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Effect and changes in PD-1 expression of CD19 CAR-T cells from T cells highly expressing PD-1 combined with reduced-dose PD-1 inhibitor

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Abstract. CD19 chimeric antigen receptor (CAR) T cell therapy has changed the outcomes of relapsed/refractory B-cell leukemia and lymphoma. However, its efficacy in patients with relapsed/refractory non-Hodgkin lymphoma (NHL) has been less impressive compared with that in patients with acute lymphoid leukemia. Furthermore, immune checkpoints have a critical role in the immune system. Several clinical trials have confirmed the dramatic effects of programmed death-1/programmed death-ligand 1 (PD-1/PD-L1) inhibitors in numerous malignancies, but the immune-associated adverse events of PD-1/PD-L1 inhibitors may occur in a number of systems. The aim of the present study was to investigate the combination of CD19 CAR-T cells with a reduced dose of PD-1 inhibitor. This method is expected to overcome the side-effects of PD-1 inhibitors, while maintaining therapeutic efficacy. The findings demonstrated that a reduced dose of PD-1 inhibitor did not affect the transfection rate, proliferation rate or cytokine secretion of CD19 CAR-T cells. An interesting finding of the present study was that the number of PD-1-positive cells CAR-T cells, measured by flow cytometry, declined when they were cultured *in vitro*, but returned to high levels with gradual prolongation of the co-culture time of CD19 CAR-T cells with lymphoma cells; however, there was no change in the mRNA expression of T cells and CAR-T cells during this process. This phenomenon may be one of the reasons why the curative effect of CAR-T cells on B-cell lymphoma is unsatisfactory compared with B-cell leukemia. The synergistic effect of a reduced-dose PD-1 inhibitor combined with CD19 CAR-T cells from T cells highly expressing PD-1 was confirmed in a mouse trial. Mice in the combined treatment group achieved the longest survival time. In this group, the proportion of CAR-T cells and the level of interleukin-6 were higher compared with those in the CAR-T cell group. In conclusion, a reduced dose of a PD-1 inhibitor combined with CD19 CAR-T cells appears to be a promising treatment option for relapsed/refractory B-NHL exhibiting high PD-1 expression by T cells. This method may achieve good clinical efficacy while reducing the side-effects of PD-1 inhibitors.

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Key words: chimeric antigen receptor, programmed death-1 blockade, programmed death-1 inhibitor, immunotherapy, lymphoma

Introduction

Chimeric antigen receptors (CARs) are synthetic receptors that retarget T cells to tumor cell surface antigens in order to eliminate the targeted tumor cells (1). CD19 CAR-T cell therapy has led to encouraging responses in patients with relapsed/refractory B-lineage hematological malignancies, including leukemia, lymphoma and myeloma (2-5). However, despite the marked clinical response in various types of B-cell neoplasms, the efficacy of CAR-T cell therapy against relapsed/refractory lymphoma has not yet been established (6,7). There are several challenges to overcome in the therapy of B-cell non-Hodgkin lymphoma (B-NHL) by CD19 CAR-T cells. Challenges of the CAR-T cell therapy for relapsed/refractory lymphoma include selection of target antigens, management of toxicity and modulation of the tumor microenvironment (8,9).

Blocking tumor immune evasion by targeting the immune checkpoints has become a research focus in the treatment of relapsed or refractory tumors (10). Programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) are the most important immune checkpoints identified to date, and have introduced a modern era of cancer immunotherapy (11). A significant correlation between the level of PD-1 (expressed on T cells)/PD-L1 (expressed on tumor cells) and the immunosuppression of T cells has been reported (12). Several clinical trials have confirmed the effects and clinical application value of PD-1/PD-L1 inhibitors (13-15). The PD-1 expression on T cells or PD-L1 expression on tumor cells may compromise the efficacy of CD19 CAR-T cell therapy. Therefore, treatment with CD19 CAR-T cells in combination with PD-1 inhibitors may overcome the immunosuppression of the PD-1/PD-L1 axis and improve the therapeutic effect on relapsed/refractory B-NHL. In a previously reported case, a PD-1 inhibitor was administered to a patient with refractory diffuse large B-cell lymphoma (DLBCL) and progressive disease on day 26 after therapy with CD19 CAR-T cells (16). The patient

exhibited a clinically significant response, an expansion of CD19 CAR-T cells and decreased expression of PD-1.

It was hypothesized that CD19 CAR-T cells from patient T cells with high PD-1 expression in combination with a PD-1 inhibitor may overcome the immunosuppression induced by the PD-1 pathway. The aim of the present study was to improve the therapeutic efficacy in relapsed or refractory B-lineage hematological malignancies, particularly lymphoma.

Materials and methods

Primary cells, cell lines and PD-1 inhibitor. Informed consent was provided by 7 patients with lymphoma and healthy donor agreed to participate in this experiment within a clinical trial at the Department of Hematology at Tianjin First Central Hospital (Tianjin, China) with autologous CAR-T 19 cells (ChiCTR-ONN-16009862; Tianjin First Central Hospital Medical Ethics Committee). All animal procedures were approved by the institutional animal and care use committee of Tianjin First Central Hospital. Human T cells with high PD-1 expression were derived from the peripheral blood of seven patients with lymphoma (males:females, 3:4; age: 25-68 years old). PD-1 normal expression of human T cells was isolated from the peripheral blood of seven healthy donors (males:females, 1:6; age, 22-45 years old). Raji lymphoma cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10-20% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin/streptomycin, 2 mmol/l L-glutamine, and 1 mmol/l sodium pyruvate at 37˚C in a humidified incubator with a 4% CO_2 atmosphere. The PD-1 inhibitor used was OPDIVO (nivolumab).

