Nanobody Armed T Cells Endow CAR-T Cells the Ability to Against Lymphoma Cells

Hongxia Wang

Ningxia Medical University <https://orcid.org/0000-0002-6927-2384>

Liyan Wang

Ningxia Medical University

Yanning Li

Ningxia Medical University

Guangqi Li

Ningxia Medical University

Xiaochun Zhang

General Hospital of Ningxia Medical University

Dan Jiang

Ningxia Medical University

Yanting Zhang

Ningxia Medical University

Liyuan Liu

Ningxia Medical University

Yuankui Chu

Ningxia Medical University

Guangxian Xu (✉ 599040064@qq.com)

Guangdong Medical University

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Abstract

Background

Taking advantages of nanobody (Nb) in immunotherapy, here we investigate the cytotoxicity of Nb based Chimeric antigen receptor T cells (Nb CAR-T) against Lymphoma cells.

Methods

CD19 Nb CAR-T, CD20 Nb CAR-T, and Bispecific Nb CAR-T cells were generated by panning anti-human CD19, CD20 specific nanobodies sequences from naive phage display library, then integrating Nb genes with lentiviral cassette that included other CARs elements, and finally transducing T cells that were expanded under optimization system with above prepared CARs lentiviruses. Prepared Nb CAR-T cells were co-cultured with tumor cell lines or primary tumor cells for 24 hours or 5 days to evaluate the biological function.

Results

Obtained several Nb sequences specific to CD19 and CD20. Optimized culture conditions of T cells that expand 87.5 folds after 7 days of activation. Generated Nb CAR-T cells that could recognize Burkitt lymphoma cell lines (Raji and Daudi), induce activation, proliferation, and therefore kill target cells specifically. Furthermore, same results were also obtained from patient samples with cytotoxicity about 60%.

Conclusions

Our study demonstrated that nanobody based single and bispecific CAR-T cells have certain killing ability against both tumor cell lines and patient-derived tumor cells in vitro.

Background

Cancer immunotherapy has yielded an excellent clinical therapeutic effect against many cancers[1-3]. CAR-T cells therapy, as one of the most promising immunotherapy approaches, already have five drugs (Kymriah, Yescarta, Tecartus, Breyanzi, Abecma) been approved by FDA to treat B-cell precursor acute lymphoblastic leukemia (B-ALL), r/r large B-cell lymphoma, r/r Mantle-Cell Lymphoma or multiple myeloma, all of them target CD19 excepted Abecma target BCMA[4-6]. Classical CARs consist three parts: extracellular antigen recognition region composed of single chain variable fragment (scFv), transmembrane domain like CD8a, intracellular activation domains including costimulatory molecules 4-1BB and/or CD28 and CD3 ζ signaling domain. The most widely studied and mature CAR is 2nd-generation[7-9]. scFv usually derived from monoclonal antibody (mAb), which are composed of a heavy-chain variable fragment connected to a light-chain variable fragment by a flexible linker. While scFv often lead to recurrence in some patients that owing to its large size, high immunogenicity, feeble affinity, easily

aggregation, tonic signaling, and often not fold efficiency[10]. Therefore more studies need to be optimized, including orders of scFv, suitable linker, and reasonable length of linker[11, 12].

Recent studies have elaborated that use nanobodies substitute of scFv as part of the antibody recognition can also induce T cells exert antitumor effect[13-15]. Nanobodies, also known as variable domain of heavy chain of heavy chain antibody (VHH), were found firstly in dromedaries by Hamers Castermans in 1993 and then also found in Camelidae and sharks. Nanobodies belong to the variable region of the heavy chain antibodies (HcAbs), which only contain variable region of heavy chain and CH2, CH3, but devoid of light chain and CH1[16, 17]. The nanobodies only need three CDRs to binds antigen, while without any influence in affinity and specificity to antigens that compared with mAb which need six complementarity-determining region (CDRs)[18]. In addition, because most sequence identity to the human VH gene family III that result in weakly immunogenicity[19]. Therefore, may be more safe for human than mAb derived from mouse, and more importantly, with the help of mature surface display platform, it's feasible to obtain several Nbs that recognize various epitopes of the same antigen, which was hindered as for mAb[20, 21]. Furthermore, Nbs have been applied in Antibody-Drug Conjugates owing to its small molecular weight(15KDa), stable and strong penetrating power[22, 23]. Above all, nanobody show a promising future in therapeutic applications for those favorable characteristics[17, 24].

