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Construction of Anti-CD20 Single-Chain Antibody-CD28-CD137-TCRζ Recombinant Genetic Modified T Cells and its Treatment Effect on B Cell Lymphoma

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Immunotherapy has been explored as a new therapy for B cell lymphoma, which is a non-Hodgkin's lymphoma. Because CD20 is a B lymphocyte-specific marker, anti-CD20 single chain-tagged T lymphocytes have already begun to be experimentally used in B cell lymphoma treatment, but its use is still limited because of its unspecific targeting. T cells transfected with CD28 and CD137 can significantly improve the ability of cytokines secretion and anti-tumor effect, as well as extending T cell survival time and improving their proliferation ability.

Material/Methods: Genes containing anti-CD20-CD28-CD137-TCRζ were constructed. After cloning and sequencing, the plasmid was constructed and packaged by lentivirus. It was transfected to the peripheral blood T lymphocyte after identification transfection to induce the fusion protein expression. The cells were incubated with Raji cells and the LDH test was performed to detect the cytotoxic effect of CAR-T cells; the tumor volume and survival rate were measured to observe its inhibitory effect on B cell lymphoma in nude mice.

Results: Gene with anti-CD20-CD28-CD137-TCRζ was successfully constructed and transfected to the T cell surface. LDH assay revealed that CAR-T cells can kill the Raji cells with a killing rate of 32.89±6.26%. It can significantly inhibit B cell lymphoma growth in nude mice.

Conclusions: T lymphocytes transfected with anti-CD20-CD28-CD137-TCRζ fusion gene can kill B cell lymphoma, which could provide a new strategy for tumor treatment.

MeSH Keywords: CD4-Positive T-Lymphocytes • Human T-lymphotropic Virus 1 • Sleep Arousal Disorders

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Background

B cell lymphoma is a non-Hodgkin's lymphoma. In recent decades there has been an increase the incidence of non-Hodgkin's lymphoma [1]. Traditional treatment for B cell lymphoma includes chemotherapy, radiotherapy, and hematopoietic stem cell transplantation. However, for many refractory, relapse, or elderly lymphoma patients who cannot tolerate chemotherapy and stem cell transplantation, immune treatment has attracted more attention in recent years [2,3].

T lymphocytes have an important role in antitumor immunity [4,5]. Although adoption of autologous T lymphocytes with the same MHC antigen to normal tissues can avoid rejection [6], it may produce immune tolerance for lymphoma cells that still have tumor-related antigen immune tolerance on this part of autologous T cells. Thus, the clinical curative effect of the abovementioned method is still far from being able to cure lymphoma [7].

In recent years, with the development of genetic engineering technology, the antibody that identifies the tumor associated antigen could be connected to the costimulatory molecule gene sequence required for T cell activation to form the carrier. Then, it could be transfected to T lymphocytes *in vitro*, which combines the high affinity of the antigen antibody with the cell-killing mechanism of cytotoxic T cells. This develops the T cell targeted killing effect, which become a new exploration for tumor cell immune therapy [8]. Since most non-Hodgkin's lymphoma originated from B cells [9], and more than 90% of patients expressed CD20 antigen [10], anti-CD20 antibody fusion genes are widely used for B cell lymphoma treatment.

Induction of T cell activation, proliferation, and differentiation to effector cells needs a costimulatory signal. CD28 and CD137 are 2 important costimulatory molecules; the former is expressed stably on the T cells, and the latter is able to activate T lymphocytes independent of CD28. Some reports suggested that overexpression of CD137 and CD28 in T cells can significantly improve its cytokines secretion and anti-tumor effect, extend T cells survival time, and improve T cell proliferation ability [4,11,12].

Our study aimed to construct an anti-CD20-CD28-CD137-TCRζ fusion gene and transfect it to the peripheral blood T lymphocyte by lipofection transfection method to observe its cytotoxic effect *in vitro* and the therapeutic effect on lymphoma in a nude mouse model.