Isolation of peripheral blood mononuclear cells (PBMCs) and transduction of T cells. Ethical approval and informed consent were obtained. Patients with lymphoma and healthy donors agreed to participate this experiment as part of a clinical trial at the Department of Hematology at Tianjin First Central Hospital with autologous CAR-T 19 cells (ChiCTR-ONN-16009862). PBMCs of 7 patients and healthy donors were isolated from buffy coat by Ficoll density gradient centrifugation (500 x g for 10 min at room temperature). $CD3^+$ T cells were selected by MACS using CD3 microbeads (Miltenyi Biotec, Inc., Cambridge, MA, USA) from the PBMCs. Then, $CD3^+$ T cells were stimulated with anti-CD3/anti-CD28 mAb-coated Human T-Expander beads (cat. no. 11141D; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and cultured in T-cell medium X-Vivo 15 (Lonza Group, Ltd., Basel, Switzerland) supplemented with 250 IU/ml interleukin-2 (IL-2; Proleukin®; Novartis International AG, Basel, Switzerland) at 37˚C in a humidified incubator with a 4% CO_2. The manufacturer's instructions were followed and performed as described by Kochenderfer *et al* (5). At 4 days after isolation and culture, T cells ($3x10^6$) were transduced with a lentiviral vector encoding CD19 CAR constructs (5 μg; lenti-EF1a-CD19-2rd-CAR; Creative Biolabs, Inc., Shirley, NY, USA) and cultured in media containing recombinant human IL-2 (30 U/ml). After 12 days in culture, T cells were analyzed by flow cytometry to detect the CD19 CAR-T expression.

Flow cytometry. Flow cytometric analysis of PBMCs was performed using $2\text{-}5x10^6$ total cells/condition. To quantify transgene expression in transduced T cells, cells were isolated and stained with antibodies (1:200) for 15 min at room temperature. CD19 expression on lymphoma cells was determined using anti-CD19-phycoerythrin (PE; 1:200; cat. no. 560992; Beckman Coulter, Inc., Brea, CA, USA). CD3 expression on lymphoma cells was analyzed using anti-CD3-allophycocyanin (1:200; cat. no. 561800; Beckman Coulter, Inc.). The expression of PD-1 on $CD3^+$ T lymphoma cells was analyzed using anti-CD297-fluorescein isothiocyanate (1:200; cat. no. 558694; Miltenyi Biotec, Inc.). To assess phenotypes of CAR-T cells, data were analyzed based on gated CAR^+/GFP^+ cells using BD AccuriC6 software (BD Biosciences, San Jose, CA, USA).

CAR-19 T cell proliferation in vitro. The proliferation of CD19 CAR-T cells was detected using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). CCK-8 was added to the medium and cultured simultaneously. The absorbance at 450 nm was determined as the blank control. Absorbance was detected using an enzyme standard meter at 450 nm at 0, 24 and 48 h.

Cytotoxicity of Raji lymphoma cells in vitro. Each group of CD19 CAR-T cells ($4x10^5$) combined with a PD-1 inhibitor (36 μg/ml; Bristol-Myers Squibb, New York, NY, USA) were co-cultured with Raji lymphoma cells ($1x10^5$; or not co-cultured) at a 4:1 ratio for 48 h in the absence of supplemented cytokines. CD19 CAR-T cells were isolated from the co-culture by MACS using CD3 microbeads (as described above). Cytotoxicity was detected using a lactate dehydrogenase (LDH) cytotoxicity test kit (Dojindo Molecular Technologies, Inc.) at 490 nm at 0, 24 and 48 h.

Cytokine release assays. Cytokine release assays to detect tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) were detected using ELISA kits (cat. nos. 555268 and 550612; BD Biosciences). The absorbance detection value was detected at 450 nm at 0, 12, 24 and 48 h. The level of IL-6 (cat. no. 200-06; Wuhan Merck Biotechnology Co., Ltd.) in the serum was detected by electrochemiluminescence analysis.

PD-1 mRNA expression of T cells or CAR-T cells. The expression of PD-1 mRNA in T cells or CAR-T cells was detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. At 24 or 48 h after treatment/co-culture, total RNA extracted from the cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used as the template for all RT reactions. The cDNA was synthesized with random priming from 10 μl total RNA with the aid of the Revert Aid™ First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. RT-qPCR was performed to characterize the mRNA levels of specific genes using Fast SYBR-Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) in a Biosystems StepOne Real-Time PCR machine (Applied Biosystem; Thermo Fisher Scientific, Inc.). Expression level of PD-1, normalized to GAPDH and relative to a calibrator, was expressed as $2^{-\Delta\Delta Cq}$ (fold difference). The primers used in

