Potential Efficacy of Human IL-1RAP Specific CAR-T cell in Eliminating Leukemic Stem Cells of Chronic Myeloid Leukemia

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Abstract

The purpose of this report was to evaluate the effect of IL1RAP sepecifc CAR-T cell on inducing IL1RAP positive chronic myeloid leukemia cell apoptosis.

Leukemic stem cells (LSCs) as the main initiating factor of leukemia relapse still cannot be eradicated thoroughly. Even though Tyrosine kinase inhibitors (TKI) revolutionized the therapy of chronic myeloid leukemia (CML), it alone does not cure this disease for the no response to quiescent LSCs. Human IL1RAP identified by Landberg N and we can be used as a specific surface marker and tumor burden indicator of LSC in CML. Therefore, IL1RAP chimeric antibody receptor (CAR) T cell specific targeting LSCs might be a novel strategy to CML therapy. Here, we demonstrated that IL1RAP CAR T cell showed more powerful cytotoxicity to kill IL1RAP positive CML cell lines than that to directly block IL1RAP expression by shRNA. Furthermore, compared with shRAN group and blank vector treated group, higher rate of apoptosis and lower proliferation of leukemia cells were showed in IL1RAP CAR T cell co-cultured group. In conclusion, in the present study a potential creative therapeutic target to eliminate LSCs and assistant strategy to cure CML was proved.

Keywords: CML; LSC; IL1RAP; CAR-T

Introduction

Dramatic advances on the molecular pathogenesis of chronic myeloid leukemia (CML) have been deciphered in recent years [1]. Tyrosine kinase inhibitors (TKI) as the BCR-ABL-specific targeted drugs have improved 10-year survival to more than 80% in CML [2]. However, it alone does not cure this disease, because TKI does not kill quiescent leukemia stem cells (LSCs), which thus persist in a majority of patients and may cause disease relapse [3]. In the previous studies, Landberg and we both have proved that IL-1RAP as a measure of LSCs burden is a specific surface maker for distinguishing LSCs and normal HSCs in CML [4,5]. Furthermore, research showed that IL1RAP antibody could kill CML CD34+CD38– progenitor cells through antibody-dependent cell mediated cytotoxicity (ADCC), but not corresponding normal cells [6]. From the above evidence, we believe that a previously unexplored avenue for therapy of CML has been opened up based on IL1RAP. However, given to drawbacks of IL1RAP Ab used in patients including neutralization by soluble IL1RAP and continuously injection, more novel strategies need to be explored. The success of chimeric antigen receptor modified T cells (CAR-T) in leukemia patients provide us encouraging potential therapeutic approaches, demonstrating a unique concept for the possible eradication of CML stem cells [7,8].

Here, IL1RAP CAR-T cells with specificity for human CML LSCs antigen IL1RAP, coupled with CD137 (a costimulatory receptor in T cells) and CD3-zeta (a signal-transduction component of the T-cell antigen receptor) signaling domains were established. These IL1RAP specific CAR T cells were used and their function was evaluated in CML cell lines.

Materials and Methods

Cell lines

293FT cell, KU812, K562 and SUP-B15 leukemia cell lines were stored by Key Laboratory of Bone Marrow Stem Cell, Xuzhou Medical University, Jiangsu Province, China. 293FT cells were cultured in DMEM and other leukemia cells were cultured in RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA). 3H6E10 and 10D8A7 human IL1RAP hybridoma were constructed and stored by Key Laboratory of Bone Marrow Stem Cell, Xuzhou Medical University.

Antibodies and reagents

Complete RPMI 1640 medium, H-DMEM medium and FBS were purchased from GIBCO (USA). Lipofectamine 2000, TRIzol® Plus RNA Purification System and SuperScriptTM First-Strand Synthesis System were supplied by Invitrogen (Grand Island, NY). PE conjugated anti-hIL-1RAcP monoclonal antibody was purchased from R&D Company (USA). OKT3、CD28.2 Annexin V and 7-AAD were brought from eBioscience (USA). The cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Japan). IL1RAP shRNA(31892-1, 31893-2 and 31894-1) were designed and produced by Invitrogen (Shanghai, China).