Material and Methods

Material

HD-Lai plasmid was provided by the GeneArt Company (Grand Island, NY). pLenti6.3 _MCS_IRES2 – EGFP vector and Ligase were provided by the NEB Company (Ipswich, GK). Stbl3 cell was purchased from Invitrogen (Grand Island, NY). BamHI, AscI, BglII was provided by MBI Company (Fermentas, Canada). Lentiviral vector was prepared by Invitrogen Shanghai (Shanghai, China). Packaging plasmid pLP1, pLP2, pLP/VSVG, DMEM +; FBS, lipofectamine2000, Opti-MEM, 0.05% Trypsin were all from Invitrogen (Grand Island, NY). Primers were synthesized by Takara (Dalian, China). Gene sequencing was performed by Kunming Expression Technology Co., LTD. (Kunming, China); CytoTox® 96 Non-Radioactive Cytotoxicity Assay kit was bought from Promega (Beijing, China); Nude mice was purchased from Beijing Vital River Co., LTD (Beijing, China). 293T cell and Raji cell were derived from the ATCC Cell Bank (Manassas, VA).

Recombinant plasmid construction

After HD-Lai plasmid was double-digested by BglII and AscI and after pLenti6.3_MCS_IRES2-EGFP vector was double-digested by BamHI and AscI, the fragment and vector were connected by ligase. Gene sequencing was performed to validate the insertion into the recombinant clone fragment.

Lentivirus packaging and transfection

Peripheral blood was collected from lymphoma patients, and a COBE machine was used to collect the mononuclear cells. Immune magnetic beads were used to separate CD4+ CD8+ T cells. The CD4+ CD8+ T cells were seeded in the cell culture bottles and maintained at 37°C and 5% CO_2 in an incubator overnight. The transfection was performed on the second day, when the Packaging Mix and lentivirus plasmid were mixed in the 37°C preheated Opti-MEM, while lipo2000 was added to Opti-MEM. After standing at room temperature for 5 min, the plasmid solution and lipo2000 diluent solution were mixed and reacted at room temperature for 20 min. And then, the solution was added to the cell culture bottle and incubated for 6 h. After changing the medium and incubating for 48 h, the cell supernatant was collected and centrifuged to get the anti-CD20-CD28-CD137-TCRζ fusion gene modified T cells. The transfection effect was observed under fluorescence microscope and flow sorting was performed to obtain the CD20-CD28-CD137-TCRζ CAR T cells.

Cytotoxic effect

The LDH test was performed to detect the *in vitro* cytotoxic effect of CAR-T cells by co-culture of CAR-T cells and Raji cells.

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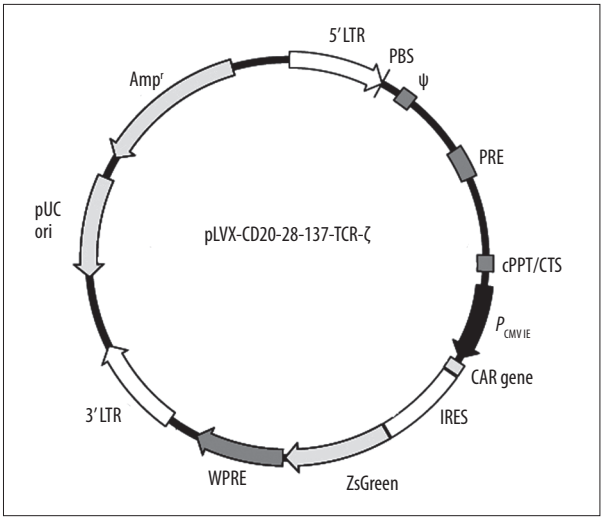

Figure 1. CD20-28-137-TCR-ζ single chain lentivirus vector model.