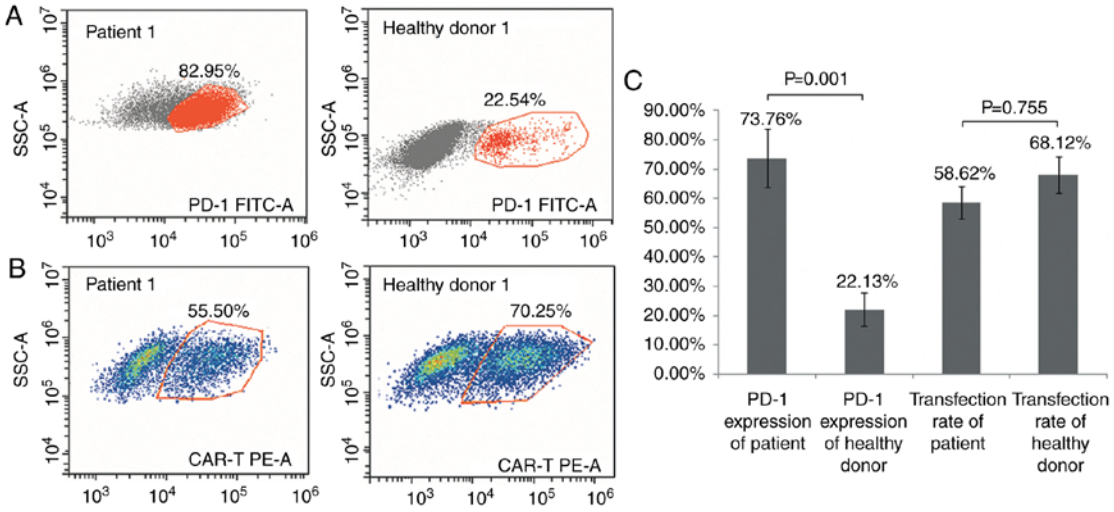

Figure 1. (A) The highest expression of PD-1 of the lymphoma patients was 82.95%; meanwhile the expression of healthy donor 1 was 22.54%. (B) The transduction efficiency of the lymphoma patient and healthy donor cells with CD19 CAR constructs. (C) The average expression (percentage of positive cells) of PD-1 of the lymphoma patients was 73.76±9.89%; meanwhile the average expression of the healthy donors was 22.13±5.74% (P=0.001). The average transfection efficiency of the lymphoma patients was 58.62±5.58%; meanwhile the healthy donors was 68.12±6.26% (P=0.755). PD-1, programmed death-1; CAR, chimeric antigen receptor.

for qPCR experiments were as follows: PD-1, forward 5'AGA CGGAGTATGCCACCATT3' and reverse 5'CACTGTGGG CATTGAGACAT3'; GAPDH, forward 5'-ATTCAACGGCAC AGTCAAGG-3' and reverse 5'-GCAGAAGGGGCGGAG ATGA-3'.

Mouse tumor models. In a lymphoma animal model, 6-8 week old female CAnN.Cg-Foxn1nu/CrlVR (BALB/c) mice weighing 20±1.8 g (n=24; Beijing Vitonlihua Experimental Animal Technology Co., Ltd., Beijing, China) were housed a rat facility with light and dark cycle (10 h light and 14 h darkness each day) and access to food and water. The room temperature was 26-28˚C. They were injected with $1x10^7$ Raji lymphoma cells transduced with luciferase (purchased from Shanghai Suer Biotechnology Co.) through the tail vein. Mice were monitored for established tumors with bioluminescent imaging using a multifunctional *in vivo* imaging system. After 25 days, mice were randomized and treated tail vein injection as follows: PD-1 high expression T cells from lymphoma patient ($5x10^6$); transduced CD19 CAR-T cells ($5x10^6$) from lymphoma patient (high expression PD-1); PD-1 high expression T cells ($5x10^6$) with PD-1 inhibitor (3 mg/kg) or CD19 CAR-T cells ($5x10^6$) from lymphoma patient (high expression PD-1) with PD-1 inhibitor (1.5 mg/kg). At 14, 21, 28 and 35 days, mice were monitored with bioluminescent imaging for disease progression following intraperitoneal injection with luciferin (150 mg/kg). The peripheral blood was taken from the tail vein of mice to analyze. The proportion of CD19 expression on lymphoma cells and CD19 CAR-T cells in mice were analyzed by flow cytometry.

Statistical analysis. SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) statistical software was used for statistical analysis. Data are expressed as the mean ± standard error and analyzed by one-way ANOVA, with Student-Newman-Keuls method used for pairwise comparison. P<0.05 was considered to indicate a statistically significant difference.

Results

Transduction efficiency of CD19 CAR-T cells from T cells with high PD-1 expression. The titer of CD19 CAR cell virus was $3x10^8$ TU/ml. The mean expression of PD-1 on T cells from the seven patients with lymphoma by flow cytometry was 73.76±9.89% and the highest expression of PD-1 was 82.95% prior to transduction. The mean transduction efficiency of T cells with high PD-1 expression in the lymphoma patients was 58.62±5.58%. In addition, the mean expression of PD-1 on the T cells of the seven healthy donors was 22.13±5.74%, and the mean CD19 CAR transduction efficiency was 68.12±6.26% (P=0.001; Fig. 1).

Effect of different doses of PD-1 inhibitor on the cytotoxicity of CD19 CAR-T cells. CD19 CAR-T cells produced from T cells with high PD-1 expression were combined with72, 36 and 18 μg/ml PD-1 inhibitor and cultured *in vitro*. In addition, transduced CD19 CAR-T cells from the healthy donors, CD19 CAR-T cells from T cells with high PD-1 expression without PD-1 inhibitor, and the T cells with high PD-1 expression combined with PD-1 inhibitor, were used as the control groups. An LDH assay was used to determine cytotoxicity. The cytotoxicity of CD19 CAR-T cells produced from T cells with high PD-1 expression was lower than PD-1 inhibitor treated CAR-T cells (high PD-1) and healthy donor CAR-T cells at 24 and 48 h after co-culture with Raji lymphoma cells. At 24 h after co-culture with Raji lymphoma cells, there was no difference between the cytotoxicity (LDH activity) of CD19 CAR-T cells from T cells with high PD-1 expression combined with different doses of PD-1 inhibitor, and those from the healthy donor CAR-T cells. However, at 48 h after co-culture, the cytotoxicity of CD19 CAR-T cells from T cells with high PD-1 expression combined with 18 μg/ml PD-1 inhibitor was lower than all other groups of combined PD-1 inhibitor treatment and healthy donor CD19 CAR-T cells. The cytotoxicity of CD19 CAR-T cells from T cells with high PD-1 expression combined