Additional, studies report that one possible reasons for a poor prognosis of CAR-T therapy is that multiple tumor antigens expressed on tumor cells[25, 26]. Based on the promise of this theory, some groups have shown that bispecific/multiple targets therapy is an idea to decrease this phenomenon in some extent, mainly include Tadem CAR-T, Bispecific CAR-T, or mixing two single targeted CAR-T[8]. Shah NN shown that single CAR-T cells can lysis tumor cells and Tadem CAR-T that target CD19 and CD20 simultaneously could further enhance cytotoxicity[27].

Here, we acquired Nbs that specific bind human CD19 and CD20, optimized T cell activation and expand conditions, generated CD19 Nb CAR-T, CD20 Nb CAR-T and Bispecific Nb CAR-T cells that with the ability to accurate recognize, activate and proliferate upon tumor cells stimulate, and those Nb CAR-T cells also possessed the ability to effective against Raji and Daudi cells in vitro. Furthermore, the cytotoxicity was verified by primary patient-derived tumor cells of Acute lymphoblastic leukemia (PD-ALL).

Methods

Generation of nanobodies (Nbs)

The anti-human CD19 and CD20 nanobodies were selected from the bactrian camel naïve phage display library. First, 500ul phage and 500ul prepared biotinylated CD19 or CD20 antigen(Shanghai Anyan, China) using EZ-Link Sulfo-NHS-LC-Biotin (Thermo) were put into 1.5 ml centrifuge tube together, rotated for 1h at room temperature. Then added 50ul streptavidin magnetic beads (invitrogen) and incubated for 30 min, phage were eluted with 800ul pH 2.7 Tris-HCl after several times washing. Finally, the neutralized eluent were added to TG1 bacteria and incubated at 37°C for 30min, then plated on 2YT plates supplemented with 2% glucose, 100ug/ml ampicillin, 50ug/ml and cultured overnight at 37°C. Collect the

colonies, named the first round of panning library and stored in glycerine for later use. Apart of the colonies were retained to precipitated phage, the second round of panning is carried out with the same methods as first, repeated for 4 times. Several single colonies from 3 and 4 round of panning plates were picked and identified. The identified Nb sequence linked with IgG Fc, His-tags, and then recombinant to pCZN1 vector, the purified Nbs were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Fluorescent semiconductor quantum dots (QDs) immunofluorescence

Daudi cells were mounted on the cell slide by centrifuge with 1000rpm, 15min, and fixed with 4% paraformaldehyde for 10 minutes, washed the cells with TBS and permeabilized with 0.1% Triton X-100 for 20 minutes, blocked by 5% bovine serum albumin for 30 min. Then stained with a 1:250 dilution of Nbs in TBS or TBS alone at 4°C for 12h, next day, incubated with a 1:200 dilution of biotinylated anti-his-tag secondary antibody at RT for 1 h, blocked again and incubated with QD-streptavidin for 30min, followed by imaging under a fluorescence microscope.

Surface Plasmon Resonance (SPR)

To detect the affinity of the obtained nanoantibodies, SPR were used. The experiment was operated using a Biacore T200 system (Healthcare Life Sciences, GE). Mouse anti-human IgG (FC) antibodies were immobilized on a sensor chip (GE Healthcare) to capture the nanobody. Next, the nanobody was injected into the experimental channel at a flow rate of 10µL/min. Finally, 7 groups of serial twofold dilutions of 10µg/mL human CD19/CD20 antigen were successively injected into the test channel and reference channel at the flow rate of 30µL/min, the association time was 120s, and the separation time was 300s. Kd values were calculated using Biacore T200 analysis software.