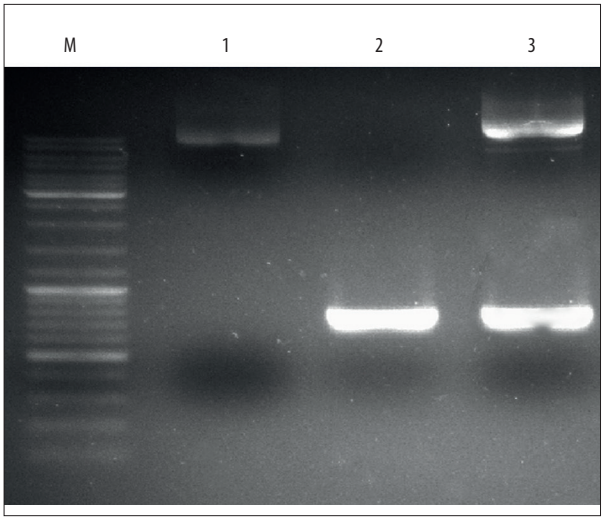

Figure 2. pLVX-CD20-CD28-CD137-TCRζ plasmid construction agarose gel electrophoresis. M, Marker; 1, pLVX empty vector; 2, PCR product of CD20 scFv; 3, pLVX-CD20-CD28-CD137-TCRζ.

Raji cells were seeded in 96-well plates and maintained at 37°C for 24 h. CAR-T cells were added to Raji cells at the ratio of 1:2, 1:1, 3:1, and 10:1. After 4 h, the cells was added with lysis and incubated at 37°C for 45 min. After centrifugation, 50 μL of supernatant was moved to a new 96-well plate and 50 μL of assay buffer resuspended substrate mixture was added and incubated at room temperature away from light for 30 min. After adding 50 μL of terminated liquid, the absorbance value at 490 nm was read for statistical analysis.

Case selection

Lymph node biopsy was performed on suspected lymphoma patients after they signed written informed consent, and part of the lymph node was stored at –80°C. After pathological diagnosis was clear, 2 cases of chronic B cell lymphoma/chronic lymphocytic leukemia (we saved the lymph nodes and chronic lymphocytic leukemia cells at the same time), 2 cases of diffuse large B lymphoma, and 2 cases of follicular B lymphoma were selected for experimentation.

Animal experiments

A small piece of lymph node tissue from malignant lymphoma was cut into 1×1×1 mm for transplantation. According to the literature, a lymphoma tissue *in situ* transplantation model was established in nude mice [13]. We anesthetized 4–5 weeks old Balb/c nude mice by caudal vein injection of sodium pentobarbital (30 mg/kg). After open the abdomen, an incision 3 mm deep to mucosa was performed on the rectum, and 2 pieces of 1×1×1 mm tumor tissue were fixed on the mucous layer of the rectum. The mice continued feeding after surgery. The mice were randomly grouped with 10 in each group. CAR-T cells were injected for treatment. Diet and mental state of the nude mice were observed every day, tumor volume was measured every 5 days, and the survival time was observed.

Statistical analysis

All statistical analyses were performed using SPSS19.0 software (Chicago, IL). Numerical data are presented as means and standard errors (±SEM). Differences between means were analyzed using Student's t test or 1-way ANOVA, when necessary. $P<0.05$ was considered as a statistically significant difference.

Results

Recombination lentivirus vector construction, virus packaging and identification

The target fragment was used to synthesize CD20-CD28-CD137-TCRζ gene by Gene-On-DemandTM technology and connected into pLVX lentivirus vector (Figure 1). Meanwhile, anti-CD20 scFv gene was constructed by the same method as the positive control. Agarose gel electrophoresis was used to detect target fragment connection to the pLVX lentivirus vector (Figure 2). Compared with the known sequence, the tested sequence and insertion direction were correct (Figure 3). After transfection with lentivirus vector, green fluorescent protein was found expressed in T cells under the fluorescence microscope (Figure 4). These findings show that our vector can successfully transfect to T cells with relative high transfection efficiency.

