Coexpression of IL7 and CCL21 Increases Efficacy of CAR-T Cells in Solid Tumors without Requiring Preconditioned Lymphodepletion

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ABSTRACT

Purpose: T-cell recruitment, survival, and proliferation are the important limitations to chimeric antigen receptor (CAR) T cells therapy in the treatment of solid tumors. In this study, we engineered CAR-T cells to coexpress cytokines IL7 and CCL21 (7 × 21 CAR-T), a cytokine combination in order to improve proliferation and chemotaxis of CAR-T cells.

Experimental Design: CLDN18.2-specific second-generation CAR-T cells coexpressing cytokines were prepared using retroviral vector transduction. The proliferation and migration of genetically engineered CAR-T cells were evaluated in vitro. The antitumor activities of genetically engineered CAR-T cells were evaluated against multiple solid tumors in C57BL/6 mice in vivo.

Results: In vitro, the proliferation and chemotaxis of 7 × 21 CAR-T cells are significantly improved when compared with those of the conventional CAR-T cells. In vivo, 7 × 21 CAR-T cells revealed superior therapeutic effects to either conventional CAR-T cells or 7 × 19 CAR-T cells which coexpress IL7 and CCL19 as previously reported in three different solid tumors without cyclophosphamide precondition. Interestingly, 7 × 21 CAR-T cells could also suppress the tumor growth with heterogeneous antigen expression and even induce tumor complete remission. Mechanistically, IL7 and CCL21 significantly improved survival and infiltration of CAR-T cells and dendritic cells in tumor. In addition, CCL21 also inhibited the tumor angiogenesis as proved by IHC.

Conclusions: Coexpression of IL7 and CCL21 could boost CAR-T cells’ antitumor activity, and 7 × 21 CAR-T cells may be served as a promising therapy strategy for solid tumors.

Introduction

The successful antitumor activities of second-generation (conventional) chimeric antigen receptor (CAR) T cells have been proved for the treatment of B-cell leukemia and lymphoma (1). However, limited success has been made in using CAR-T cells to treat solid tumors. The hypoxic and extracellular matrix–rich tumor microenvironment prevents T cells from infiltrating the tumor tissue. Furthermore, inhibitory surface proteins, cytokines, or soluble products of disrupted cell metabolism within the tumor can impair the activation and persistence of T cells (2). Recently, in order to improve the infiltration, accumulation, and survival of CAR-T cells in solid tumors, CAR-T cells engineered to express IL7 and CCL19 (7 × 19 CAR) have been developed to increase their antitumor activities through enhancing the CAR-T cell survival and T/dendritic cell (DC) infiltration (3). The combination showed remarkable efficacy in cancer immunotherapy, but whether other combinations of the cytokines could be equally or more effective remains to be explored.

Chemokines provide specific signaling to leukocytes for extravasation from the blood and direct locomotion as well as microenvironmental homing of leukocytes within tissues (4). In addition to CCL19, CCL21 is another homeostatic chemokine ligand for chemokine (C-C motif) receptor 7 (CCR7), expressed constitutively in endothelial cells of high endothelial venules and in stromal cells within the T-cell zone of secondary lymphoid organs and strongly attract naïve T cells and antigen-presentation cells (APCs), such as DC (5, 6). Structurally, CCL21 differs from CCL19 in that it has an extra 32 amino acid C-terminus of basic amino acids that may mediate the distinct binding of CCL21 to other molecules (7, 8). CCL21 can also bind to the receptor CXCR3 that possesses the angiostatic activity to inhibit tumor growth independent of leukocyte recruitment (9, 10).

Previous studies have shown that CCL21/CCR7 plays a central role in coordinating the meeting between mature DCs and naïve T cells to initiate a pathogen or tumor antigen–specific T-cell–mediated immune response (9, 11, 12). It was recently shown that the presence of CCL21 in tumor induces infiltration of DC and CD8+ T cell and leads to immune-mediated inhibition of melanoma (12), lung (11), and colon (13) carcinomas in experimental animals. The chemotactic properties of CCL21 were also used in the development of the DC-based vaccines (14). Treatment of tumor-bearing mice with these genetically altered DCs resulted in tumor growth inhibition and remission in some of the experimental tumors (12, 15).

