Inducible secreting TLR5 agonist CBLB502 enhances antitumor activity of CAR133-NK92 cells for colorectal cancer

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Article

Keywords: TLR5 agonist, CBLB502, CAR133, NK92 cells, endogenous immune response, colorectal cancer

Posted Date: January 18th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1227221/v1>

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Abstract

CAR-T/NK cells have been used with minimal success against solid malignancies such as colorectal cancer (CRC) in part because of the heterogeneous nature of tumour-associated antigens and the emergence of antigen loss variants that lead to negative antigen relapses after the initial response. Here, we firstly engineered a CAR133-NK92 cells with inducible secretion of TLR5 agonist CBLB502 at the tumour site, and demonstrated that CAR133-i502-NK92 cells *in vitro* exhibit enhanced proliferation, cytokines expression and antitumor activities compared with CAR133-NK92 cells. In addition, CAR133-i502-NK92 cells has more potent antitumor activities not only for hCD133+ but also hCD133+/hCD133- mixed colon cancer xenograft mouse models, and also has well tolerated *in vivo*. Notably, CBLB502 secreting from CAR133-i502-NK92 cells can effectively activate endogenous immune cells to eradicate tumor cells. Taken together, our study demonstrates that the CAR133-i502-NK92 cells not only specific directly eliminate CD133 positive colon cancer cells in a CAR133-dependent manner, but also can indirectly eradicate CD133 negative heterogeneous colon cancer cells in an CBLB502-elicited endogenous immune activation manner, which might offer a promising therapeutic new strategy for colorectal cancer.

Introduction

Colorectal cancer (CRC) is a common cause of cancer-associated mortality worldwide [1]. Despite the significant advances that have been made in the treatment of CRC, the morbidity associated with this disease is rapidly increasing, and its 5-year survival rate is low [2]. This highlights the need to investigate modified or novel strategies for the treatment of patients suffering from colorectal cancer. Adoptive immunotherapy based on genetic engineering of lymphocytes to create chimeric antigen receptor-modified T (CAR-T) cells has become one of the most promising approaches in the field of cancer therapy [3]. Several groups have reported preclinical studies in which CRC was treated using CAR-T cells, such as targeting epidermal growth factor receptor- (EGFR-) [4], EpCAM [5] and EGP40 [6] CAR T cells and targeting carcinoembryonic antigen (CEA) CAR cytokine-induced killer (CIK) cells [7]. In addition to preclinical studies, clinical trials using CAR-T cells targeting HER2 [8], TAG-72 [9], CEA [10] and CEACAM5 [11] have also been conducted in patients with CRC. Unfortunately, the therapeutic effects of CAR-T cells in CRC have been unsatisfactory. This is thought to be due at least in part to the heterogeneity of tumor association antigen (TAA), which induces cancer progression and recurrence, as CAR-T cells recognize only one target molecule expressed on tumor cells [12]. It was recently reported that enhanced accumulation and persistence of endogenous immune cells in addition to transferred CAR-T cells can overcome this obstacle and improve the potent therapeutic effects of CAR-T cells in solid tumors such as CRC.

As a key activator of the immune response, Toll-like receptors (TLRs) have been regarded as potential agonists for cancer treatment [13, 14]. However, owing to their acute inflammatory response, which causes substantial safety issues, only two TLR agonists have been approved by the FDA; these are the TLR4 agonist monophosphorylated lipid A and the TLR7 agonist imiquimod [15]. Currently, there is strong interest in developing the TLR5 agonist flagellin, the only known ligand of TLR5, as an excellent nontoxic

candidate adjuvant for immunotherapy because acute inflammation and cytokine storms do not occur during the physiological response induced by flagellin [16, 17]. On binding to the extracellular domain of TLR5, which is expressed on the surfaces of numerous types of immune cells including dendritic cells (DCs), macrophages, lymphocytes, and NK cells, flagellin rapidly induces a MyD88-dependent signalling cascade and activates nuclear factor (NF)- κ B and mitogen-activated protein kinase pathways that regulate the innate and adaptive immune response, a response that is considered important for the development of the adaptive immune response [18, 19]. Flagellin has also been shown to prolong the survival of tumor-bearing animals [20, 21]. Over the past several years, a series of engineered TLR5 agonists have been reported to activate the immune response; among them, CBLB 502 has been frequently used in both animal studies and clinical studies. CBLB502 was derived from *Salmonella* flagellin by deletion of its hypervariable and highly immunogenic D2 and D3 domains and was expected to reduce flagellin immunogenicity. The function and safety of CBLB502 has been supported by the results of two clinical studies in which cancer patients and healthy individuals were administered the CBLB502, which proved to be well tolerated and effective in antitumor applications [22] (<http://clinicaltrials.gov/ct2/show/NCT02654938>). These findings prompted us to investigate whether the TLR5 agonist CBLB502 can improve the therapeutic effects of CAR-T cells against CRC.

In addition to mobilizing endogenous immune cells, eliminating cancer stem cells (CSCs) is also an important strategy for improving the therapeutic effects of CAR-T cells in CRC because colon cancer stem cells (CCSCs) are considered the main triggering factor of cancer progression and recurrence [23, 24]. As a specific molecular biomarker for CCSC, CD133 is regarded as a reasonable target for immunotherapy [25, 26]. Several clinical trials have shown that targeting of CD133 CAR-T cells is safe and well tolerated [27, 28]. This provided us with a rationale for exploring the potential of anti-CD133 CAR-T cells modified to release the TLR5 agonist CBLB502 to enhance the therapeutic efficacy of CAR-T cells against colon cancer.

Compared with CAR-T cells, CAR-NK cells are potential competitors with many advantages and have sparked significant interest related to cancer immunotherapy [29]. NK cells are acceptable CAR drivers that are not restricted to autologous cells. CAR-engineered NK cell lines such as NK92 have been investigated thoroughly and tested in multiple studies [30–33]; among them, CARHer2-NK92 cells are ready for phase I clinical trials [34]. However, their therapeutic potential in CRC has never been explored. These encouraging results pave the way for further combining CAR133-NK92 cells with the TLR5 agonist CBLB502 to develop an attractive modality for cancer therapy in CRC.