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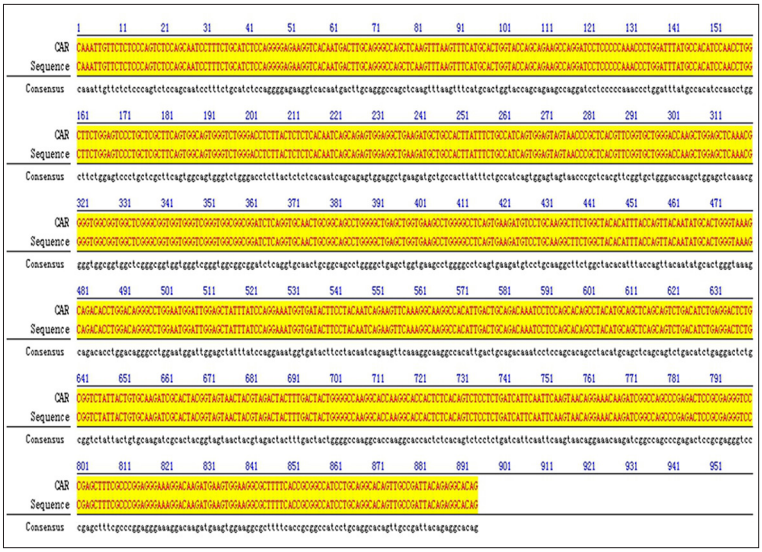

Figure 3. CD20-CD28-CD137-TCRζ gene comparison after sequencing. CAR, gene sequencing result; sequence, primary gene.

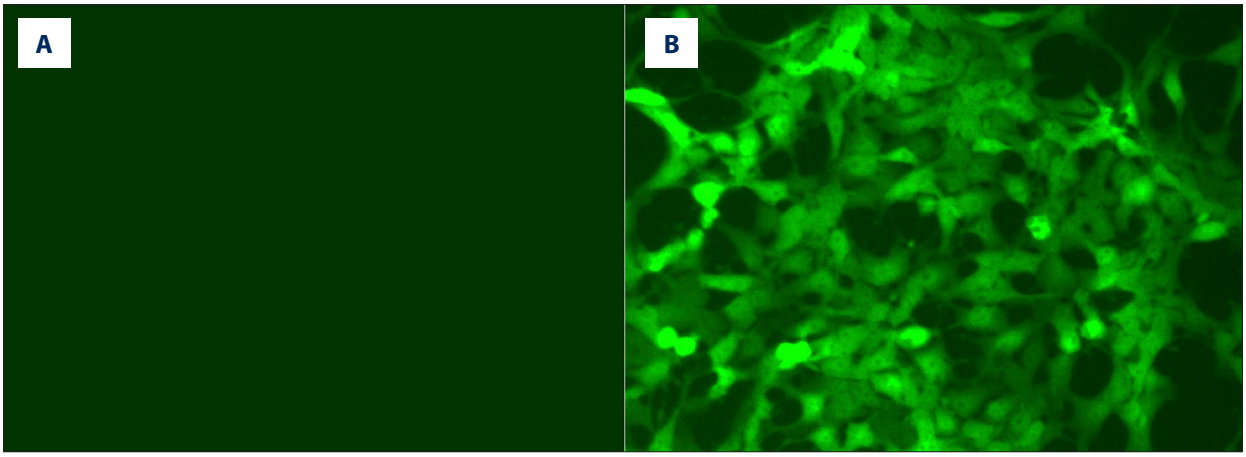

Figure 4. pLVX-CD20-CD28-CD137-TCRζ transfected T cells. (**A**) T cells transfected with empty vector; (**B**) T cells transfected with pLVX-CD20-CD28-CD137-TCRζ.

CAR-T cells exhibited cytotoxic effect *in vitro* after transfection

The LDH test was performed to detect CAR-T cytotoxic effect. Cell refraction and third-dimension fade away gradually under the microscope. These effects show that at the ratio of 10: 1, CAR-T cells can kill Raji cells effectively (Figure 4).