IL1RAP CAR-T cell design and construction

The VH and VL DNA of IL1RAP single chain antibody were amplified by RACE and overlap extension PCR from total RNA that were extracted from 3H6E10 and 10D8A7 hybridoma and they were ligated into specific IL1RAP single-chain variable fragment(scFv). CD8αtransmembrane domain, CD137 intracellular domain, TCR ζ chain, human CD8αsignal peptide and scFv-anti-IL1RAP were cloned into plasmid LV-lac named as LV-Gene. Vector just carried with CD8α, CD137 and TCR ζ, but without scFv-anti-IL1RAP was used as LV-Control. Recombinant lentivirus was generated by co-transfected recombinant plasmid LV-lac, pMD2.G, and psPAX2 helper vectors into 293FT packing cells.

Isolation of human CD3+ T cell

PBMCs from healthy volunteers were harvested by density centrifugation with Ficoll-Hypaque (GE Healthcare Bio-Sciences). Cells were collected and washed twice in PBS. Then PBMCs were labeled with human CD19-PE (Miltenyi Biotec), CD56-PE (BD Biosciences) and CD33-PE (BD Biosciences) antibodies for 10 min, RT. After wash, cells were isolated by magnetic activated cell sorter (MACS) magnet according to the manufacturer's protocol. Briefly, anti-PE MicroBeads (Miltenyi Biotec) were added and incubated in dark for 15 min. Then cells were put into magnet fitted with a MACS LS column. Unlabeled cells were collected and used as CD3+ T cells.

Cell transfection

According to the manufacturer's protocol, K562 and KU812 cells were treated with three kinds of viral shRNA (31892, 31893 and 31894) for different time periods (24, 48 and 72 hours) following with cell lysis for WB detection. Purified CD3+T cells at a concentration of 1×10^6 cells/mL were cultured with solid-phase coating antibody CD3 and CD28 for 48h. Lentivirus solution with multiplicity of infection (MOI) of 20 was applied with polybrene of final concentration of 8 μg/mL into activated CD3+T cells. After culture for 24 h in incubator and CD3+T cells were transfected again. Then cells were harvested after repeated infection for 48 h.

Flow cytometry

KU812, K562 and SUP-B15 cells were stained with anti-hIL-1RAP-PE antibodies for 15min, RT. Cells were washed, resuspended and detected by flow cytometry (LSRII, BD, USA). Normal CD3+T cells (Black control group, BC group), CAR-T cells with black vector (LV-Control group), CAR-T cells with anti-IL1RAP gene (LV-Gene group) and T cells with shRNA group were incubated with KU812 cells for 24 h respectively. After washing cells were stained with Annexin V for 15min in dark. Then washed again and incubated with 7-AAD antibody for 10min. Cells were acquired on Flow cytometer and data were analyzed with the program Cellquest BD, USA.

Cell proliferation assay

Black control group, LV-Control group, LV-Gene group and shRNA group were incubated with KU812 cells for 24 h respectively. Then CCK-8 solution was added for 4 h before measuring OD value using microplate reader (BIO-RAD680, Bio-Rad Co, USA) at 450 nm wavelength according to the manufacturer's protocol.