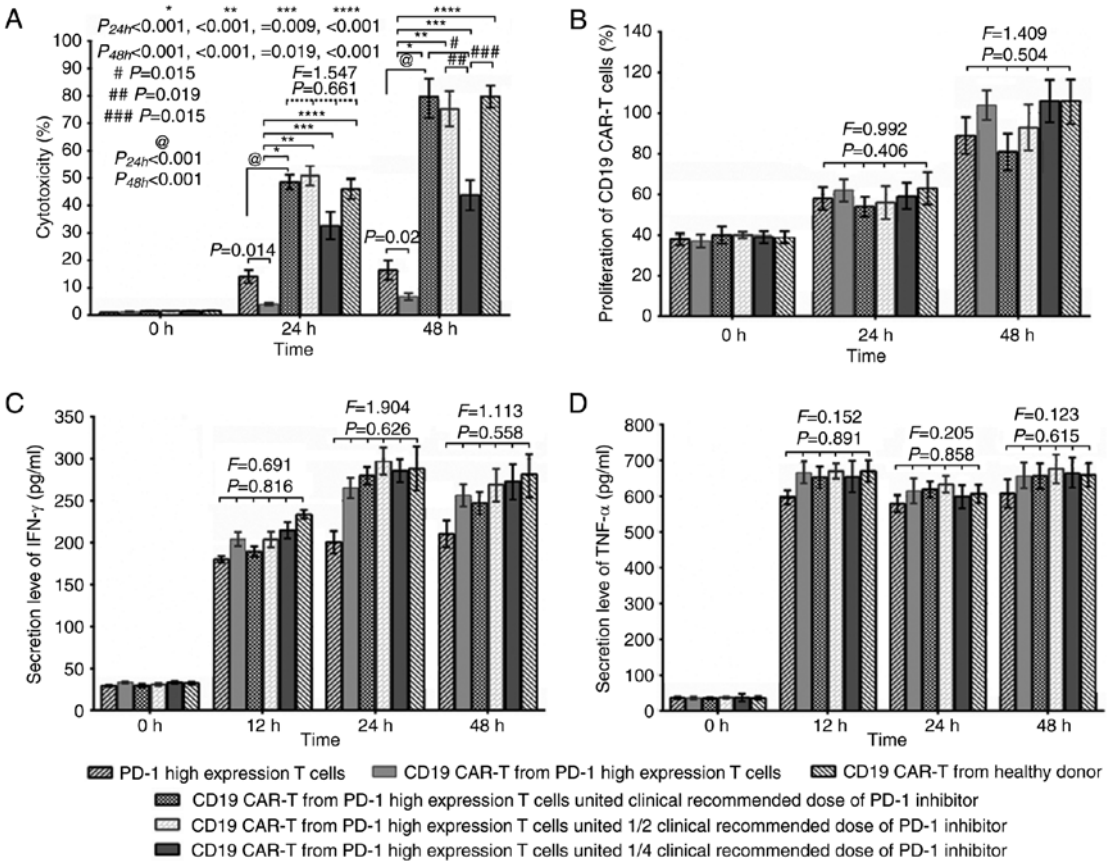

Figure 2. (A) The cytotoxicity (LDH assay) of T cells with high PD-1 expression transduced with CD19 CAR was lower transduced cells from healthy donors and high PD-1 expression transduced cells treated with PD-1 inhibitor at 24 and 48 h after co-culture with Raji lymphoma cells ($P_{24\,h}$ <0.001, <0.001, =0.009, <0.001; $P_{48\,h}$ <0.001, <0.001, =0.019, <0.001). At 24 h after co-culture with Raji lymphoma cells, there were no differences in the cytotoxicity (LDH assay) of CD19 CAR-T cells from T cells with high PD-1 expression combined with different doses of PD-1 inhibitor and those from healthy donors (P=0.661). However, at 48 h after co-culture with Raji lymphoma cells, the cytotoxicity (LDH assay) of CD19 CAR-T cells from T cells with high PD-1 expression combined with 18 μg/ml of PD-1 inhibitor was lower compared with the other high PD-1 combined treatment T cells (PD-1 inhibitor and CD19 CAR transduction) and the CD19 CAR transduced healthy donor T cell group (P=0.015, 0.019 and 0.015, respectively). (B-D) There were no differences in (B) cell viability of CAR-T cells (isolated using MACS CD3 Microbeads), or the (C) IFN-γ and (D) TNF-α secretion level among all groups at each time point. CAR, chimeric antigen receptor; PD-1, programmed death-1; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α.

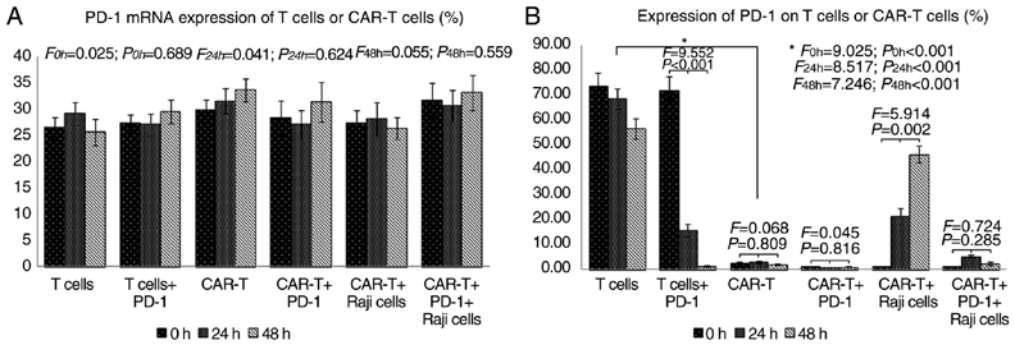

Figure 3. (A) PD-1 mRNA expression in different groups and at different time points. The control is healthy donor cells. (B) After *in vitro* culture, the PD-1 expression on CD19 CAR-T cells (high PD-1) declined significantly compared with from non-transduced T cells (high PD-1) by flow cytometry ($P_{0\,h}$<0.000; $P_{24\,h}$<0.001; $P_{48\,h}$<0.001. PD-1 inhibitor also could reduced the expression of PD-1 on T cells inhibitor (P<0.001). Co-culture with Raji lymphoma cells increased the expression of PD-1 on CD19 CAR-T cells with the extension of exposure time to tumor cells (P=0.002). Expression of PD-1 on CD19 CAR-T cells co-cultured with tumor cells did not increase when PD-1 inhibitor was present (P=0.285). PD-1, programmed death-1; CAR, chimeric antigen receptor.

with different doses of PD-1 inhibitor was higher compared with that of T cells with high PD-1 expression (Fig. 2A).