Thus, we developed CAR133-NK92 cells that display activation-induced release of the TLR5 agonist CBLB502, thereby resulting in the release of CBLB502 upon NK92 cell activation triggered by specific tumor antigen recognition. To selectively deposit CBLB502 in the tumor tissue while avoiding systemic immune cell activation, we engineered an inducible CBLB502 (i502) expression cassette under the control of the nuclear factor of activated T cells (NFAT)-IL2 minimal promoter. Our results showed that incorporating the TLR5 agonist CBLB502 into CAR133-NK92 cells enhanced their expansion and activation and thereby suppressed tumor growth and prolonged survival not only in hCD133+ ,but also in

hCD133+/hCD133- mixed colon cancer xenograft mouse models. In addition, we found that CBLB502 releasing from CAR133-i502-NK92 cells elicited a stronger immune cell activation effect.

Material And Methods

CD133 expression analysis in tissue microarrays and colon cancer cell lines

To measure the expression of CD133 in normal tissues and colon cancer tissues, the HOrg-N090-PT-02 microarray (Outdo Biotech, Shanghai, China) containing 24 human normal tissue samples and the HCoLA-180Su11 microarray (Outdo Biotech) containing 76 colon cancer tissue samples were used to examine the expression of CD133 by IHC as previously described [35]. Briefly, after deparaffinization and rehydration, sections were exposed to 3% H₂O₂ in methanol to eliminate endogenous peroxidase activity. Bovine serum albumin (1%) was used to block the sections for 30 min at room temperature (RT). The sections were then incubated with anti-human monoclonal antibodies against CD133 (Abcam, ab19898) overnight at 4°C. The sections were then washed with PBS and incubated with peroxidase-conjugated secondary antibodies (ChemMate™ DAKO EnVision™ Detection Kit, Peroxidase/DAB, Rabbit/Mouse, DAKO) for 45 min at room temperature (RT). CD133 immunoreactivity was detected on the cytoplasmic and cytoplasmic membranes. The expression level of CD133 was evaluated semiquantitatively using the H score, which is calculated by multiplying the intensity of the signal by its relative extent. Low and high CD133 expression were scored using the median value as the cut-off threshold. The staining results were independently evaluated by 2 trained investigators. As a negative control, slides were processed as described above without the addition of primary antibodies. Slides that were used as positive controls for detection of the primary antigens were provided by Outdo Biotech.

To evaluate the surface expression of CD133 in colon cancer cell lines (SW620, SW480 and HCT116), the cells were washed with PBS containing 4% BSA and incubated with PE-conjugated anti-CD133 antibody (Abcam, ab253271). IgG isotype controls were included. The stained cells were analysed using a NovoCyte flow cytometer (ACEA Biosciences, Hangzhou, China) and CellQuest Pro software (BD Biosciences) according to the protocol provided by the manufacturer.

Cell lines and culture conditions

Human colorectal cancer cell lines (SW480 and SW620) and human monocytic THP-1 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The human colorectal cancer cell line HCT116 was kindly provided by Jian Yu, University of Pittsburgh. The human NK cell line NK92 was provided by Jianhua Yu, Ohio State University. Human monocytic THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI 1640, Invitrogen) medium containing 10% FBS and 2 mmol/L L-glutamine (Gibco) and differentiated into M0 macrophages by incubation for 24 h with 150 nM phorbol 12-myristate 13-acetate (PMA) (Sigma, USA). NK92 cell lines were cultured in alpha minimum essential medium (α-MEM, Gibco, USA) supplemented with 12.5% heated-inactivated foetal bovine serum (FBS) (Gibco, USA), 12.5% horse serum (Gibco), and 150 IU/ml recombinant human interleukin-2 (IL-2)

(Genscript). The colorectal cancer cell lines SW480 and SW620 were transduced with a retroviral vector encoding the firefly luciferase (FFluc) gene as described previously [36]. All the above cell lines were cultured at 37°C in a 5% CO₂ incubator.

Human T cells were isolated from healthy donors with their consent and expanded ex vivo as previously described [37]. Briefly, peripheral blood mononuclear cells (PBMCs) were collected from patients by leukapheresis. Primary T cells derived from PBMCs were activated using Dynabeads Human T-Activator CD3/CD28 magnetic beads (Invitrogen) at a bead:cell ratio of 1:1 and cultured in X-VIVO 15 medium (Lonza) supplemented with 300 U/ml recombinant human IL-2 (PeproTech).

Generation of CAR133-i502-NK92 cells

All CAR designs are 4-1BB (CD137)-based second generation. CAR.133 containing anti-CD133 scFv was derived from GenBank accession no. HW350341.1, human CD137 and CD3ζ signalling domains as described previously [28]. The TLR5 agonist CBLB502 with a C-terminal 6×His-tag was synthesized by Integrated DNA Technologies and Invitrogen. The sequence of a CBLB502 composed of the conserved N- and C-terminal domains required for TLR5 activation is from flagellin of *Salmonella enterica* serovar Dublin (GenBank accession no. M84973.1) [38]. For inducible expression, CBLB502 was expressed under the control of the NFAT/IL-2 minimal promotor. The CAR construct was verified by DNA sequencing. CAR133-i502-NK92 cells were generated using standard techniques as previously described [31, 39, 40].

The surface expression of CARs on the transduced NK92 cells was analysed by flow cytometry, which was conducted as described previously [32, 33]. Briefly, transduced NK cells were washed with PBS containing 4% BSA and incubated with biotin-labelled goat anti-mouse F(ab')₂ polyclonal antibody or with normal polyclonal goat IgG antibody as an isotype control.

Proliferation, cytotoxicity and exhaustion analysis

To measure the proliferation of effector cells, CAR133-NK92 cells and CAR133-i502-NK92 cells were cultured with IL-2, and the expansion of CAR133-NK92 cells and CAR133-i502-NK92 cells was calculated on days 3, 5 and 7 using the Cell Counting Chamber slide (Invitrogen) according to the manufacturer's instructions.