Cell lines, patient samples

The following cell lines were used: K562 (a chronic myelogenous leukemia line), Daudi, Raji (burkitt lymphoma cell line), and 293T (an embryonic kidney cell line). All cell lines were purchased from ATCC and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (BI) and 1% penicillin-streptomycin (Solarbio), while 293T cells were cultured in DMEM. To generated the luciferase-expressing cell lines, wild-type tumor lines were transduced with lentiviral vector encoding firefly luciferase, then followed by puromycin selection of luciferase-positive cells up to 14 days. ALL cells were obtained by gradient centrifugation using Lymphocyte Separation Medium (Solarbio), cultured in RPMI 1640 supplemented with 10% FBS.

CARs construction

For CD19 Nb CAR and CD20 Nb CAR, nanobodies were incorporated into basic CARs that were composed of IgG4 hinge, CD8 transmembrane domains, 4-1BB and CD3ζ signaling domain. For Bispecific Nb CAR, the anti-CD20 and anti-CD19 nanobodies were linked by (EAAAK)₃, then combined with basic CARs. All

CARs included a fluorescent protein tracer linked to a P2A sequence. Then complete CARs sequences were cloned into a lentiviral plasmid backbone under the regulation of a human EF-1 α promoter. The lentiviruses were produced by transfecting 293 T cells. concentrated by ultracentrifuging, concentrated CAR lenti-virus was immediately stored at -80°C.

Western blot

Proteins of 293T cells were harvested after 72h of transduction, then western blots were performed using mouse anti-human CD3 ζ primary antibody at 500:1 (Thermo).

Primary T cells isolation, expansion and transduction

Peripheral blood that collected using Heparin or Ethylene Diamine Tetraacetic Acid (EDTA) anticoagulant blood vessel were purified by density gradient centrifugation (TBD), then CD3 positive T cell were enriched by positive selection using the magnetic bead separation (Miltenyi Biotec).

For optimize T cell expand conditions, isolated T cells were cultured in X-VIVO15 medium (Lonza) supplemented with 200 or 400U/ml IL-2, with or without 10% FBS or autologous plasma, at a density of 1×10^6 cells/ml, then activated with immobilized 2ug/ml anti-human CD3/CD28 antibody (Peprotech) or together with additional 5ug/ml RetroNectin for 24h, 48h, 72h, respectively.

For transduction, a 48-well cell culture plate that coated with 20ug/ml RetroNectin (Takara Bio) were used. First, according to multiplicity of infection (MOI) of 3, calculated relevant volume of virus, virus were added to prepared 48-well plate and centrifuged at 32°C (2000g, 2h). Then, T cells were collected from the plates that post 48h of activation, centrifuged with viral supernatants at 32°C (1000g, 1h) in the presence of 8ug/ml polybrene (Solarbio), 200 U/ml IL-2, 1% penicillin-streptomycin, followed by incubated overnight at 37°C, 5% CO₂. After 24h, add equal volume of medium, then T cells were expanded at a density of $0.7-1 \times 10^6$ cells/ml, cell numbers were counted every 2 days. CAR expression was detected by flow cytometry, expand cells were used for in vitro assay on day 10.

Flow cytometry

For tumor cells expressing CD19 and CD20, 5×10^5 tumor cells were harvested and washed twice with PBS. Then, tumor cells were stained with 20ul of APC-conjugated mouse anti-human CD19 (BD) and 20 ul of PE-conjugated mouse anti-human CD20 (BD) at room temperature and protected from light for 30 min, washed with PBS once, and resuspended in FACS buffer for assessment. For CAR expression, T cells were stained with PE-conjugated mouse anti-human CD3, CAR-T cells were gated on FITC+PE+. For viability and CD8/CD4 ratio, T cells were stained with 7-AAD, APC-Cy7™-conjugated mouse anti-human CD8, BV510-conjugated mouse anti-human CD4 (BD). For T cell activation, after overnight incubation at the E: T of 2:1, the CD69 were detected using the PE-conjugated CD69 antibody (Biolegend). All data were recorded using a FACSCelesta™ Flow cytometer and analysed by Flowjo 10.