Effect of different doses of PD-1 inhibitor on the proliferation of CD19 CAR-T cells. CD19 CAR-T cells from T cells with high PD-1 expression co-cultured with Raji lymphoma cells were treated with 72, 36 and 18 μg/ml PD-1 inhibitor, and cultured *in vitro*. CD19 CAR-T cells were isolated from the co-culture by MACS using CD3 microbeads. There were no differences in the proliferation of all groups at 24 and 48 h of culture (Fig. 2B).

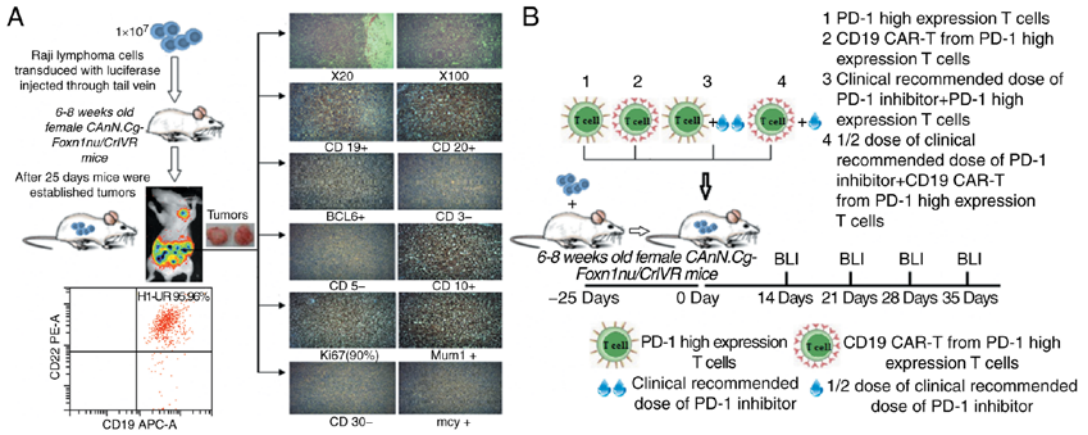

Figure 4. (A) Lymphoma establishment was confirmed in mice. (B) The mice were randomly allocated to four groups: 1) Patient T cells with high PD-1 expression; 2) transduced CAR-T cells generated from patient T cells with high PD-1 expression; 3) patient T cells with high PD-1 expression combined with the clinical recommended dose of PD-1 inhibitor (72 μg/ml); and 4) transduced CAR-T cells generated from patient T cells with high PD-1 expression combined with 1/2 the clinical recommended dose of PD-1 inhibitor (36 μg/ml). PD-1, programmed death-1; CAR, chimeric antigen receptor.

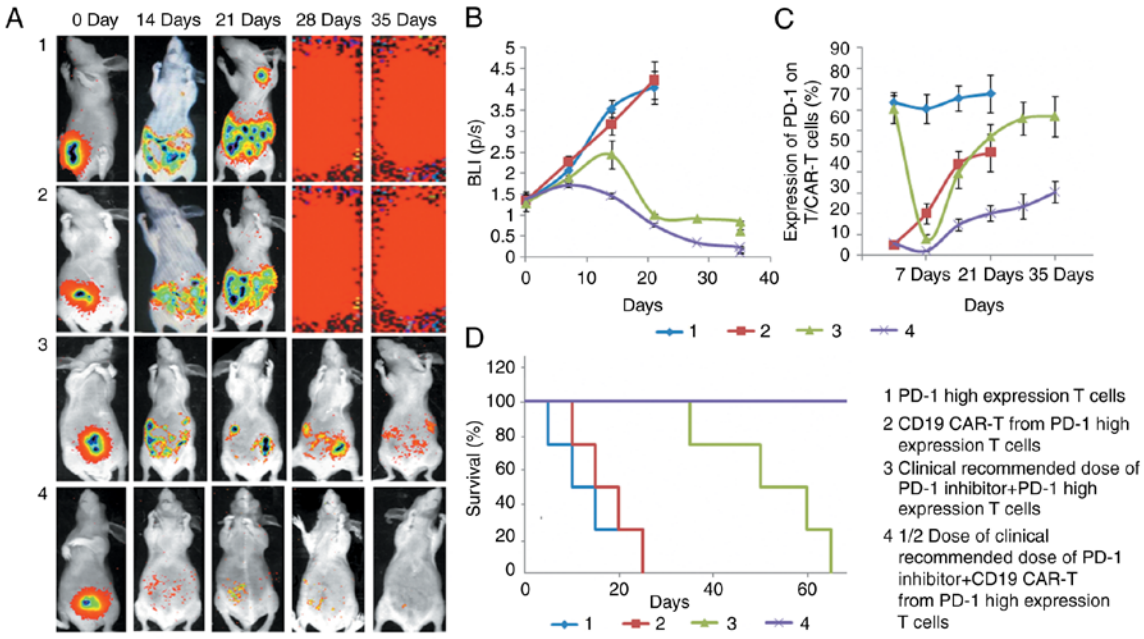

Figure 5. (A) *In vivo* imaging of lymphoma progression in experimental mice. (B) BLI in each group of mice. (C) Expression of PD-1 on CAR-T cells in each group of mice by flow cytometry. (D) Survival of mice in each group. BLI, bioluminescence imaging. PD-1, programmed death-1; CAR, chimeric antigen receptor.