To assess the cytotoxicity of effector cells, two CD133-positive colon cancer cell lines (SW620 and HCT116) and one CD133-negative colon cancer cell line (SW480) were cocultured with effector cells at an effector-to-target (E:T) ratio of 10:1. After incubation of the cells for 24 hours, culture supernatant was obtained from each sample, and LDH levels were measured using an LDH cytotoxicity kit (Promega, USA) according to the manufacturer's instructions. The cytotoxicity of effector cells was also evaluated by RTCA using the xCELLigence RTCA TP system (ACEA) as previously described [41]. Briefly, CAR133-NK92 cells and CAR133-i502-NK92 cells were added in a 10:1 ratio to the wells of an E-Plate 16 that had been seeded 24 hours earlier with various target cells, and target cell proliferation was monitored by measuring the conductivity of the cell surface area in contact with the gold electrodes covering the plate surface. In

addition, degranulation of the effector cells was analysed by detecting the cell-surface expression of CD107a using the BD FastImmune CD107a Kit (BD Biosciences). Briefly, anti-human CD107a antibody was added to the medium in which CAR133-NK92 cells and CAR133-i502-NK92 cells were being cocultured with target cells at an E:T of 10:1. One hour later, 1 μ L of GolgiStop (BD Biosciences) was added, and the cultures were incubated for an additional 4 hours. Effector cells were collected, and CD107a expression was analysed using a BD FACS Canto II flow cytometer and CellQuest Pro software. All experiments were performed in triplicate.

To analyse genes encoding activity receptors, inhibitory receptors and exhaustion gene, CAR133-NK92 cells and CAR133-i502-NK92 cells were cocultured with SW620 cells at an effector-to-target (E:T) ratio of 10:1 for 3 days. The effector cells were then collected and washed, and total RNA was extracted using the RNAsimple Total RNA Kit (Tiangen) according to the manufacturer's instructions. cDNA was synthesized using a PrimeScript® RT Reagent kit (Takara), and the expression of the exhaustion markers PD-1, TIM-3 and LAG-3, the activity receptors NKp46, NKp44, NKG2D, CD69, CD137 and CD16, the inhibitory receptors NKG2A was measured in triplicate using SYBR Green qPCR Master Mix (Thermo Fisher) and the Bio-Rad CFX Manager system. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The primers used in this study are listed in Supplemental Table 1.

Collection of conditioned medium (CM) and stimulated immune cells (T cells, NK92 cells and macrophages)

CAR133-NK92 cells and CAR133-i502-NK92 cells were cocultured with mitomycin C-treated SW620 cells at an effector-to-target (E:T) ratio of 10:1 without IL-2 in RPMI 1640 medium containing 10% FBS and 2 mmol/L L-glutamine (Gibco). 3 and 5 days later, conditioned medium from the two groups was collected and stored at -20°C for use in further stimulation of immune cells. THP-1 cells were differentiated from M0 macrophages by exposure to 150 nM PMA for 24 h. T cells, NK92 cells and macrophages were seeded in 6-well plates (1×10^5 cells/well) and cultured in X-VIVO 15 medium, α -MEM and RPMI 1640 medium, respectively. After 24 hours, the medium was replaced with conditioned medium, and the cells were cultured for an additional 24 hours and propagated without further stimulation in the absence of exogenous cytokines or feeder cells. Then, T cells, NK92 cells and macrophages were collected and washed twice with PBS containing 4% BSA. Immunostaining using anti-human Ki67-APC, CD69-APC and CD86-PE and isotype controls were performed according to a standard surface immunostaining flow cytometry protocol. The stained cells were measured using a NovoCyte flow cytometer (ACEA Biosciences, Hangzhou, China) according to the manufacturer's protocol. The data were analysed using CellQuest Pro software. The expansion of NK92 cells was calculated using the Cell Counting Kit-8 (CCK-8) (Beyotime) according to the manufacturer's instructions.

Microarrays for cytokine release

Human cytokine array C5 (Ray Biotech) was used according to the manufacturer's instructions to qualitatively measure the cytokines and chemokines released by CAR133-NK92 cells and CAR133-i502-

NK92 cells cocultured with mitomycin C-treated SW620 cells for 3 and 5 days. Briefly, the array membranes were blocked for 30 min, incubated with 100- μ L samples at 4°C overnight, and then incubated with biotin-conjugated antibodies at room temperature for 2 hours. The membranes were then washed and incubated with HRP-conjugated secondary antibody for 2 hours. Finally, the membranes were incubated with chemiluminescent substrate and exposed to X-ray film for 15 min. Chemiluminescence measurements of the arrays were quantified using ImageJ; the fold change over the control is reported in heatmaps. The ratios between the two groups were calculated in 3 experiments. Individual quantification of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-7 and IL-6 was performed using specific ELISA kits.

In vivo antitumor studies

Xenogeneic mouse model

A xenograft colon cancer model was established in 6- to 8-week-old BALB/c nude mice (nu/nu) purchased from the Model Research Center of Nanjing University. The mice were inoculated subcutaneously in their left flanks on day 0 with 1×10^6 SW620 cells labelled with firefly luciferase (FFluc) or with 5×10^5 SW620 cells in combination with 5×10^5 SW480 tumor cells labelled with firefly luciferase (FFluc). The tumor-bearing host mice were not subjected to preconditioning or lymphodepletion before effector cell treatment. On day 14 after transplantation, the tumor volume reached approximately 100 to 200 mm^3 , and the mice were randomly divided into three groups for injection with NK92 cells, CAR133-NK92 cells or CAR133-i502-NK92 cells ($n = 5$ each) based on tumor bioluminescence signal intensity (BLI) to ensure similar tumor burden among the experimental groups. The mice were injected intravenously (i.v.) with the effector cells at the indicated dose and time intervals. For SW620 cells and SW480 cells mixed mouse model, the mice were randomly divided into two groups for injection with CAR133-NK92 cells or CAR133-i502-NK92 cells ($n = 5$ each). before injected with effector cell, the mice were preinjected with the 1×10^5 T cells. Mouse tumor volume and body weight were measured twice weekly. Tumor growth was measured using calipers, and tumor volumes were calculated using the following formula: volume = length $\times$ (width) $^2 \times 0.5$. Bioluminescence imaging of tumor-bearing mice was performed by injecting the mice intraperitoneally with D-luciferin potassium salt (150 mg/kg) (Beyotime) dissolved in PBS and quantifying luminescence using the FUSION FX imaging system (Vilber Lourmat). Luminescence images were collected and analysed using Fusion software (Vilber Lourmat). The mice were monitored for survival and euthanized when they showed signs of distress. All of the mice were euthanized when the tumor volume in the control mice was larger than 1,500 mm^3 .