CAR-T cell proliferation

For proliferation assays, tumor cells were treated with 20µg/mL mitomycin C (MCE) for 12h. CAR-T cells were labeled with 1µM CellTrace Far Red (Life Technologies) in PBS at 37°C for 20min, then 4-5fold complete medium were added, some CAR-T cells were collected for flow cytometry analysis after 1 hours incubated. Then, labeled T cells were co-cultured with treated target cells at a 2:1 effector/target ratio in X-VIVO15 medium without IL-2 for 5days, followed by flow cytometry analysis, CAR T cells were gated on GFP signals.

ELISA

CAR-T cells and tumor cells were co-cultured at ratio of 2:1 in X-VIVO medium without additional cytokines in 24-well plates, after 24 hours, the supernatant was collected and used for IL-2 ELISA measurements (Biolegend).

Cytotoxicity assay

For tumor cell lines, cytotoxicity was evaluated by luciferase report assay. CAR-T cells and tumor cells were co-cultured at ratio of 2:1 for 24 h in 96-well plates. The same volume of assay buffer was added into the wells and incubated for 10 min at room temperature, then luminescence values were obtained by Microporous Plate Detector. Percentage of specific lysis was calculated by the following formula:

$$1 - \left(\frac{\text{experiment values}}{\text{max values}} \right) \times 100\%.$$

For tumor cells derives from ALL patient, cytotoxicity was evaluated by lactate dehydrogenase (LDH, Dojindo). Primary ALL cells were isolated through gradient centrifugation and then some cells were used to test the expression of CD19 and CD20 by Flow cytometry. Primary ALL cells were incubated with CAR-T cells for 18h, experiment setting was followed to instruction of test kits. Before added assay buffer, lysis buffer was put to Target Maximum Release and Volume Correction Control wells, after 30min incubated at room, the OD490nm were obtained by Thermo Scientific Microplate Reader. Percentage of specific lysis was calculated by the following formula:

$$\left(\frac{\text{Experimental - Effector Spontaneous - Target Spontaneous}}{\text{Target Maximum Release - Target Spontaneous}} \right) \times 100\%.$$

Statistical analysis

All statistical analyses were performed using SPSS 26.0 and GraphPad Prism version 7. Data variance were analyzed by one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ns, not significant (P>0.05). All data were shown as means ± SD with three independent replicates.

Results

Select Nbs that have high affinity to human CD19 and CD20

To obtain nanobodies that specific target human CD19 and CD20, we adopted magnetic beads-based selection (Fig. 1A). After 4 rounds of panning, we identified three nanobodies that bound CD19 and two nanobodies that bound CD20. There were absolutely different sequences in CDR3 domain, which mean that those Nbs could bind distinct regions of CD19 and CD20 (Fig. 1B). The 3D structural were also simulated (Fig. 1C). To determine the best sequence for CARs construction, we first assessed the Molecular weight and Grand average of hydropathicity of each sequence due to hydrogen-bond interaction influence the affinity between antigen and antibody, preliminary analysis indicated that both Anti-CD19 Nb1 and Anti-CD20 Nb1 have the highest value, therefore we decided to utilize those two sequences in all subsequent experiments (Fig. 1D). Then, those two Nbs were expressed and purified, an obvious band about 50kDa were detected by SDS-PAGE, which is consistent to expected molecular size (Fig. 1E). Furthermore, take the advantage of QDs immunofluorescence in imaging, we using QDs to analysis the cell binding capacity of Nbs. It revealed that only in the presence of Nbs, the red fluorescence was seen on the surface of the Daudi cells, while with no specific fluorescence when use TBS as control (Fig. 1F-G). Finally, we tested the affinity of those nanobodies by SPR, the SPR revealed that the equilibrium dissociation rate constant K_D of CD19 is $0.29\mu\text{M}$, CD20 is $0.147\mu\text{M}$, which could meet the needs of highly binding ability to CD19 and CD20 (Fig. 1H-I). Taken together, these results indicate that we selected specific nanobody sequences for generation of CARs.