Effect of different doses of PD-1 inhibitor on inflammatory factor secretion by CD19 CAR-T cells. CD19 CAR-T cells from T cells with high PD-1 expression co-cultured with Raji lymphoma cells were treated with 72, 36 and 18 μg/ml PD-1 inhibitor and cultured *in vitro*. There were no differences in the IFN-γ and TNF-α secretion level among the groups at 12, 24 and 48 h of culture (Fig. 2C and D).

PD-1 expression and PD-1 mRNA expression of T cells or CAR-T cells. T cells and mature CAR-T cells were used to investigate the effect of PD-1 inhibitor on the PD-1 mRNA expression and PD-1 expression of T cells or CAR-T cells. Following culture *in vitro* for 48 h, data from T cells with high PD-1 expression with or without PD-1 inhibitor, CD19 CAR-T cells from patient T cells with high PD-1 expression with or without PD-1 inhibitor, and Raji co-culture of CD19 CAR-T cells from patient T cells with high PD-1 expression and tumor cells with or without PD-1 inhibitor. There were no differences in the PD-1 mRNA expression among different groups and at different time points (Fig. 3A). The number of CD19 CAR-T cells expressing PD-1 declined significantly after *in vitro* culture, but it increased with the prolonged exposure time to Raji cancer cells (Fig. 3B).

Effect of CD19 CAR-T cells combined with different doses of PD-1 inhibitor in mice. After 25 days, tumor establishment in mice was confirmed following intravenous injection of Raji lymphoma cells (Fig. 4). Having demonstrated that PD-1 inhibitor can reverse the low cytotoxicity of CD19 CAR-T cells from patient T cells highly expressing PD-1 *in vitro*, the

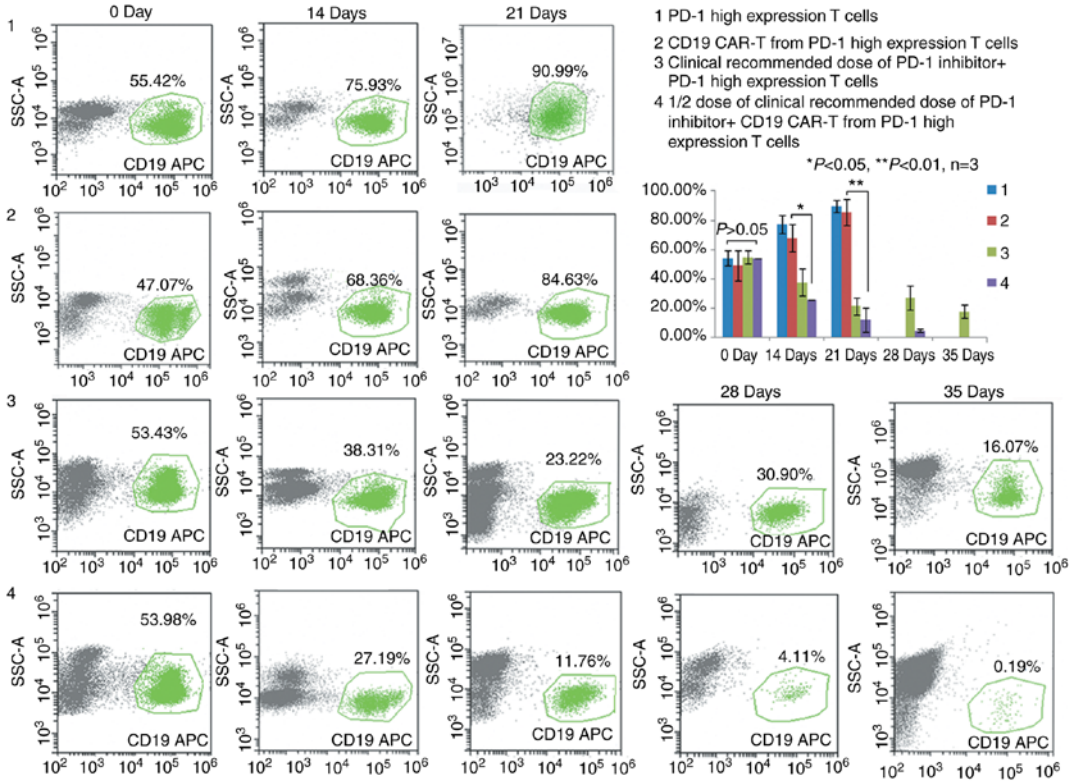

Figure 6. Percentage of lymphoma cells in the each group determined by flow cytometry (CD19-positive). APC, allophycocyanin; PD-1, programmed death-1; CAR, chimeric antigen receptor.

synergistic effect of CAR-T cells and PD-1 inhibitor was investigated *in vivo*. To lower the incidence of side-effects of the PD-1 inhibitor in mice, a reduced dose of PD-1 inhibitor was applied in combination with CAR-T cells. The mice were then randomly allocated to receive patient T cells with high PD-1 expression, CAR-T cells from patient T cells with high PD-1 expression, patient T cells with high PD-1 expression combined withPD-1 inhibitor, and CAR-T cells from patient T cells with high PD-1 expression combined with 1/2 the clinical recommended dose of PD-1 inhibitor (indicated as 1, 2, 3 and 4, respectively, in Fig. 4).