Analysis of effector cell persistence in mouse peripheral blood

Mouse peripheral blood samples were collected by retroorbital bleeding. Cell pellets were isolated by centrifugation of the collected blood at 5000 rpm for 5 minutes. The red blood cells were lysed in 0.45 mL 1 $\times$ BD FACS Lysing Solution (BD Biosciences), and 50 μ L of each sample was then incubated with the

CD56-PerCP antibody while protected from light for 15 minutes at room temperature. The proportion of effector cells was analysed using a BD FACS Canto II flow cytometer and CellQuest Pro software.

H&E staining and immunohistochemistry (IHC) analysis

Tumor tissues and organs, including the heart, liver, kidneys, lungs, and stomach, were harvested from the indicated tumor-bearing mice, fixed with formalin, embedded in paraffin and prepared as 2-mm-thick sections. The sections were mounted on slides and histopathologically stained with HE. The tumor tissue sections were stained for infiltration by human T cells using a mouse monoclonal antibody against human CD3 ϵ (Thermo Scientific) and for tumor angiogenesis using a mouse monoclonal antibody against human CD31 (Abcam). Following overnight incubation with the primary antibody at 4°C, the secondary antibody was added, and the results were visualized using a ChemMate Envision Detection Kit (DakoCytomation).

Study approval

This study was performed in accordance with the Declaration of Helsinki and was approved by the ethics committee for human subject research of Southwest University (Chongqing, China) and Shanghai Outdo Biotech Company (NO. YB M-05-02). All donors gave written informed consent to the use of their PBMCs for research purposes. All animal experiments were performed in accordance with the Animal Care and Use Committee guidelines of the NIH (Bethesda, MD, USA) and were conducted under protocols approved by the Institutional Animal Care and Use Committee of Daping Hospital and Research Institute of Surgery.

Statistical analysis

The data are presented as mean+SD or SEM. A two-tailed t test was utilized to compare significant differences between means. A P value <0.05 was considered statistically significant. For microarray data, nSolver analysis software was used to identify proteins and cytokines that showed statistically significant differential expression using a t test that assumes unequal variance. For in vivo bioluminescence intensity, the Kruskal–Wallis test was utilized to compare the median of the CAR133-i502-NK92 group to that of the CAR133-NK92- and NK92-treated groups. For survival data, Kaplan–Meier curves were plotted, and the differences between groups were analysed using a log-rank test. Prism 7.0 (GraphPad) software was used to perform the statistical calculations.

Results

1. Expression profile of CD133 in normal tissues and colorectal cancer tissues

We first assessed the expression of CD133 in primary colon cancer tissues by performing immunohistochemical staining of a tissue microarray containing 76 samples. As expected, CD133 appears to be positively expressed in all tissues, and it is highly expressed in 43.4% of the samples (Fig. 1A, B). We next evaluated CD133 expression in normal human tissue by performing

immunohistochemical staining of a tissue microarray containing 25 types of tissue samples; we observed positive staining only in the stomach tissues (Fig. 1C). Additionally, we used flow cytometry to examine CD133 expression in the colon cancer cell lines HCT116, SW480 and SW620. We observed that CD133 is highly expressed in HCT116 and SW620 cells (99.73% and 89.27%, respectively) but not expressed in SW480 cells (Fig. 1D). In the subsequent experiments, we used SW480 cells as the control group and SW620 and HCT116 cells as the experimental groups.

2. Generation of CBLB502-secreting CAR133-NK92 cells

A second generation of CD133-specific CAR (conv. CAR133) containing of anti-CD133 scFv, the CD8 α -hinge, the transmembrane domain of CD8 α (CD8 α -TM), the costimulatory molecule 4-1BB, and the human CD3 ζ domain, was successfully constructed as described previously [27, 28] (Fig. 2A and B). To design an anti-CD133 CAR that produces flagellin -based agonist 502s “on demand” and to selectively deposit it in the tumor tissue while avoiding systemic immune cell activation, the conv. CAR133 construct was further engineered to include an inducible TLR5 agonist CBLB502 (i502) expression cassette under the control of the nuclear factor of activated T cells (NFAT)-IL2 minimal promoter. The use of such an expression construct means that CBLB502 is released by the engineered NK92 cells following CAR engagement of cognate antigens; to facilitate their analysis, CBLB502 is expressed as a translational fusion with an oligo-His-tag, and the oligo-His-tag has been shown to have little, if any, effect on the protein’s binding affinity or on its killing capability [42]. The two CARs with CD133 specificity were inserted into a pWPXL lentiviral vector using BamHI and NdeI enzymes (Fig. 2C). The entire sequence was then confirmed by direct sequencing.

3. Flagellin derivative CBLB502 enhances CAR133-NK92 cell proliferation, cytokines expression and antitumor activity in vitro

NK92 cells were expanded according to the standard procedure and stably transduced with the two constructs, conventional CAR133 (conv. CAR133) and inducible TLR5 agonist CBLB502-secreting CAR133 (i502-CAR133), to generate CAR133-NK92 cells and CAR133-i502-NK92 cells, respectively. The transduction efficiency was determined at 3 days post-transduction by staining with anti-F(ab)2 and conducting flow cytometry. The results showed that CAR expression in CAR133-i502-NK92 cells is almost equivalent to or slightly lower than that in CAR133-NK92 cells (Fig. 2D). Secretion of TLR5 agonist CBLB502 was assessed by ELISA of the culture supernatant of CAR133-i502-NK92 cells following 5-day coculture of the cells with CD133+ SW620 cells and CD133-SW480 cells. We observed that CBLB502 is secreted by CAR133-NK92 cells engineered with CBLB502 when the cells were cocultured with CD133+ SW620 cells (Fig. 2E) but not with CD133-SW480 cells, indicating that the CAR133-i502-NK92 cells secretes the flagellin -based agonist CBLB502 in an antigen-dependent manner.