Optimization primary T cell culture and expand condition

In order to quickly and effectively expand T cells that meet the needs of lentivirus transduction, we combine several groups of different culture and stimulate methods. After optimization, we first found that T cells purified from heparin sodium blood shown higher activity after 24h stimulated in immobilized CD3/CD28 plates that compared to EDTA (Fig. 2A). Then, with regard to Retronectin that also could help T cells to proliferation, we then coated anti-human CD3/CD28 together with Retronectin, and different culture medium were used, we observed that T cells from heparin sodium blood also shown strongly proliferation after removed from 48h activation plates, and therefore we had to divided cells (groups: autologous plasma (10%), IL-2(200U/ml)/IL-2(400U/ml)) in order to keep suitable cell density on day5(Fig. 2B). Additional, we also extend activation time to 72h, and numbered cells on day7, the results indicated that T cells under immobilized CD3/CD28 stimulated for 48h, supplanted with 10% autologous plasma, 200U/ml IL-2 shown best proliferation (Fig. 2C).

Generation of CD19, CD20, and Bispecific Nb CAR T Cells

Similar to conventional CAR constructs, all CARs we generated here were composed of three parts: binding domain, hinge and transmembrane domain, intracellular signaling domain. Given the length of the hinge play a major role in CAR-T cells effector functions, we used a long hinge in CD20 Nb CAR, a short in CD19 Nb CAR and Bispecific Nb CAR[27, 28]. Furthermore, the green fluorescent protein (GFP) gene driven by an internal ribosome entry site (IRES) promoter was inserted to downstream of the CAR sequences by P2A, aim to guestimate transduction efficiency (Fig. 3A). The lentiviral vector only encoding

GFP was set as control. In order to primary assess whether Nb CARs were successfully construct, we delivered lentivirus to 293T cells and observed high level expression of GFP (Fig. 3B). Then, the cell proteins were collected to detect the molecular weights of CARs protein by probing of CD3ζ. Western blot showed specific bands about 40kDa, 70kDa, 60kDa, the expected target band size of the CD19 Nb CAR, CD20 Nb CAR, and Bispecific Nb CAR, respectively, which proved that CAR gene fragment had been integrated into the the 293 T cells (Fig. 3C). Together, we successfully constructed three nanobody based CARs.

Then CAR-T cells were generated and expanded as describe in (Fig. 4A). At 72h post transduction, we observed that all groups indicated bright GFP signals (Fig. 4B). And the CD8 to CD4 ratio keep equally among groups (Fig. 4C), the positive rate of Nb CAR-T cells ranged from 20% to 80% (Fig. 4D). Additional, the numbers of Nb CAR-T cells were recorded, Nb CAR-T cells showed similar expand ability compared to Mock group, nearly increased 150 fold after transduction (Fig. 4E). Together, we successfully prepared Nb CAR-T cells for subsequent assay study.

Nb CAR-T cells specifically recognize target cells in vitro

To evaluate whether these Nb CAR T cells have the ability to specifically recognize target tumor cells, we first used tumor cell lines as targets. Raji and Daudi were used for positive target cells as they endogenous express both CD19 and CD20, K562 used for negative control as it lacks those antigens (Fig. 5A). Using the Cell Trace Far Red, we evaluated the proliferation potential of Nb CAR-T cells in response to tumor cells. The results revealed that Nb CAR-T cells underwent robust proliferation in reaction to Raji and Daudi, but not K562 cells. In contrast, the Mock group only have somewhat proliferation potency compared to Nb CAR-T cells (Fig. 5B). And meanwhile, the cells were collected for CD69 staining after 24h co-incubated, resemble to proliferation, the Flow cytometry showed that both Raji and Daudi, but not K562 could stimulate these Nb CAR-T cells activation, with CD69 up-regulated in all Nb CAR-T cells groups (Fig. 5C). Together, the Nb CAR-T cells have potential ability to reactive with target cells in vitro in an antigen-dependent way.