Mice treated with CAR-T cells from patient T cells with high PD-1 expression combined with 1/2 the clinical recommended dose of PD-1 inhibitor exhibited the fastest lymphoma regression and the longest survival (Fig. 5). High tumor load was observed in the lymphoma cells in the four groups of mice on day 0. Lymphoma cells were reduced in the PD-1 inhibitor group and the CAR-T cells combined with PD-1 inhibitor group. At 35 days after the injection of CD19 CAR-T cells, the number of lymphoma cells in the CAR-T cells combined with PD-1 inhibitor group was very low (Fig. 6).

Mice treated with CAR-T cells combined with PD-1 inhibitor exhibited the highest ratio of CAR-T cells 7 days after injection of CD19 CAR-T cells. PD-1 inhibitor promoted the proliferation of CAR-T cells in mice. In addition, the IL-6 level was the highest at 4 days after injection of CD19 CAR-T cells in the combination therapy group (Fig. 7). However, no such results were observed in the CAR-T cells without PD-1 inhibitor group. The body weight of mice in group 1 and 2 decreased by 3-6 g before death, but the weight loss of mice was not significant in group 3 and 4 (data not shown).

Discussion

CAR-T cells can improve remission rates with a favorable outcome for relapsed/refractory B-cell acute lymphoblastic leukemia (ALL) (4,5,17,18) and other B-lineage hematological malignancies (6,19). A meta-analysis investigated the effect of CD19 CAR-T cells in 119 patients with refractory B-cell malignancies from 14 phase I clinical trials (20). The overall response rate to CD19 CAR-T cells was 73%. ALL patients had higher response rates, but chronic lymphocytic leukemia and B-NHL patients had comparatively lower response rates. CD19 CAR-T-cell therapy for B-lineage hematological malignancies is associated with several problems, including the insufficient activity of CAR-T cells (21), as repeated antigen exposure may result in CAR-T cell exhaustion. The resolution of this problem requires novel strategies to enhance CAR-T cell function and persistence (22).

The immunosuppressive pathways are promoted by the combination of tumor cells expressing PD-L1 and T cells expressing PD-1, preventing T cells from entering the tumor area or inducing T-cell apoptosis, thereby blocking the effect of immunotherapy mediated by T cells (4,23,24). The marked upregulation of PD-1 expression on CAR-T cells results in a reduction of the anti-tumor immune response and failure of CAR-T cell therapy (25,26). These results were confirmed by our experiments *in vivo* and *in vitro*. Checkpoint inhibitors specifically blocking these immunosuppressive pathways may improve the immune function of T cells. Thus, the identification of PD-1 expression on T cells and PD-L1 expression on tumor cells revolutionized cancer immunotherapy (10). The clinical use of these antibodies is rapidly expanding as

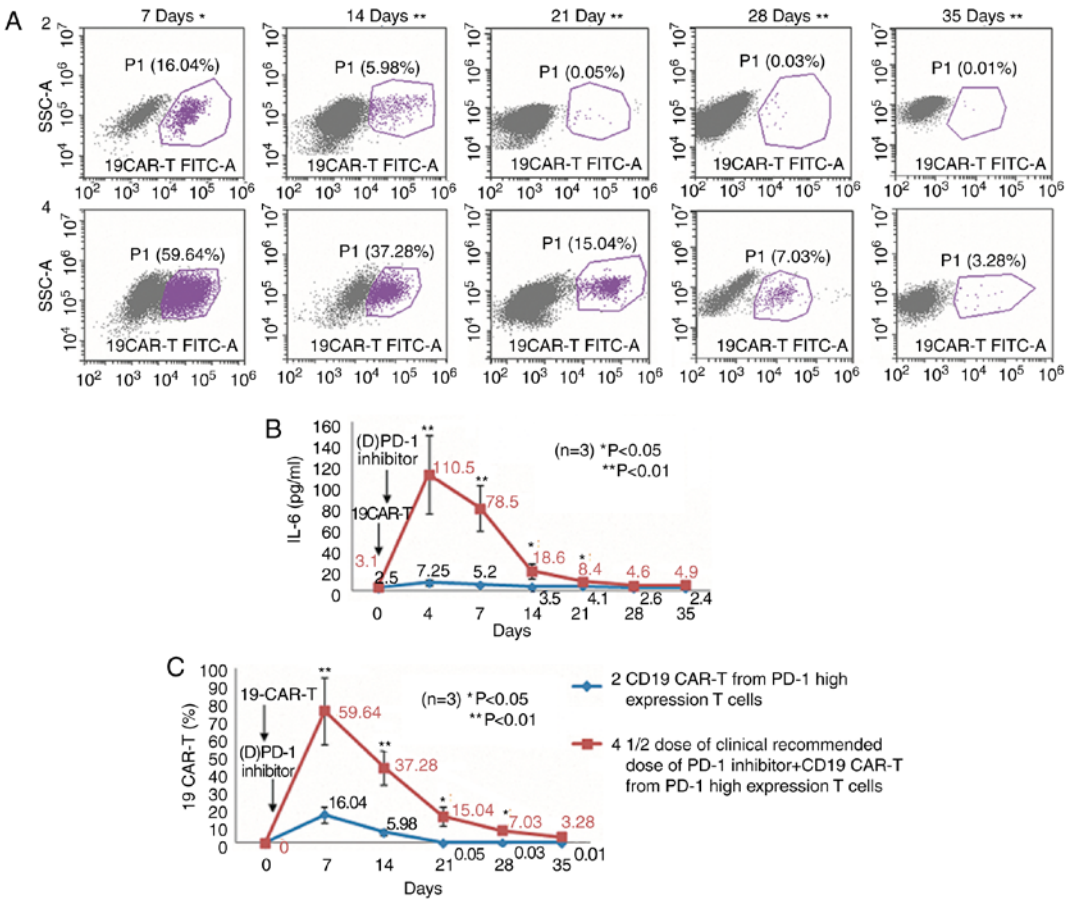

Figure 7. (A) The proportion of CAR-T cells in mice that received transduced CAR-T cells generated from patient T cells with high PD-1 expression (group 2) and mice that received transduced CAR-T cells generated from patient T cells with high PD-1 expression combined with 1/2 the clinical recommended dose of PD-1 inhibitor (group 4). *P<0.05; **P<0.01, n=3. (B) The level of IL-6 in serum throughout the experiment in both groups. *P<0.05; **P<0.01, n=3. (C) The percentage of CAR-T cells throughout the experiment in both groups 2 and 4 of mice. *P<0.05; **P<0.01, n=3. CAR, chimeric antigen receptor. FITC, fluorescein isothiocyanate; PD-1, programmed death-1.