It has been reported that T cells engineered to secrete flagellin exhibit increased cell proliferation and cytotoxicity to tumor cells [43]; therefore, we evaluated NK92 cell proliferation, and we observed that both CAR133-NK92 cells and CAR133-i502-NK92 cells were significantly expanded compared to non-transduced NK92 cells (Fig. 3A). The cell number is substantially higher for CAR133-i502-NK92 cells than

for CAR133-NK92 cells at days 5 and 7; CAR133-i502-NK92 cells were also found to display enhanced expression of the proliferation marker Ki67 when cocultured with CD133-positive SW620 (Fig. 3B), suggesting that the genetically encoded TLR5 agonist CBLB502 may act as a signalling cytokine that interacts synergistically with CAR activation to drive CAR133-NK92 cell proliferation.

Further characterization of the cytokine profile of 502s-secreting CAR133-NK92 cells indicated that the production of cytokines was generally comparable between CAR133-NK92 cells and CAR133-i502-NK92 cells stimulated with hCD133 positive tumor cell lines (treated with mitomycin C) for 3 days (data not show), however, CBLB502 significantly augmented granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6 and IL-7 production after in vitro stimulation with hCD133-positive tumor cell lines for 5 days, indicating that enough concentration of TLR5 agonist CBLB502 might further derive CAR133-NK92 to secrete a pro-inflammatory-like profile cytokine (Fig. 3C). GM-CSF, IL-6 and IL-7 concentrations in the culture supernatants were further determined by ELISA (Fig. 3D). CAR133-i502-NK92 cells were also found to display enhanced expression of the activation marker CD69 when cocultured with hCD133-positive SW620 for 3 days (Fig. 3E). Consistent with this, expression of the genes encoding activity receptors such as NKp46, NKp44, NKG2D, CD69 and CD137 was significantly enhanced, and inhibitory receptors such as NKG2A was significantly decreased, whereas no difference in the expression of markers associated with NK cell exhaustion (PD-1, TIM-3 and LAG-3) was observed between CAR133-NK92 cells and CAR133-i502-NK92 cells (Fig. 3F). Furthermore, we cocultured CAR133-NK92 cells and CAR133-i502-NK92 cells with colorectal cancer cells at E:T ratios (10:1) and found that CAR133-i502-NK92 cells showed enhanced tumor lytic potential against hCD133+ colon cancer cells compared to CAR133-NK92 cells, while neither caused lysis of hCD133- colon cancer cells (Fig. 3G, H), the antitumor activity was further confirmed at analysis the degranulation of the effector cells (Fig. 3I), confirming that expression of CBLB502 enhances the lytic function of CAR133-NK92 cells. Overall, these data suggest that co-expression of CAR133 with CBLB502 enhances NK92 cell proliferation, cytokines expression and antitumor activity.

4. CAR133-i502-NK92 cells delay tumor growth in mice with subcutaneous hCD133-expressing colon tumors

Encouraged by our observation that CAR133-i502-NK92 cells exhibit enhanced antitumor activity in vitro, we investigated their antitumor activity in a mouse xenograft model using nude mice engrafted with colorectal tumor cells expressing hCD133 labelled with FFluc. When tumors had become established (defined as day 0), the mice were randomized based on the tumor BLI to ensure that the three experimental groups had similar tumor burdens and treated with intravenous (i.v.) injection of CAR133-NK92 cells with or without engineered CBLB502 (5×10^6 cells/mouse/dose) on days 0, 3 and 6. Mice treated with NK92 cells were used as the control group. The experimental design was shown in Fig. 4A. Compared with control cells, CAR133-i502-NK92 and CAR133-NK92 cells both significantly suppress tumor growth at day 20, and CAR133-i502-NK92 cells show a more potent antitumor effects (Fig. 4B, C). Moreover, CAR133-i502-NK92 cell therapy prolongs survival at the time of sacrifice (Fig. 4D). Serum collected on day 20 was analysed by ELISA for the presence of CBLB502. Mice injected with CAR133-

i502-NK92 cells display a high level of CBLB502 (Fig. 4E). Consistently, the number of CAR133-i502-NK92 cells in the serum of mice injected with those cells was elevated 3-fold to 1680 cells/ μ L compared to the number of CAR133-NK92 cells in the serum of mice injected with CAR133-NK92 cells ($p < 0.001$; Fig. 4F), suggesting that CAR133-i502-NK92 cells might exhibit antigen-dependent secretion of CBLB502 and thereby promote their proliferation. Further analysis indicated that mice treated with CAR133-i502-NK92 cells show a transient weight loss at day 8, but their weight return to the baseline level and remain stable thereafter (Fig. 4G).

Detailed pathological examination of mice euthanized on day 20 after CAR133-i502-NK92 cell infusion was also performed. Various organs and tissues, including the heart, liver, spleen, lungs, and kidneys, were extracted and subjected to H&E staining, and no pathological abnormalities were found in any of the mice (Fig. 4H). The experimental results *in vivo* showed that treatment with CAR133-i502-NK92 cells enhance antitumor activity in tumor-bearing nude mice, but not cause any obvious toxicity to important organs.

5. CAR133-i502 construct-engineered NK92 cells mediate the effective activation of NK cells, macrophages and T cells

Flagellin is known to activate immune response by recognized receptors expressed on lymphocytes, NK cells, macrophages and other cells. Therefore, we characterized the endogenous immune cell response to CBLB502 secreting from CAR133-502s-NK92 cells. As we reported that the production of cytokines was generally comparable between CAR133-NK92 cells and CAR133-i502-NK92 cells stimulated with CD133 positive tumor cell lines (treated with mitomycin C) for 3 days, however, the finding of detectable levels of CBLB502 in the culture supernatant of CAR133-i502-NK92 cells was observed to be significantly elevated. Therefore, NK cells, T cells and macrophages were stimulated with the above supernatant. As expected, administration of conditioned medium from CAR133-i502-NK92 cells significantly enhances the proliferation and activation of NK92 cells *in vitro* compared with conditioned medium from CAR133-NK92 cells, showing the expression of proliferation marker Ki67 and activation marker CD69 was significantly increased (Fig. 5A-C). Consistent with NK92, conditioned medium derived from CAR133-i502-NK92 cells also significantly enhances CD69 and Ki67 expression on T cell (Fig. 5D, E), CD86 and Ki67 expression on macrophages (Fig. 5G, H), subsequently, the number of CD3⁺, CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells was significantly increased (Fig. 5F), indicating that CBLB502 releasing from CAR133-i502-NK92 also enhanced proliferation and activity of T cells and macrophages. Taken together, these results suggest that TLR5 agonist CBLB502-engineered CAR133-NK92 cells may trigger the endogenous immune response and that immune cells might further responsible for clearing antigen-negative tumor cells *in vivo*.