In the previous study, the cyclophosphamide (CPA) preconditioned chemotherapy was applied to potently increase the 7 × 19 CAR-T cells’ function in vivo owing to differences in the susceptibility of tumors to CAR-T cells and/or in the tumor growth rate. However, some patients are intolerant of lymphodepletion chemotherapy, and this study had also shown that the potent antitumor ability of 7 × 19 CAR-T cells was...
IL7 and CCL21 Enhance Antitumor Efficacy of CAR-T Cells

**Translational Relevance**

There is limited success in using chimeric antigen receptor (CAR) T cells to treat solid tumors. One of the major reasons is the suppressive tumor microenvironment that impairs the infiltration, expansion, and persistence of CAR-T cells in tumor tissues. In this study, we engineered CLDN18.2-specific CAR-T cells to coexpress cytokines IL7 and CCL21 (7 × 21 CAR-T). Our studies indicated that IL7 and CCL21 can enhance the survival of CAR-T cells and infiltration of T cells and dendritic cells in tumor tissues. Interestingly, 7 × 21 CAR-T cells can efficiently destroy different solid tumors with or without preconditioned lymphodepletion chemotherapy and suppress the tumor growth with heterogeneous antigen expression in vitro and in vivo. These findings suggest that 7 × 21 could boost CAR-T cells antitumor activity and may be served as a potential therapy strategy for solid tumors.

**Materials and Methods**

**Cell lines**

PANC02 mouse pancreatic carcinoma cell and E0771 mouse breast cancer cell were conserved by our laboratory. Hepa1-6 mouse hepatocellular carcinoma cell and 293T cell were purchased from the Chinese Academy of Sciences (Shanghai, China). These three cancer cell lines have C57BL/6 genetic background and were lentivirally transduced to stably express murine CLDN18.2 using pWPT-CLDN18.2 lentiviral vectors (designated as PAN02-A2, E0771-A2, and Hepa1-6-A2). 293T, PAN02, and Hepa1-6 were cultivated in DMEM medium ( Gibco) supplemented with 10% FBS (GIBCO). E0771 were cultivated in RPMI 1640 supplemented with 10% FBS (GIBCO).

**CARs construction**

The second generation of the CLDN18.2 CAR is comprised of the anti-CLDN18.2 scFv (hu8E5-2I; ref. 22) linked in-frame to the hinge and transmembrane regions of the murine CD8α chain and intracellular murine 4-1BB and CD3ζ signaling domains, which was then cloned into the EcoRI/Sall sites of the retroviral vector MSCV-IRE5-GFP for expression. To generate CARs expressing murine cytokine IL7 and CCL21, 2A peptide sequences (P2A and P2A) were intercalated among the second-generation CAR genes.

**Retrovirus production and generation of CAR-T cells**

293T cells were transfected with the CAR-expressing plasmid together with the retroviral packaging plasmid Pol-Eco (Addgene) by using polyethylenimine. Retroviral supernatant was collected 2 days later and filtered through a 0.45-μm syringe (Millipore). Murine T cells were stimulated with anti-mouse CD3/CD28 magnetic beads (STEM-CELL Technologies) for 24 hours and then infected with retrovirus in RetroNectin (Takara)-coated plates. Infected cells were incubated overnight at 37°C and 5% CO2.

**Cytokine release assays**

Mouse CAR-T cells were stimulated with different tumor cell lines at an effector-to-target ratio of 1:1 for 24 hours. The supernatant was harvested to test the secretion of cytokines. The concentrations of IL7, CCL21, CCL19, IFNγ, granzyme B, TNFα, and IL2 in the culture supernatants were measured by ELISA kits (R&D Systems; Multi Sciences Biotech).

**In vitro cytotoxicity assays**

To study the cytotoxicity of CAR-T cells, different target tumor cells were cocultured with CAR-T cells at effector-to-target ratio of 3:1, 1:1, and 1:3. The cells were mixed in 96-well plates and analyzed in triplicate. After 18 hours of coculture, the specific cytotoxicity of CAR-T cells was monitored by the lactate dehydrogenase release in the supernatants using the CytoTox 96 Nonradioactive Cytotoxicity Kit (Promega).

**Cell migration assay**

Chemotaxis of the responder T cells or DCs was measured by migration through a polycarbonate filter of 5-μm pore size in 24-well transwell chambers (Corning). CAR-T cells were stimulated with PAN02-A2 cells, and the coculture supernatant was collected. Then the supernatant was placed in the lower chambers, and the responder cells were incubated in the upper chambers. After 2, 4, or 6 hours, the cells migrated from the upper chamber to the lower chamber were counted by blood counting chamber.
In vivo antitumor activities