Statistical analysis

Data are shown as means ± SD. All statistical analyses were conducted using GraphPad Prism software (version 6.0). Statistical significance between 2 treatment groups was analyzed using unpaired Student's t-tests with 2-tailed P values. Multiple comparisons were performed by one-way ANOVA tests. P<0.05 was considered statistically significant.
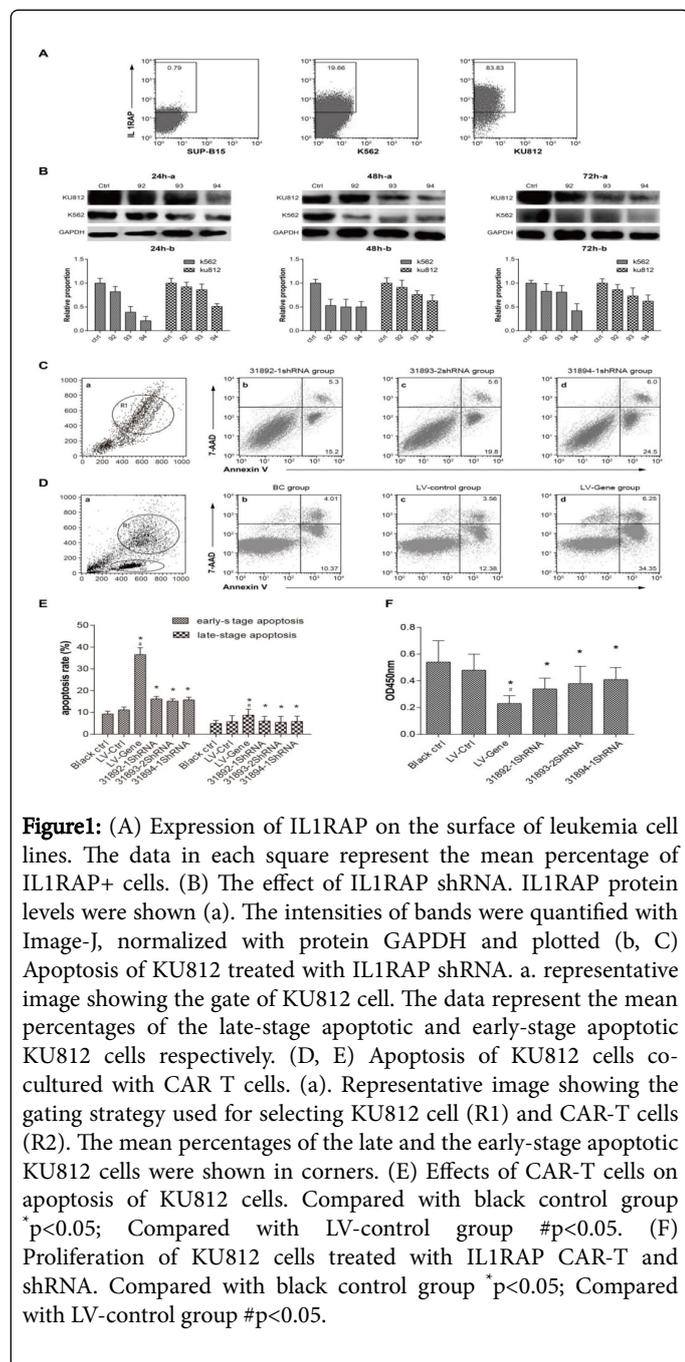
Results