6. Flagellin signalling significantly enhances antitumor activity of CAR133-NK92 cells on hCD133- and hCD133+ mixed colon cancer cells in a mouse model of subcutaneous transplanted tumor

Based on the effect of flagellin signalling *in vitro*, we next determined whether CAR133-i502-NK92 cells can simultaneously eliminate antigen-positive and negative tumor cells *in vivo*. We inoculated syngeneic

nude mice with a mixed population of equal amounts of hCD133+ and hCD133- colon cancer cells labelled with FFluc, followed by treatment with CAR133-i502-NK92 cells and CAR133-NK92 cells. The experimental design is shown in Fig. 6A. In mice treated with CAR133-NK92, the tumors grew rapidly, whereas treatment with CAR133-i502-NK92 cells suppress tumor growth and improve survival (Fig. 6B-D). The enhanced antitumor activity of CAR133-i502-NK92 cells was also demonstrated by the fact that the expression of CD31, a marker of tumor angiogenesis, was significantly decreased in the tissues of mice treated with CAR133-i502-NK92 cells (Fig. 6E). This might be attributed to mobilization by the flagellin signal of endogenous immune cells that targeted the antigens of either CD133+ or CD133- tumor cells. To test this hypothesis, we conducted HE experiments with tumor tissues derived from CD133+ and CD133- (at a 1:1 ratio) tumor-bearing mice treated with either CAR133-i502-NK92 cells or CAR133-NK92 cells and found that tumor tissues derived from mice treated with CAR133-i502-NK92 cells induced massive CD3+ T cell infiltration, substantially higher than that observed in CAR133-NK92 cells (Fig. 6F). We also measured the weights of the mice that underwent the therapeutic regimens and found that there were no significant differences in body weights among the two groups (Fig. 6G), suggesting that CAR133-i502-NK92 cells are not systemically toxic to the mice. Our findings suggest that the use of an appropriate combination of CAR133 with the immune-regulatory factor TLR5 agonist 502s can enhance the antitumor potential of NK92 cells in coordination with the activation of tumor-reactive endogenous immune cells.

Discussion

In the current investigation, CAR-expressing NK92 cells are shown to be a promising cellular platform for cancer immunotherapy; however, as in the case of CAR-T cells, negative antigen relapses after initial response are a major limitation to the magnitude and durability of CAR-NK92 cell therapy efficacy in solid tumours settings, as demonstrated in several trials.

Flagellin, as a key agonist of the immune response, has been shown to activate innate and adaptive immune responses by recognizing toll-like receptor 5 expressed on the surface of numerous immune cells, including NK cells, macrophages, lymphocytes and dendritic cells [19]. Due to their potent immunostimulatory capability, TLR5 agonists have been approved for the treatment of various malignancies; among them, Mobilan and entolimod are currently used in the treatment of cancer and have been shown to induce innate and adaptive antitumor immune responses in the tumour microenvironment. Both agonists are derived from *Salmonella* flagellin by deletion of its hypervariable and highly immunogenic D2 and D3 domains (named CBLB502), a modification that was expected to reduce its immunogenicity.

Based on the provided proof of principle, here we take advantage of CBLB502 and utilize it as an immune adjuvant to engineered CAR133-NK92 cells to recruit a second wave of immune cells to the tumor microenvironment, which enhances an antitumor response to tumors that escape targeted killing by CAR133-NK92 cells. We demonstrated that CAR133-NK92 cells secreting the immune-modulatory TLR5 agonist CBLB502 exhibit enhanced proliferation and cytokines expression in vitro and more potent

antitumor cytotoxicity in vivo compared to NK92 cells modified to express tumor-targeted CAR alone. Recent evidence has indicated that T cells engineered to secrete flagellin not only increase T cell proliferation, survival, and cytotoxicity to tumor cells in vitro but also improve survival in xenogeneic mouse models and that this is might associated with elevated levels of several cytokines and chemokines [43]. When we analysed the cytokines and chemokines secreted by CAR133-i502-NK92 cells, we found that the production of cytokines was generally comparable between CAR133-NK92 cells and CAR133-i502-NK92 cells stimulated with CD133 positive tumor cell lines (treated with mitomycin C) for 3 days, actually, the levels of IL-6, IL-7 and GM-CSF in the culture medium were significantly elevated when CAR133-i502-NK92 cells stimulated with CD133 positive tumor cell lines (treated with mitomycin C) for 5 days compared to those in the culture medium of CAR133-NK92 cells without CBLB502, indicating that enough concentration of TLR5 agonist CBLB502 secreting from CAR133-502s-NK92 cells might further derive CAR133-NK92 to secrete a pro-inflammatory-like profile cytokine. Indeed, the induction of the pro-inflammatory cytokines GM-CSF and IL-6 has been verified to be TLR5-dependent and occurs in a dose-dependent manner in response to CBLB502, a pharmacologically optimized flagellin derivative, and Feinstein.,et al also suggested that G-CSF and IL-6 might be candidate biomarkers of CBLB502 efficacy in vivo [44]. This idea was further supported by the work of Eremina., and colleagues, who reported that Mobilan (consisting of human Toll-like receptor 5 and CBLB502) induced elevation of G-CSF and IL-6 levels [45]. Beyond that, our results also indicated that IL-7 production by CAR133-i502-NK92 cells was elevated; and IL-7 is critical in promoting cell proliferation and inhibiting apoptosis of NK cells [46, 47], therefore, this might explains why CAR133-502s-NK92 cells showed enhanced proliferation compared to CAR133-NK92 cells without CBLB502.