Nb CAR-T cells specifically kill target cells in vitro

To determine whether Nb CAR-T cells possess the functional properties that specifically kill target cells in vitro, we performed cytotoxicity and examined cytokine production. We first established cell lines that stable expressed luciferase by transfer firefly luciferase gene to K562 and Daudi (Fig. 5D-E). Mirror to activation and proliferation, Nb CAR-T cells could kill Daudi-luc cells but incapable to K562-luc cells, while Mock T cells have no difference when incubated with Daudi-luc cells or K562-luc cells (Fig. 5F-G). ELISA results showed that the levels of IL-2 in the supernatants of co-cultured Nb CAR-T cells and Daudi-luc cells were obvious higher than that of the Mock group, and all of those groups have no significance when K562-luc cells were used (Fig. 5H-I). Together, we concluded that the Nb CAR-T cells have robust activity against tumor cells in vitro

Nb CAR-T cells can recognize and kill primary ALL tumor cells in vitro.

Based on above results, we wondered whether those Nb CAR T cells also have the potential cytotoxicity to PD ALL cells. We collected patient PBMC, and the Flow cytometry results showed that a majority of lymphocyte population express CD19 and about 20% express CD20 (Fig. 6A). Consistent with the cytotoxicity assay using cell lines as targets, after 5 days incubated with Nb CAR-T cells, we found that PD ALL cells could stimulate Nb CAR-T cells proliferation (Fig. 6B). And we also observed that increased expression of activation marker CD69 (Fig. 6C). Furthermore, we conducted LDH assay to estimate cytotoxicity and the results showed that PD ALL cells were lysed when incubated with Nb CAR T cells rather than Mock T cells, Bispecific Nb CAR-T cells showed higher cytotoxicity than single Nb CAR-T cells (Fig. 6D), and elevated IL-2 were also observed (Fig. 6E). Together, those result demonstrated that Nb CAR-T cells could specifically recognize and kill primary ALL tumor cells in vitro.

Discussion

Despite the curative effect of CAR-T cells in hematologic malignancy is remarkable, there do have some patients relapse after treatment, and the study in solid tumor treatment is also limited for multiple reasons[29-31]. Giving straitened circumstances, many measures were proposed, among them, nanobodies therapy reveal a good prospect for their unique advantages[32-34]. Recently, Matthew A. Nix, et. described that CD72 Nb CAR-T cells show robust activity against B-cell malignancy models, and several studies further indicated that Nb CAR-T exert robust cytotoxicity to solid tumor[35, 36].

In this study, based on our prior generated naive phage display libraries, we obtained several specific Nbs sequences. Compared to immunize llama, this kind of panning is more convenient and time-saving, in order to avoid nonspecific binding and improve specificity at utmost, we obtained Nbs using biotinylated CD19, CD20 antibodies, for the strong affinity between biotin and streptavidin[37]. We used monovalent nanobody for CARs construction, and there have little clearly study demonstrated that whether bivalent Nb CAR-T is superior to monovalent Nb CAR-T. With the encourage of several study indicated robust cytotoxicity of CD19/CD20, BCMA/CS1, CD70/B7-H3 bispecific CAR-T cells against tumors[38-41]. Therefore, in addition to Nb-derived single CAR-T, like other studies, we also constructed Nb-derived bispecific CAR-T cells[42-44].

Additional, if unsuitable anticoagulation, medium, time and ways of activation were applied, it's not easy to expand enough numbers of primary T cells for subsequent study. Therefore, taken all those conditions into consideration, we found that no matter activated with how many hours or what ways were took, T cells in blood that anticoagulation by Heparin sodium always tend strong proliferation than EDTA. The reason maybe is that EDTA belong to Calcium ion chelating agent, which block the panels that delivery activation signals. And we also found that additional autologous plasma help T cells expand quickly than FBS. Furthermore, to our surprise, under activation both in Retronectin and anti-human CD3/CD28, T cells shown obvious expand among groups at first few days, but decreased by degrees after 5days of activation, while solely anti-human CD3/CD28 activated cells shown continued growth, the complete mechanism need further study.