T lymphocytes modified with anti-CD20-CD28-CD137-TCRζ recombination gene exhibited an inhibitory effect on tumors in B lymphoma transplantation on BALB/c nude mice.

From the second day after the tumor inoculation surgery, diet and mental conditions of the nude mice were good. Angiogenesis on the tumor surface could be found on the third day through the skin. Tumor volume increased obviously on the eighth day. Tumor volume was recorded every 5 days to depict the tumor growth curve. The results showed that the tumor formation rate was 86.67% in 30 mice, of which mice in the blank control group and the negative control group all had tumor formation. Tumor growth in the test group mice was obviously inhibited, with only 6 mice having tumor formation. Mice in the negative control group and blank control group became thinner, lost appetite, appeared depressed, and were less active, while the mice in the experimental group appeared to be in better condition. The mean tumor volume (208±29 mm^3) was significantly smaller than in the negative control group (2100±239 mm^3) and blank control group (2311±361 mm^3) ($p<0.05$), while there was no statistical difference between the negative control and blank (Figure 5).

Discussion

B cell lymphoma is a non-Hodgkin's lymphoma. Non-Hodgkin's lymphoma (NHL) is a group of malignant hyperplastic diseases,

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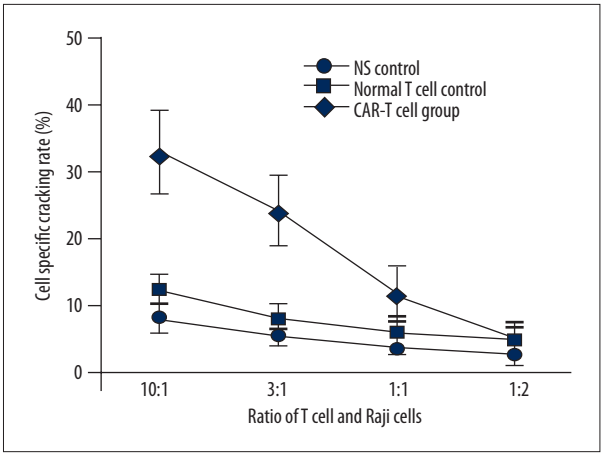

Figure 5. LDH test detect CAR-T cells cytotoxic effect on Raji cells.

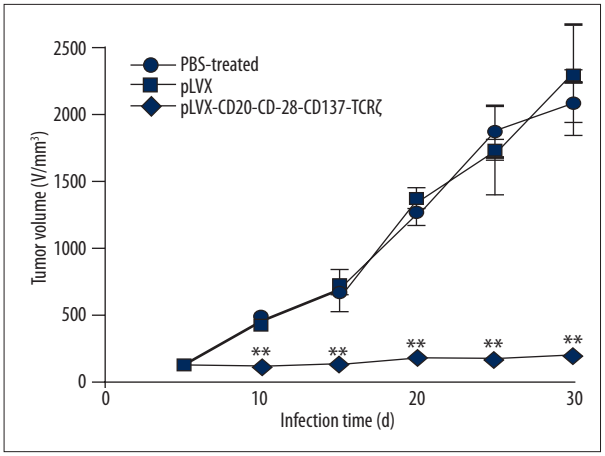

Figure 6. T lymphocytes modified with anti-CD20-CD28-CD137-TCRζ recombination gene exhibit inhibitory effect on tumor in B lymphoma transplantation on BALB/c nude mice.** p<0.01 *vs.* PBS or pLVX treated group; n=10, mean ±SEM.

including various different clinical manifestations and biological characteristics, which accounts for about 90% of all lymphoid neoplasms [14]. Although the appearance of cetuximab, represented by the anti-CD20 monoclonal antibody drug, greatly improved patient prognosis, allogeneic hematopoietic stem cell inhibition could be a treatment choice for resistance or recurrence patients [15,16], but its use has been limited by the high probability of graft-versus-host disease after allograft transplantation and non-recurrence mortality rate. Autologous T lymphocytes overcome the differences of MHC antigen, and thus avoid rejection. It allows T lymphocytes to remain alive in the body without clearance by the immune system, but still cannot avoid tumor-related antigen immune tolerance. Thus, the clinical curative effect of the abovementioned method is still far from lymphoma healing [7]. Autologous T lymphocytes transfected through CD20 antibody exhibited better curative effect on B cell lymphoma in a nude mouse model [17].

