Construct a chimeric antigen receptor Jurkat cell which carried a humanized anti-CD20 scFv based on Rituximab.

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Abstract

The clinical trials demonstrate that CAR-T cells make a great success for tumors, haematologic malignancies especially. The third generation CAR induces the higher level of secretion of cytokine and the stronger ability against tumor cells and prolongs the life span to cells. The single-chain fragment variable isolated from murine exists potential higher immunogenicity in term of the humanized of full human scFv. Based on the human-mouse chimeric anti-CD20 rituximab monoclonal antibody, the murine part, the variable fragments of ritiximab, was humanized to reduce the immunogenicity. And the humanized scFv is utilized in the CAR-T to recognize the CD20 antigen for the relapsed indolent B-cell lymphomas. We verified the humanized scFv with biological activity. And then the jurkat cells were successfully transduced with the lentivirus which were encoding the CAR with the anti-CD20 humanized scFv..

Keywords

Chimeric antigen receptor, humanization, CD20.

1. Introduction

Chemical antigen receptor (CAR) T cells therapy obtains enormous clinical benefit for haematologic malignancies, for solid cancers as well. CAR, the recombination receptor targeting antigen, is consisted of a single chain fragment variable（scFv）which is derived from the monoclonal antibody (mAb) or the scFv libraries and recognizes the tumor associated antigen (TAA), a reasonable length of hinge, a transmembrance region, one or more costimulatory domains and a immunoreceptor tyrosine-based activation motif, such as CD3ζ chain[1-5]. Compared with other nature immune cells, T cells transduced with CAR show a great advantage that CAR-T cells against tumor cells in the manner of circumventing the major histocompatibility complexI(MCHI)[6-8]. After recognizing the cell-surface tumor antigen, CAR-T cells are specifically activated and then lead to the CAR-T cells proliferation. The tumor cells are eradicated by the means of directly killing or the secretion of various cytokine by CAR-T cells in mouse xenograft models and clinical trials[9-13].

The statistic showed that the rate of new cases with non-Hodgkin lymphoma (NHL) and the rate of death in all cancers was 2.8% and 2.6% respectively in 185 countries in 2018[14]. For the NHL, in particular, the indolent B-cell lymphomas, got a long remission based on the allogeneic stem cell transplantation therapy[15, 16]. In 2007, it is inspiring that two kinds of CAR-T cells products targeting CD19 B-cell lymphomas had been on the market. Rituximab (rituxan), an human-mouse chimeric anti-CD20 mAb, have been obtained significant clinical outcome for the CD20 positive B-cell lymphomas, however, it would prove challenging for refractory and relapsed B-cell lymphomas[17-20]. Thus, the CD20 antigen is a well-established immunotherapy target for B-cell lymphomas except the CD19 antigen for CAR-T cells therapy[21-23].

Some preclinical trails and the two kinds of CD19+CAR-T cells show that the CARs their used were the second generation CAR which only contain a co-stimulation molecule, for example, CD137, CD28 or OX40 and so on. Though it can enhance the activity and the capacity of the antitumor compared to the first generation CAR in vitro and vivo, it is not superior as the third which includes two co-stimulation molecules in tandem. The CARs share with the common region rather than the scFv. The scFv of a great number of CARs are derived from the murine. There is a potential high immunogenicity in the murine scFv. Based on the human-mouse chimeric rituximab, of which scFv is murine as well, so the murine scFv derived from the rituximab is essential to humanize to reduce the potential immunogenicity. We therefore construct a CAR which contained the humanized anti-CD20 scFv based on the rituximab and CD137 and CD28 two co-stimulation domains.

2. Methods

2.1 Cell line

HEK293T cells were cultured in 10% fetal bovine serum (FBS, Gibco, USA) in DMEM (Gibco, USA) with penicillin, streptomycin, and glutamine. The Jurkat cells were cultured in 10% FBS in RPMI 1640 with penicillin, streptomycin. All cells were incubated at 37°C humidity with 5% CO_2.