By evaluating the biological function of those Nb CAR-T cells, we found that all Nb CAR-T groups could activation and proliferation in respond to target cells, which mean that those Nb CAR-T cells have the ability to accurately recognize antigen. And furthermore, we also found that those CAR-T cells could lysis Daudi cells. But to our surprise, CD20 Nb CAR-T cells show relatively low cytotoxicity compared to CD19 Nb CAR-T, although the difference is reversed in bispecific Nb CAR-T, the killing efficiency of bispecific Nb CAR-T is only somewhat higher than CD19 Nb CAR-T. We consider that the possible factors were that we use longer hinge in CD20 Nb CAR, which leading to a relatively lower positive rate and maybe lead to low density of CARs that displayed on T cell surface, even we equilibrated CAR-T cells positive among groups, the specific reason remains to be further explored. Recently, some findings indicated that CD22 and CD123 were also co-expressed in B-line hematologic tumors, we considered that targeting these targets simultaneously might also enhance the CAR-T killing effects[45, 46].

Finally, using patient tumor cells as target cells could more appropriate to reflect cytotoxicity[42, 47]. Therefore, we collected ALL patients PBMC sample to further validate our prepared Nb CAR-T, notably, the results demonstrated that those Nb CAR-T cells could also recognize tumor cells, exhibit obvious proliferation, and lysis tumor cells in vitro.

Although we acquired promising results, several limitations of our study should be mentioned. First, we must take individual differences into account, there still needs more patient samples to be used. Second, difference between conventional CAR-T cells and Nb CAR-T cells also need to be verified, therefore to highlighted the obvious advantage of nanobody based CAR-T cells. At last, despite the obvious cytotoxicity against tumor cell lines and primary ALL tumor cells were shown in our study, there needs further study to be carried out in vivo to verified Nb CAR-T cells anti-tumor effects.

Conclusions

In conclusion, we successfully generated Nb-based CAR-T cells based on the combination of nanobody technology and CAR T technology, and its killing effect against tumor cell lines and patient primary tumor cells were verified in vitro, our study providing a good foundation for the application of nanobodies technology in CAR-T cells.

Abbreviations

CAR-T: Chimeric antigen receptor T cells

Nb: nanobody

Nb CAR-T: Nb based Chimeric antigen receptor T cells

B-ALL: B-cell precursor acute lymphoblastic leukemia

mAb: monoclonal antibody

scFv: single chain variable fragment

VHH: variable domain of heavy chain of heavy chain antibody

HcAbs: heavy chain antibodies

CDR: complementarity-determining region

PD-ALL: patient-derived tumor cells of Acute lymphoblastic leukemia

QDs: Fluorescent semiconductor quantum dots

SPR: Surface Plasmon Resonance

Declarations

Ethics approval and consent to participate

All studies involving human blood cells have been approved by the Ethics Committee of General Hospital of Ningxia Medical University (number: KYLL-2021-216) and obtained the consent from patient.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed in the current study are available from the corresponding author in response to reasonable requests.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contribution

All listed author took part in this study. HW, LW designed experiments, generated CAR-T cells, implemented function assays, analyzed data and wrote the manuscript draft. YL, GL obtained Nbs sequence and identified Nbs affinity. XZ provided patient blood samples and clinical disease related knowledge. DJ, YZ, LL and YC assisted with the CARs design, prepared lentivirus. GX designed experiments, interpreted the data, wrote and edited the final manuscript.

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Figures

Figure 1

Generation of Nbs. (A) Schematic diagram of generation Nbs. First, a nanobody library were prepared for obtain Nbs (a), then bio-human CD19 or CD20 were added to bind Nb (b), finally, streptomycin labeled magnetic beads were mixed with those complex (c) and followed by several times washing (d). A total of 4 rounds of panning were proceed. (B) Amino acid sequence of anti-CD19 and anti-CD20 Nbs. The framework (FR) and complementarity-determining region (CDR) sequences are defined according to the IMGT. The CDR1, CDR2 and CDR3 are highlighted in green, blue and red, respectively. (C) Simulate the three-dimensional crystal structure of Anti-CD19 Nb1-3 and Anti-CD20 Nb1-2 using Swiss-model. (D) Nb's Molecular weight (Mw) and Grand average of hydropathicity (GRAVY) were analysed by online soft of ExPASy-ProtParam Tool. (E) SDS-PAGE analysed molecular weight of purified Anti-CD19 Nb1 and Anti-CD20 Nb1. (F-G) Cell binding capacity of nanobodies that detected by QDs immunofluorescence.