T lymphocyte proliferation and differentiation into effector T cells needs 2 signals for stimulation. The first signal is produced by the combination of antigen and T cell surface receptor, and the second signal is generated by the combination of T cell surface receptors with costimulatory molecules that exist on antigen-presenting cells. To increase the antitumor activity and tumor antigen-specific cytokines production of CAR-T cells, a costimulatory area was often added to the new generation of CAR components [18]. CD28 is an important type of costimulatory molecular receptor that is expressed on most of the T cells with relatively stable expression. It is a member of the immunoglobulin superfamily, and also is a type of glycoprotein [19]. CD28 combined with B7 molecule on antigen-presenting cells can provide a positive stimulus signal for T lymphocytes activation. At the same time, it can promote cytokines such as interleukin 2 secretion to strengthen the function of T lymphocytes [20]. It has been reported that CD28 monoclonal antibody plays an important role in the process of CD4-CD25-T lymphocyte proliferation. Single application of CD28 monoclonal antibody within 3 days can make CD4-CD25-T lymphocyte group amplify 20 times [21]. It was also found in the transplant that lack of CD28 costimulatory signal may lead no immune response to the donor T cells, which showed that CD28 also plays an important role in maintaining the balance of immune activity.

CD137 is also a kind of costimulatory molecule. It is expressed on the surface of the activated T lymphocytes, monocytes, activated eosinophils, eosinophils, follicular dendritic cells, NK cells, and microglial cells. It not only participates in the innate immune response, but also can participate in the adaptive immune response, and plays an important role in immune adjustment and immune response. It can activate T lymphocytes independent of CD28 and initiate T cell-mediated immune response [22]. Its biological activity can be summarized as promoting T cell proliferation, inhibiting apoptosis, and extending the survival time of activated T cells. Overall, it is of great significance in improving immune function [12,23].

Lentivirus vector is a kind of gene therapy vector, which was developed based on the human immunodeficiency virus (HIV) 1 [24]. It not only has the infection ability to the divisive cells, but can infect the undivided cells. It can integrate the foreign genes into the host cell's chromosome and achieve persistent expression [25]. This research used the efficient lentivirus vector to transfect CD20-CD28-CD137-TCRζ single-chain antibody gene to modify T cells, which solves the problem of low transfection efficiency in the CAR-T cell immune therapy. We introduced CD28, CD137 cytoplasm signal domain to the CD20 monoclonal antibody gene to give the transfected T cells sustained survival and long-term proliferation in the body, so

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as to achieve the immune monitoring to tumor cells. At the same time, we set up multiple controls to certify the results.

Our results show that T lymphocytes have specific recognition and killing effect of the B lymphocyte tumor cell line Raji cells that expressed CD20 antigen after transfection for 24 h. When the ratio of CAR-T cells and Raji cells reached 10: 1, CAR-T cells exhibited greater killing effect on Raji cells. The tumor formation rate of the nude mice treated with T lymphocytes transfected with pLVX-CD20-CD28-CD137-TCRζ fusion protein (60%) was significantly lower than the blank control group (100%) and the negative control group (100%). The mean tumor volume in the treatment group was also obviously smaller than in the negative control group and blank control. These findings indicate that T lymphocytes transfected with pLVX-CD20-CD28-CD137-TCRζ fusion protein can significantly inhibit the growth of B lymphocyte tumors in nude mice *in vivo* and *in vitro*.

Conclusions

In summary, transfecting monoclonal antibody gene to autologous T lymphocytes provides a new strategy for tumor-targeted therapy, while enhancing T cell function and prolonging its action time through gene fusion provides new support for immune gene therapy.

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