As shown in Figure 1A, we have examined the expression of IL1RAP protein on the surface of leukemia cell lines KU812, K562 and SUP-B15 respectively. The results of flow cytometer detection showed that the expression of IL1RAP protein on the surface of KU812 cells was the highest and the level of IL1RAP in K562 cells was a little lower, whereas SUP-B15 cell was almost IL1RAP negative. Therefore, KU812 and K562 cells were used in the next experiment. Here, shRNA of IL1RAP was designed as a positive control for the effector of IL1RAP specific CAR-T cells. Results in figure B showed that IL1RAP protein was significantly down-regulated both in KU812 and K562 cell lines after infected for 24 h. Following the time passing by, more obviously and lower IL1RAP expression was investigated, especially in shRNA94 group. Then cells were infected with shRNA and co-cultured with IL1RAP specific CAR-T cell respectively. Given the high expression of KU812, it was used as the effector cells in the next targeting-cell killing experiment of CAR-T cell with IL1RAP-specificity. Normal CD3+T cells (Black control group), CAR--T cells (LV-Control group), CAR+-T cells (LV-Gene group) and virus (shRNA group) were cultivated with KU812 cells for 24 h. According to the results of flow cytometry using AnnexinV/7-AAD maker, the percentages of viable apoptotic cells in the LV-Control group (11.16 ± 1.27%) and the BC group (9.26 ± 1.30%) were similar (Figures 1C, 1D and 1E). However, the percentage of viable apoptotic cells in the LV-Gene group (36.56 ± 3.17%) increased significantly compared with that of BC group and LV-control group (*P<0.05, #P<0.05 respectively). Importantly, the apoptosis rate of LV-Gene was obviously higher than that in all of the shRNA groups. The results of the CCK-8 test in Figure F showed that the OD value of the LV-Gene group (0.23 ± 0.06) was significantly lower than that of Black control (BC) group (0.54 ± 0.16) and LV-Control group (0.48 ± 0.12), in which the differences were statistically significant (p<0.05). Also, compared with the shRNA groups (0.34 ± 0.08, 0.38 ± 0.13, and 0.41 ± 0.09) decreased cell proliferation was detected in LV-Gene group. All the above results suggest that CAR+-T cells can express IL1RAP specific fragment that is capable of identifying and binding IL-1RAP protein on the target cell surface and then kill positive cells. Besides, compared with IL-1RAP-shRNA virus infected cells, the suppression ratio and apoptosis rate of IL-1RAP specific CAR-T cells are significantly higher, which means more specific effect of CAR-T cells against IL-1RAP on KU812cells.

Discussion

In the present experiment, the target cell damage effect of IL1RAP CAR-T cell on CML cell lines KU812 with high expression of IL1RAP protein has been proved. Furthermore, collections of CML patient samples are ongoing now to investigate the response of CAR-T cell with IL1RAP specificity on primary LSC. Though specific killing effect of CAR-T cells against KU812 cells has been investigated *in vitro*, the killing mechanism is unknown yet. We could believe that in the present study IL1RAP specific induced TCR signal combined with CD137 secondary signal were involved. However, during the process of

IL1RAP specific CAR-T mediated target cell apoptosis cytokine secretion has not been detected. Besides, as we known, T memory cells play very important roles in immunological surveillance on newborn or relapsed tumor cells [9]. Thus, further research is also needed to examine the effect and survival of IL1RAP specific CAR-T cells *in vivo*.

Figure1: (A) Expression of IL1RAP on the surface of leukemia cell lines. The data in each square represent the mean percentage of IL1RAP+ cells. (B) The effect of IL1RAP shRNA. IL1RAP protein levels were shown (a). The intensities of bands were quantified with Image-J, normalized with protein GAPDH and plotted (b, C) Apoptosis of KU812 treated with IL1RAP shRNA. a. representative image showing the gate of KU812 cell. The data represent the mean percentages of the late-stage apoptotic and early-stage apoptotic KU812 cells respectively. (D, E) Apoptosis of KU812 cells co-cultured with CAR T cells. (a). Representative image showing the gating strategy used for selecting KU812 cell (R1) and CAR-T cells (R2). The mean percentages of the late and the early-stage apoptotic KU812 cells were shown in corners. (E) Effects of CAR-T cells on apoptosis of KU812 cells. Compared with black control group *p<0.05; Compared with LV-control group #p<0.05. (F) Proliferation of KU812 cells treated with IL1RAP CAR-T and shRNA. Compared with black control group *p<0.05; Compared with LV-control group #p<0.05.

Based on the limitation of TKI which is unable to kill the most primitive and quiescent leukemic stem cells, current research and attractive targets that have emerged with potential for eradicating CML are exploring. Several new targets have recently been investigated as potential modulators in myeloid leukemia pathogenesis, including the multiple gene regulators miRNAs, the apparently leukemia-specific cell surface marker IL1RAP and CD49f, transcription factors such as BMI1 and FOXOs, the tumor suppressors PML and PP2A, and the tyrosine kinase JAK2 [10]. It's undeniable that CAR-T cell targeting IL1RAP is an important innovative strategy in effectively eliminating LSCs in CML. In addition, it is also an important alternative therapeutic way for patients with primary TKI resistance.

Acknowledgements

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