Fluorescence was captured by fluorescence microscope, TBS was used as control. (H-I) Affinity between CD19/CD20 and the Nbs was determined by SPR binding assay.

Figure 2

Optimization culture condition of T cells. (A). Cell vitality that under stimulated by anti-human CD3, CD28 after 24h. (B). T cell proliferation in different culture and activate groups. (C). Cell expand folds after one week of activation.

Figure 3

Nb CARs construction. (A) Schematic diagram of Nb CARs, including CD8 signal peptide (SP), Nb, IgG4 Hinge, CD8TM, 4-1BB, and CD3 ζ domain. (B-C) Nb CARs expression on 293T cells. 293T cells were transduced with control or different Nb CARs, GFP expression was observed after 72h, cell lysates were collected and blotted with first antibody mouse anti-human CD3, the specific band were seen.

Figure 4

Nb CAR-T cells generation and expansion. (A) Schematic diagram of Nb CAR-T cells generation. primary T cells were activated, transduced and then expanded with IL-2 up to day 10. (B) The expression of GFP in different groups observed by fluorescence microscope. (C-D) Nb CAR-T expression and phenotype. Modified T cells were collected on day7, T cells were stained with 7-AAD, PE-conjugated mouse anti-human CD3, APC-Cy7TM-conjugated mouse anti-human CD8, BV510-conjugated mouse anti-human CD4 (BD), CAR-T cells positive expression were defined as GFP+PE+. The dead cells were excluded by 7AAD. (E) Nb CAR-T cells expansion. the cells numbers were recorded by blood counting chamber every three days after primary T cells were transduced with CARs on day3.

Figure 5

Nb CAR-T cells specifically proliferate, activate and kill target cells. (A) The expression of CD19, CD20 on Raji, Daudi, and K562 cells. (B) Nb CAR-T cells experience proliferation under tumor lines stimulate. Cell Trace labeled Nb CAR-T cells were incubated with mitomycin C-treated Daudi, Raji or K562 cells for 5d, then the change of Cell Trace signals on day5 were compared by Flow cytometry, CAR-T cells were gated on GFP signals. (C) The expression of CD69 on Nb CAR-T cells. Nb CAR-T cells were incubated with Daudi, Raji and K562 for 24h, then T cell activation marker CD69 were detected by flow cytometry. (D-E) Cell lines to stably expressed Luciferase. Daudi and K562 cells were transduced with lentivirus that encoding Luciferase gene, followed by puromycin selection and determined by Microporous Plate Detector. (F-G) Cytotoxicity of CD19 Nb CAR-T, CD20 Nb CAR-T, Bispecific Nb CAR T cells or Mock against Daudi, K562 at E: T of 2:1, Cell viability were measured via bioluminescence. (H-I) Nb CAR T cells and tumor cells were co-incubated overnight, then the culture supernatant were collect to measure IL-2. Data were analyzed by one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant ($P > 0.05$).

Figure 6

Nb CAR-T cells cytotoxicity on patient-derived tumor cells. (A) Expression of CD19 and CD20 on patient sample. Patient PBMC were harvested and stained with PE-conjugated mouse anti-human CD19 (BD) and APC-conjugated mouse anti-human CD20 (BD) at room temperature for 30 min, washed and resuspended in FACS buffer, Flow cytometry were used to assess CD19 and CD20 expression. (B-C) Primary ALL tumor cells induce Nb CAR-T activation and proliferation. Nb CAR T cells and tumor cells were co-incubated

overnight for 5 days, followed by Flow cytometry analyse. (D-E) Nb CAR-T perform cytotoxicity to primary ALL tumor cells. LDH-based cytotoxicity assays and ELISA were performed. Data were analyzed by one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $P < 0.0001$, ns, not significant ($P > 0.05$).