Next, we demonstrated the important role of TLR5 agonist CBLB502 secreting from CAR133-502s-NK92 cells in activation endogenous immune cells. Flagellin is reported to activate immune response by recognized receptors that are expressed on lymphocytes, NK cells, macrophages and other cells [48–51]. We utilized conditioned medium collected from CD133+ colon tumor cells cocultured with CAR133-502s-NK92 cells or CAR133-NK92 cells for 3 days (the main differences between CAR133-502s-NK92 cells and CAR133-NK92 cells are 502s,) and used it to stimulate endogenous immune cells that participate in the antitumor response, including NK cells, T cells and macrophages. We found that conditioned medium from CAR133-502s-NK92 cells not only activated these endogenous immune cells but also enhance their proliferation, suggesting that CAR133-i502-NK92 cells possess strong antitumor activity might because they are able to stimulate endogenous immune cells to recognize both antigen-positive and negative tumor cells. In a more recent study, Kaczanowska reported that intratumoral delivery of flagellin-producing T cells offered a therapeutic benefit by altering several aspects including augmenting T cell effector function and expansion as well as reduction the number of T cell expression exhaustion markers [43]. Our study extends this finding and further suggest that the elicit endogenous immune cells response by the TLR5 agonist CBLB502 secreting from CAR133-i502-NK92 cells might further results in a broadening of the antitumor response.

On the basis of these observations, we further tested the antitumor function of CBLB502-secreting CAR133-NK92 cells in a mouse model of heterogeneous population of tumor cells. We found enhanced

survival of hCD133+/hCD133- mixed colon cancer xenograft mouse models treated with CAR133-502s-NK92 cells followed by preinjection of T cells, suggesting that CBLB502-secreting CAR133-NK92 cells may effectively elicit endogenous immune cells response and thereby eradicate hCD133- colon tumor cells. Indeed, we found more CD3+ native immune cell infiltration into the tumor tissue in these mice. The utility of flagellin in eliciting antitumor immune responses has been emphasized by other groups. Among them, Rhee and colleagues demonstrated flagellin regulated neutrophil infiltration and thereby controlled immune responses against tumors [21]. Burdelya and Gudkov [52] showed that CBLB502 induced numerous immunomodulatory factors and massive recruitment of various types of immune cells and thereby inhibited the growth and liver metastasis of multiple tumors. Our study extended these findings and broadened the use of the TLR5 agonist CBLB502 to CAR-NK92 cell immunotherapy. Since colon cancer cells of DLD1 are reported to express TLR5 at high levels and to respond strongly to flagellin stimulation [21], therefore, the enhanced biological activity of the TLR5 agonist CBLB502 secreted by CAR133-NK92 cells might be due not merely to its eliciting endogenous immune response, its role of interaction by TLRs in the colon cancer cells might not be neglected. However, at this time, it is not clear whether colon cancer cells of SW620 and SW480 is expressed TLR5 or whether the TLR5 agonist CBLB502 secreted by CAR133-NK92 cells directly eradicate SW620 and SW480. This awaits clarification in future studies.

Since the CD133 antigen we used to target cancer in our study was also found to be positively expressed in stomach tissues, it is suggested that the safety of CAR133-502s-NK92 cells injection in vivo should be evaluated. In this study, pathological examination of mouse tissues, including the colon, brain and stomach, did not reveal signs of adverse events, suggesting that the CBLB502 secreted by the CAR133-NK92 cells enhanced antitumor activity and without toxicity to normal tissue. In a recent study, Eremina and Gudkov reported that Mobilan, which consists of human Toll-like receptor 5 (hTLR5) and CBLB502, was safe and well tolerated at all tested doses in early-stage prostate cancer patients [45]. Taking this recent study into account, it should be noted that the CBLB502 secreted by the CAR133-NK92 cells demonstrated satisfactory safety and tolerability in vivo.

Several limitations of this study should be considered. First, our immune deficiency mouse model lacks a functioning immune system in which to evaluate the effectiveness of CAR133-i502-NK92 cells in a situation resembling the clinical immunosuppressive tumor microenvironment. Further investigation is needed to comprehensively evaluate the interaction of CAR133-i502-NK92 cells with tumor infiltrating T cells, NK cells and macrophages and their modulation of the tumor microenvironment in mice with intact host immune systems. Second, we found that conditioned medium from CAR133-502s-NK92 cells activated immune cells, but the mechanistic explanation of this phenomenon is unclear. Flagellin triggers the immune response via the concerted action of TLR5 and the NLRC4/NAIP5 inflammasome. Blander and Medzhitov reported that TLR5 signalling was sufficient to trigger immune cell activation [53], and Lightfield and Miao reported that NLRC4/NAIP5 activation is also essential for the immune response to flagellin [54, 55]. Therefore, further studies are needed to fully characterize the mechanism of action of flagellin -based agonist CBLB502-activated immune cells. Moreover, as flagellin has been reported to

prevent preexisting tumor cells from developing into solid tumors at metastatic sites, it may be essential to explore whether CAR133-502s-NK92 cells can prevent cancer metastasis and relapse.

In summary, we successfully generated CAR133-NK92 cells that secrete the TLR5 agonist CBLB502 at tumor sites, thereby activating immune cells in the tumor microenvironment, mediating an antitumor response to tumors that escape target killing by CAR133-NK92 cells and improving antitumor activity. In addition to targeting the CD133 antigen, CAR133-i502-NK92 cells could be broadly engineered to express a wide array of other antigens that reshape the tumor microenvironment in multiple solid tumors. Our studies also suggest that CAR-NK92 cells can serve not only as direct antitumor effector cells but also as cellular vectors to convey immune-regulatory molecules to the tumor microenvironment; in this manner, antitumor immune responses in solid tumors can be triggered, augmented, and sustained.

Declarations

Acknowledgments

This work was supported by the National Key R&D Program of China (2018YFC1313400), the National Natural Science Foundation of China (No. 31540016), the Basic research and frontier exploration projects of Chongqing (No.cstc2018jcyjAX0075) and the Subsidy fund for the development of National Silk in Chongqing (No. CQ2018JSCE05).

Author contributions

X.W. designed experiments, analysed the data, created the figures and wrote/edited the manuscript. W.Q. contributed to edited the paper and analysed the data. H.L and M.H. and Z.L. performed experiments. W.G. and Z.W. assisted in the experiments. P.C., X.X. and W.H. supervised the project, contributed to the design of the experiments, and edited the paper. All authors critically read and approved the manuscript.