For lentiviral infection, Jurkat cells were collected and count. 1×10^6 Jurakt cells were plated in $25cm^2$ flask in RPMI1640 with no FBS . 1 mL of each virus' supernatant was added directly to Jurakt cells, with 10μg /mL polybrene also added to increase infection efficiency. The viral supernatant was replaced with normal growth media 12h post-infection and 10μg /mL puromycin was added to screen.

2.2 Expression humanized anti-CD20 scFv protien

The variable fragments were connected via the $(G_4S)_3$ linker and fusion with a Fc fragment. Then the humanized CD20 scFv sequence was inserted into the pCMV vector which was under the control of the CMV promoter. Then 10μg plasmid and were mixed up with 50μg PEI (Polysciences, USA) in 1mL Opti-MEM (Gibco,USA) in a 1.5mL tube 15 mins before the mixture of plasmid and PEI were added into the 293T cells which was washed with PBS and the of which meida was replaced with 5mL no FBS DMEM(Gibco,USA) in advance. 12h after incubation in the incubator at 37°C humidity with 5% CO_2, the media was replaced with no FBS DMEM media again. 72h after the transient transfection, the supernatant were collected for ELISA assay and WB.

2.3 Western blotting

The supernatant of transient transfection with humanized CD20 scFv plasmid was mixed with 5×loading buffer and then the mixture was boiled in 100°C dry bath for 5 mins. After boiling, the mixture was loaded onto gels for western blot processing. Lysates were loaded into 12% Bis-Tris, 15-well gels in running buffer and run at 80V/120V for about 2h. Gels were transferred for 2 h onto nitrocellulose membranes using a Bio-Rad transfer apparatus. And the membranes were blocked with 5% skimmed milk for 1h at room temperature. After that, the membranes were exposed to the diluted antibodies (1:5000) in 5% BSA buffer overlight, washed in 0.1%TBST for three times. Membranes were then imaged on the Bio-Rad gel image system.

2.4 Humanized CD20 scFv protien ELISA

The Raji cells was collected via centrifuging at 1000×g for 5 min at room temperature. The Raji cells were washed with PBS for three times. The the Raji cells were fixed with 25% Glutaric dialdehyde for 30 min at 37°C and then centrifuged at 1000×g for 5 min, washed with PBS for three times. The Raji cells were resuspended with 1mL coating buffer and counted. 1×10^5 fixed Jurakt cells per well per 100μL were paved overnight at 4°C. The primary antibody , the supernatant of post-transient transfection with humanized CD20 scFv plasmid and mock plasmid, was exposed to the fixed Jurakt cells for 1h at 37°C incubator after the fixed Jurakt cells was blocked with 5% BSA buffer. The plate was washed in 0.1%TBST for three times. The diluted second antibody (1:5000) was added and incubated for 1h at 37°C. The plate was washed in 0.1%TBST for three times again. The 100μL TMB

solution per well was add into the plate for 10 min at 37℃ in dark. After incubation, 100μL stop solution per well was added and the plate was read with microplate reader (Thermo, USA) at 450nm.

2.5 Lentivirus production

Lentivirus was produced by co-transfecting the pHR plasmids and vectors encoding packaging proteins (pMD2.G and pspax) using the transfection reagent PEI in HEK293-T cells plated in 10 cm^2 dish at approximately 70% confluence. Viral supernatants were collected 48h and 72h after transfection respectively, and 0.45 mm was filtered. Supernatant was used for transduction immediately, stored at 4℃ for up to 2 weeks or kept at 80℃ for long term storage.

2.6 Flow cytometry analysis

The Jurkat cells which had been infected with the lentivirus and screened with puromycin were collected and washed with PBS for three times. 1×10^6 cells were resuspended in 100μL PBS, then the 2μL protein-L-FITC (ACRO, USA) was added at 4℃ overnight. The cells were washed with PBS for three times and were measured by utilizing Calibur apparatus (BD, USA). And the data was analysed by using the software Flowjo 7.6.