an approach to cancer therapy (11,27). Although the effect of PD-1 inhibitor and PD-L1 inhibitor was notable in the lymphoma cells, persistence was limited and the majority of the patients cannot obtain a long-term benefit (28).

It was hypothesized that the problem of exhausted immune function of CAR-T cells may be overcome by combination with a PD-1 inhibitor. Previous studies on combination therapy with immune checkpoint inhibitors focused on solid tumors (29) and reports on hematological malignancies demonstrated that PD-1 inhibitors enhanced CAR-T cell activity (16). A previously reported refractory DLBCL case achieved a clinically significant response from a PD-1 inhibitor administered on day 26 after therapy failure with CD19 CAR-T cells (16). However, if the CAR-T cells are exhausted, the PD-1 inhibitor will not exert an optimal synergistic effect. Seeking to improve therapeutic efficacy, we devised the combination of CD19 CAR-T cells with PD-1 inhibitor synchronously.

PD-1 inhibitor therapy is associated with several possibly immune-related adverse events occurring in the nervous, respiratory, hematological, circulatory and musculoskeletal systems (30,31). The mechanism underlying PD-1 inhibitor-induced toxicity is unclear. Certain mechanisms of immune checkpoint inhibitor-derived toxicity have been identified (32,33), but further studies are required to elucidate the molecular mechanisms of T cell-mediated side-effects. Although these T cell-driven drug reactions are rare, they are potentially fatal.

The side-effects of CAR-T cell therapy, such as cytokine release syndrome, were also considered. It was hypothesized that CD19 CAR-T cell therapy in combination with a PD-1 inhibitor at a reduced dose may lower the risk of side-effects and improve therapeutic efficacy. In the present study, patient T cells with high PD-1 expression and CD19 CAR-T cells derived from T cells with high PD-1 expression exerted almost no effect on lymphoma cells. This phenomenon was replicated in the lymphoma animal model. Therefore, CD19 CAR-T cells from patients with high PD-1 expression were treated with different doses of PD-1 inhibitor [clinically recommended dose (72 μg/ml), and one-half (36 μg/ml) and one-quarter (18 μg/ml) of the clinical recommended dose) in order to improve the activity of the devitalized CD19 CAR-T cells. The analysis *in vitro* demonstrated, as expected, that 1/2 the clinical recommended dose of PD-1 inhibitor was able to increase the anti-tumor activity of CD19 CAR-T cells from patients with high PD-1 expression to the level of healthy donor CD19 CAR-T cells; however, the lower dose (1/4 clinical recommended dose) did not improve the activity of CD19 CAR-T cells.

The results were then verified in a lymphoma animal model, in which the combination of CD19 CAR-T cells from patients

with high PD-1 expression and 1/2 the clinical recommended dose of PD-1 inhibitor exerted satisfactory effects. The mice that received combination therapy with inhibitor had a longer survival (>35 days) compared with the CD19 CAR-T cells from patients with high PD-1 expression group. Of note, in the combination group, there was a peak of the IL-6 level and CD19 CAR-T cell proportion following injection of the PD-1 inhibitor. However, this phenomenon was not observed in the CD19 CAR-T cells from patients with high PD-1 expression group. We intend to further investigate the side-effects of this combined therapy in mice in future studies, including the body weight changes, blood cell counts, transaminase levels and LDH levels of mice. Moreover, histopathological changes of mice after this combined therapy would also bee assessed.

The present study also investigated whether the PD-1 inhibitor affected the PD-1 mRNA expression in T cells or CAR-T cells. No difference in the PD-1 mRNA expression was observed among different T-cell or CAR-T-cell groups at different time points. However, the number of CAR-T cells expressing PD-1 was markedly reduced after *in vitro* culture. This may be associated with cell proliferation in *in vitro* culture. However, at low effector-target ratio, the PD-1 expression in CAR-T cells returned to high levels with gradual prolongation of the co-culture time. In the present study, the CD19 CAR-T cells from patients with high PD-1 expression combined with 1/2 the clinical recommended dose of PD-1 inhibitor prevented the increase in the expression of PD-1 in CAR-T cells when they were exposed to tumor cells. These results may contribute to the application of CD19 CAR-T cells for the treatment of relapsed or refractory B-cell lymphoma.

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Availability of data and materials

The datasets and certain material used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

Concept and design, QD; performed the experiments, RZ; acquisition of data, YYJ and HBZ; analysis and interpretation of data, JW and MFZ; writing, review and/or revision of manuscript, RZ. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Ethical approval and informed consent were obtained. Patients with lymphoma and healthy donors agreed to participate this experiment as part of a clinical trial at the Department of Hematology at Tianjin First Central (Tianjin, China) hospital with autologous CAR-T 19 cells (ChiCTR-ONN-16009862). All animal procedures were approved by the institutional animal and care use committee of Tianjin First Central Hospital (Tianjin, China).

Patient consent for publication

All patients agreed to publication of this paper.

Competing interests

The authors declare that they have no competing interests.

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