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Figures

Fig.1

Figure 1

Analysis of CD133 expression in human normal tissue, primary colon cancer tissues and colon cancer cells

A, The HColA-180Su11 microarray (Outdo Biotech, Shanghai, China), containing 76 primary colon cancer samples was stained with an anti-CD133 antibody to determine the expression of CD133. Representative images (magnification $\times 4$) are shown for the CD133 immunostaining. Scale bar represents 500 μm .

B, The percentage of different levels of CD133 expression was indicated.

C, The HOrgN090PT02 microarray (Outdo Biotech, Shanghai, China) containing 25 human normal tissue samples was immunostained with an anti-CD133 antibody to determine the expression of CD133. Representative staining image fields (magnification $\times 20$) are shown. Scale bars represent 100 μm .

D, The CD133 expression on colon cancer cell lines was analysis by flow cytometry.

Figure 3

CAR133-NK92 cells with inducible secreting of TLR5 agonist CBLB502 enable superior ex vivo proliferation, activation and antitumor activity

A, Absolute numbers of conventional and CAR133-i502-NK92 cells on different days. Data represent the mean $\pm$ s.d. of quadruplicate wells, * P<.05; NS, not significant.

B, The expression of proliferation protein Ki67 on NK92 cells was determined by flow cytometry.

C, CAR133-NK92 cells and CAR133-i502-NK92 cells were cocultured with SW620 cells for 5 days, the cytokine and chemokine in the culture supernatant was determined by the human cytokine array C5 (Ray Biotech),* P<.05.

D, The concentration of GM-CSF, IL-7 and IL-6 in the supernatant was further determined by specific ELISA kits. Data represent the mean+SEM,* P<.05; **P<.01.

E, Activation marker CD69 was quantified by flow cytometry when CAR133 or CAR133-i502-NK92 cells were cultured with SW620 for 3 days***P<.001.

F, CAR133-NK92 or CAR133-i502-NK92 cells were cultured with SW620 cells for 3 days. mRNA expression of NKp44, NKp46, NKG2A, NKG2D, CD69, CD137, PD-1, TIM-3 and LAG-3 was assessed by RT-PCR,* P<.05.

G, Cell killing by CAR133-NK92 cells and CAR133-i502-NK92 cells was investigated by the LDH cytotoxicity assay after coculture with target cells at the indicated E/T ratios. All data are expressed as the means±SEM of triplicate samples.* P<.05; NS, not significant.

H, Cytotoxicity of CAR133-NK92 cells and CAR133-i502-NK92 cells was culculated by RTCA.

I, CD107a expression represents the degranulation of NK cells that were analyzed by flow cytometry.* P<.05; **P<.01; NS, not significant.

Figure 4

CBLB502 promote the antitumor activity of CAR133-NK92 cells in mice with subcutaneous CD133-expressing colon tumors

A, Schedule of the challenge protocol. Mice were inoculated with CD133 + SW620 cells (1×10⁶ cells/mouse) followed 14 days later by CAR133-i502-NK92, CAR133 NK92 or NK92 cells (5×10⁶ cells/mouse, n=5/group).

B, Colon cancer growth was monitored by in vivo bioluminescence imaging.

C, Tumor volumes were recorded. *P<.05.

D, Kaplan-Meier survival curves showing tumor free survival of tumor-bearing mice treated with CAR133-i502-NK92, CAR133-NK92 or NK92 cells. Significance was determined by log-rank test. * $P < .05$.

E and F, Detection of CBLB502 (E) and CAR133-i502-NK92, CAR133-NK92 or NK92 cells (F) in peripheral blood at days 20 after NK92 cells treatment. *** $P < .001$.

G, Body weight of the individual mice from each treatment group. Data are presented as the mean $\pm$ SD of three independent experiments. Error bars represent the standard deviation.

G, Quantification of CAR133-i502-NK92, CAR133-NK92 and NK92 cells (CD56+) in peripheral blood at days 20 after NK92 cells treatment. * $P < .05$; ** $P < .01$.

H, Histopathological analysis of mouse organ tissues by H&E staining. Magnification $\times 20$. Each scale bar represents 100 μm .

Figure 5

CBLB502 secreting from CAR133-i502-NK92 cells mediates effective activity of NK, macrophages and T cells

A and B, The expression of activation marker CD69 (A) and proliferation marker Ki67 on NK92 cells were recorded by flow cytometry. Data represent the mean $\pm$ SEM.* $P < .05$; *** $P < .001$.

C, Expansion of the NK92 cells in the presence conditioned medium from CAR133-NK92 cells or CAR133-i502-NK92 cells cocultured with CD133 positive tumor cell lines (treated with mitomycin C) using the CCK-8 assay. *P<.05.

D and E, CD69 (D) and Ki67 (E) expression on T cells was tested by flow cytometry. Data represent the mean ±SEM.*P<0.05,**P<0.01.

F, Absolute numbers of CD3+, CD3+CD4+ and CD3+CD8+ T cells when T cells were stimulated with conditioned medium from CAR133-NK92 cells or CAR133-i502-NK92 cells cocultured with CD133 positive tumor cell lines (treated with mitomycin C). Data represent the mean ± s.d. of quadruplicate wells, * P<.05.

G, CD86+ macrophages was analysis by flow cytometry.

H, Ki67 expression on macrophages was tested by flow cytometry.

Figure 6

Adoptively transferred CAR133-i502-NK92 successfully control tumor development in mice with CD133+/CD133- mixed colon tumor model

A, Schedule of the in vivo experimental design using CD133+/CD133- mixed colon tumor model.

B, BLI illustrating colon cancer growth.

C, Tumor volumes were recorded. $**P < .01$.

D, Kaplan-Meier survival curves generated from survival of mouse treatment with CAR133-i502-NK92 and CAR133-NK92. $**P < .01$.

E, The expression of CD31 protein was tested in the mouse tumor tissue using IHC assays. Representative photomicrographs are shown. magnification $\times 100$ (left) and $\times 200$ (right). Scale bar is 100 μm .

F, The representative immunostaining images of CD3+ T-cell infiltration into cancer tissues. Representative staining of tumor sections in each experimental group is shown. magnification $\times 100$ (left) and $\times 200$ (right) Each scale bar represents 100 μm .

G, Body weight of the individual mice from each treatment group. Data are presented as the mean $\pm$ SD of three independent experiments. Error bars represent the standard deviation.

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