2.7 Statistical Analysis

Statistical significance was determined by Student's t test (two-tailed). All statistical analysis and curve fitting was performed with Prism 5 (Graphpad).

3. Results

3.1 Expression of humanized anti-CD20 scFv

To verify the humanized anti-CD20 scFv (H-scfv-Fc) biological activity or not, the plasmid encoding the humanized anti-CD20 scFv was transiently transferred to the HEK293T cells. Fifure.1 (A) shows that the H-scfv-Fc protein was about 70 KDa. The supernatant which contained the H-scfv-Fc protein was boiled with reducing or non-reducing buffer, however, there is no different because the protein was in the same size position. It suggested the the Fc tag did not contribute to the dimer H-scfv-Fc protein. We speculated that the scFv of H-scfv-Fc effected the form of disulfide bond of Fc tag. The H-scfv-Fc protein was secreted in the supernatant successfully. The biological activity of H-scfv-Fc was detected by ELISA. The Figure.1 (B) shows the binding affinity to Raji cells of H-scfv-Fc and Rituximab was no difference. So it suggested the H-scfv-Fc could bind to the Raji cells and recognized the CD20 antigen.

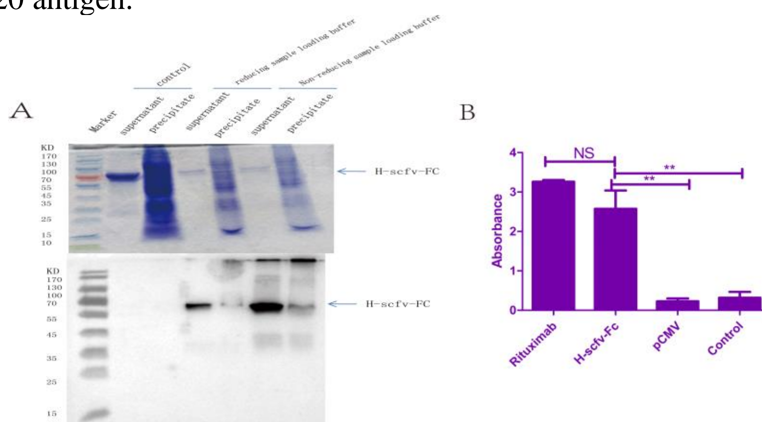


Figure.1 The expression of H-scfv-Fc and biological activity detection (A) up panel: The supernatant and lysate of the HEK293T cells which were transiently transduced transduce with pCMV-H-scfv-Fc plasmid separated by SDS-PAGE under non-reducing or reducing; down panel: Immunoblot analysis of H-scfv-Fc expression. Immunoblot analysis was performed with a HRP-conjugated anti-human IgG Fc antibody and chemiluminescent detection. (B) The biological activity detection for H-scfv-Fc was measured by ELISA. There was no difference between positive

control Rituximab and H-scfv-Fc. The OD between H-scfv-Fc and negative control had a difference ($p<0.01$)

3.2 Construction of hCARCD20 plasmid and the lentivirus production

The figure 2(A) shows sequence encoding the the humanized anti-CD20scFv, CD8 hinge and transmembrance region, CD8 and CD137 co-stimulation domain and the CD3ζ was inserted into pCDH lentiviral vector with the GFP reporter gene, named hCARCD20. The GFP was observed post-infection. The figure 2(B) shows the expression level of GFP was increased with the time goes on. The hCARCD20 lentiviral supernatant was collect at 48h and 72h. After purifying the hCARCD20 lentiviral particals, we measured the titer of lentivirus. Figure 2(C) revealed the hCARCD20 lentivirus had a high titer because the GFP was expressed when the 5×10^{-6} mL hCARCD20 lentiviral particals was added to the HEK293T cells. The FACS shows the the infection efficiency at different concentration of hCARCD20 lentiviral particals. After calculating, the titer was about 2×10^{8} TU/mL.

