to investigate the activity of tan-CAR T cells in vivo represent with immunodeficiency. On day 0, NOD/SCID/ γc-/- mice were injected via the tail vein with 7 × 106 K562 cells transduced with constructs coexpressing firefly luciferase with CD19 or BCMA or a mixture of the two kinds of tumor cells. Bioluminescent imaging (BLI) was performed on day 7 and every 4 days afterwards. Different CAR-transduced T cells (1 × 107) or

negative control T cells (1×10^7) were injected intravenously on days 8 and 10. B) BLI radiance was used as a surrogate measurement of tumor burden. C. Time course of tumor growth based on mouse whole body bioluminescence. The mean signal per mouse ± SD is plotted. Statistical analysis for Day 19 (the last time points when subjects in the no treatment control group remained alive) is shown, using one-way ANOVA followed by Tukey's multiple comparisons test vs no treatment group. Mean ± SD, ***p < 0.001. The data shown are the mean ± SD (n=3).