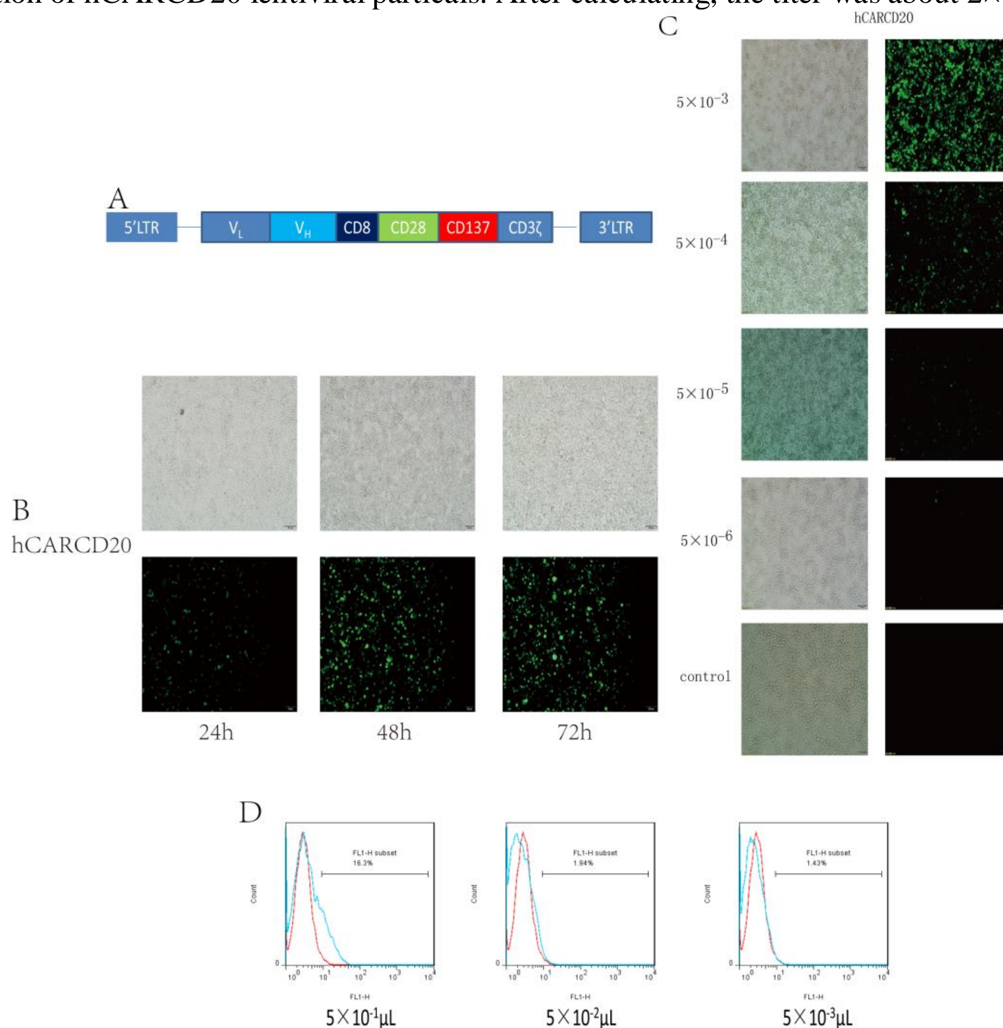


Figure 2 Construction of hCARCD20 plasmid and the lentivirus production. (A) Schematic representation of the hCARCD20 construct. The 5′long terminal repeat (LTR) controls the expression of the chimeric antigen receptor hCARCD20 which consists of IgG kappa chain leader peptide (SP), the CD20-specific single-chain antibody scFv(H-scfv-Fc), a his-tag, the hinge region of human CD8a, the CD28 and CD137 co-stimulation and CD3ζ. The puromycin-resistance gene for selection of transduced cells is driven by the CMV promoter. (B) The HEK293T packaging cells were transduced with hCARCD20 plasmid and lentivirus auxiliary plasmid pMD2.G and pspax. The reporter gene GFP was observed in fluorescence microscope at 24h, 48h, 72h after transduction. (C) The 1×105 HEK293T cells was infected with different concentration. And the the GFP expression was observed in fluorescence microscope 48h after infection. (D) The cells which could express GFP was quantified by FACS.

3.3 Construction of Jurkat-hCARCD20 cells through lentivirus infection

The Jurkat cells were infected with lentivirus which carrying the hCARCD20 sequence and puromycin-resistant gene at MOI=20, and named it as Jurkat-hCARCD20. The Jurkat-hCARCD20 cells were screened with 10μg/mL puromycin. The figure 3(A) shows the GFP reporter gene was successfully expressed at 2days and 7days after puromycin screening. Compared with 2days post-screening, the fluorescence intensity was stronger at 7days. It means the hCARCD20 sequence has steadily expressed in the Jurkat-hCARCD20 cells. The infection efficiency reached 99.5% as shown in figure 3(B) 7days after screening. However, figure 3(C) showed that the level of expression of CAR was very low, although it was the high lentiviral infection efficiency. Maybe the very low level of expression of H-scfv-Fc protein is relative to the level of expression of hCARCD20.

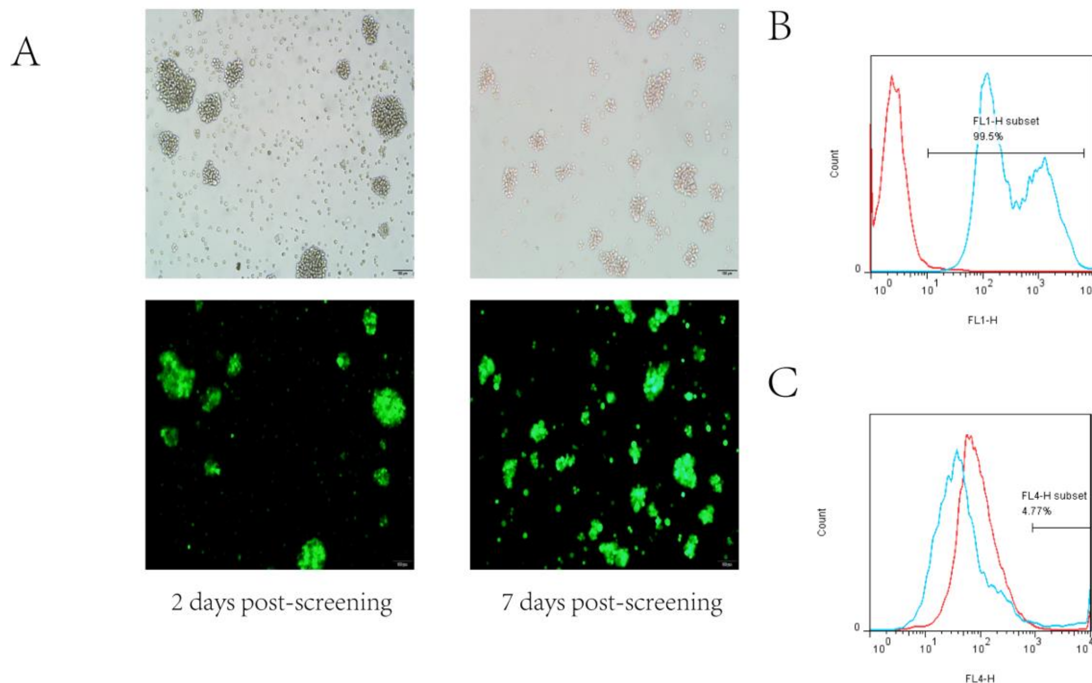


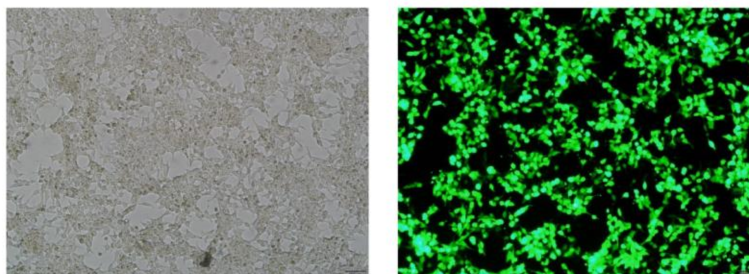
Figure.3 Construction of Jurkat-hCARCD20 cells through lentivirus infection. (A) The 1×106 Jurakt cells was infected with hCARCD20 lentivirus at MOI=20. The reporter gene GFP was observed 2days and 7days after screening with puromycin. (B) The infection efficiency was detected by FACS through measuring the reporter gene GFP. (C).The level of CAR expression was performed with a Alexa Fluor® 647-AffiniPure Fab Fragment Gaot Anti-human IgG (H+L) and FACS detection. And the data was analysed by Flowjo 7.6.

4. Discussion

The CD20 antigen was the target for NHL for CD20 is only expressed in B cells from the period of pre-B cell to late plasmablast[24]. CAR-T cells therapy provides a choose for improving the quality of live to patients. To induce the potential immunogenicity of human-mouse chimeric anti-CD20 mAb, we humanized its scFv and the regard the humanized scFv as the extracellular domain of CAR which recognized the tumor associated antigen, CD20. Even if the low level of expression of the H-scfv-Fc protein, it is biological activity for it can be bound to the Raji cells. The H-scfv-Fc nucleotide sequence which is with the CD8 transmembrance region, CD8 and CD137 co-stimulation domain and CD3ζ chain was incorporated into the lentiviral vector with reporter gene. The lentiviral titer was measured and the titer was about 2×10^{8} TU/mL. The Jurkat cells were infected with the lentivirus and screened with puromycin after infecting. The level of expression of CAR was very low, even if the infection efficiency reached 99.5% 7days after screening with 10μg/mL puromycin. The expression of CAR was measured in HEK293T which were transient transduced with the lentivirus vector carrying hCARCD20 as previously described. However, figure 4.(A,B)the level of expression is low as well. It may suggest that the CAR had a better level of expression in HEK293T than Jurkat cells.

In a whole, the level of expression of CAR depended on the level of expression of scFv and its leader. So, the leader and the expression of H-scfv-Fc would be improved.

A



B

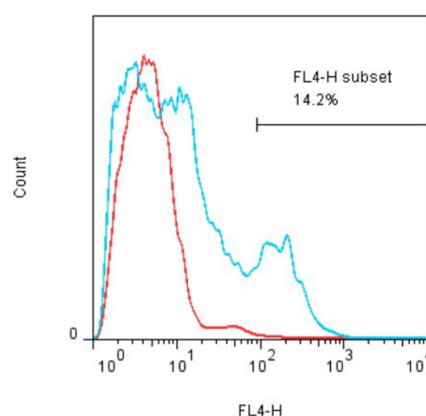


Figure.4 HEK293T were transduced with hCARCD20 plasmid and measured its CAR expression (A) The HEK293T cells were transiently transduced with hCARCD20 plasmid and the reporter gene GFP was observed after 48h. (B) The level of expression of CAR was performed with a Alexa Fluor® 647-AffiniPure Fab Fragment Gaot Anti-human IgG (H+L) and FACS detection in HEK293T which were transduced with hCARCD20 plasmid.

5. Conclusion

Chimeric antigen receptor plays a pivotal role for tumor. CD20 antigen is another target for NHL except CD19 antigen. We successfully humanized the anti-CD20 scFv based on the Rituximab. The humanized the anti-CD20 scFv protein (H-scfv-Fc) was biological activity. Then it was used to the CAR for recognizing the CD20 antigen. The CAR was firstly transduced into the CD4+ Jurkat cell for the preliminary functional verification. The Jurkat got a high infection efficiency but with low level expression of CAR.

Acknowledgements

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