All animal studies were performed in accordance with the Experimental Animal Care Commission of Shanghai Cancer Institute and housed under specific pathogen-free conditions at the Shanghai Cancer Institute Experimental Animal Center (Shanghai, China). In the PANC02-A2 pancreatic cancer model, C57BL/6 mice were inoculated subcutaneously with 2 × 10^6 tumor cells on the right flank on day 0, and in lymphodepletion condition, CPA (100 mg/kg) was administered i.p. 1 day before 2.5 × 10^6 anti-CLDN18.2 CAR-T cells' injection i.v. In the experiments of tumor rechallenging, mice with tumor complete regression after 7 × 21 CAR-T cell treatment were inoculated s.c. with PAN02-A2 cells at the left flanks of the mice. In the E0771-A2 breast cancer model, 1 × 10^6 tumor cells were inoculated in situ in C57BL/6 mammary gland on day 0, and then on day 15, 2.5 × 10^6 anti-CLDN18.2 CAR-T cells were injected i.v. into the mice. In the model of Hepa1-6-A2 tumor cells, C57BL/6 mice were inoculated subcutaneously with 1 × 10^7 tumor cells on the right flank on day 0, and then on day 7, 1 × 10^6 anti-CLDN18.2 CAR-T cells were injected i.v. into the mice. In the model using mixture of Hepa1-6 and Hepa1-6-A2 cells, C57BL/6 mice were inoculated subcutaneously with 5 × 10^6 Hepa1-6 and 5 × 10^6 Hepa1-6-A2 tumor cells on the right flank on day 0, and then on day 7, 1.5 × 10^6 anti-CLDN18.2 CAR-T cells were injected i.v. into the mice. In all experiments, tumor growth was measured by calipers twice a week, and tumor volumes were calculated on the basis of volume = length × (width)^2/2 × 0.5.

IHC analysis

Tumor tissues and organs were resected from mice. One part was fixed with formalin and embedded in paraffin. The other part was immersed in Tissue-Tek OCT compound and snap-frozen to produce cryosections. The organs were directly stained with hematoxylin and eosin. The tumor tissue sections were used for IHC. Primary antibodies used for IHC staining were as following: anti-mouse CD8α antibody (Cell Signaling Technology), anti-CD4 antibody (Cell Signaling Technology), anti-DEC205 antibody (Biolegend), anti-CD31 antibody (Abcam). Horseradish peroxidase–conjugated goat anti-rabbit was used as secondary antibody. The results were visualized using a ChemMate Envision Detection Kit (Dako/Cytomation).

Statistical analysis

All data were analyzed using GraphPad Prism software (versions 5 and 8). An unpaired two-tailed Student t test was used to determine statistical significance for two-sample comparisons in vitro experiments and one-way ANOVA and Tukey test were performed to assess differences between groups in vitro. Tumor growth data were analyzed with two-way ANOVA. Survival curves were analyzed by using a log-rank test. All data were represented as the mean ± SEM. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 were considered statistically significant.

Results

Generation of CLDN18.2-specific CAR-T cells expressing IL7 and/or CCL21

The murine second-generation CAR (mBBZ CAR) composed of the CLDN18.2-specific scFv (hu8E5-2I) fused with murine 4-1BB and CD3ζ intracellular signaling domains (Fig. 1A) was constructed. Then the murine IL7 and/or CCL21b in tandem with the mBBZ CAR (7 CAR, 21 CAR, and 7 × 21 CAR) were developed. A tandem construct encoding CAR, IL7, and CCL19 (7 × 19 CAR) was also generated as described before (ref. 3; Fig. 1A). Murine T cells were retrovirally transduced to generate CAR-T cells, and the transduction efficiency of CAR in different CAR-T cells was almost equivalent, but the mean fluorescence intensity of mBBZ CAR expression was higher than other CARs (Fig. 1B). The basic phenotype (CD4/CD8) of 7 × 21 CAR-T cells has no difference with conventional mBBZ and 7 × 19 CAR-T cells (Fig. 1C).

In vitro cytokine production and cytotoxicity of 7 × 21 CAR-T cells

The murine CLDN18.2 expression of different target cells was assayed by FACS (Supplementary Fig. S1B). We next investigated the cytokine production and cytotoxicity of CAR-T cells in vitro. As shown in Fig. 1D, 7 × 21 CAR-T cells could produce IL7 and CCL21 spontaneously in the absence of antigen stimulation. However, both cytokines’ secretion was increased when stimulated by CLDN18.2-positive PAN02-A2 cells. The cytokotoxicity assay illuminated that all CAR-T cells could specifically lyse CLDN18.2-positive target cells with equivalent efficiency in vitro (Fig. 2A). The secretion of cytokine IFNγ, granzyme B, and TNFα had no significant difference among different CAR-T cells when cocultured with CLDN18.2-positive tumor cells. For IL2 production, no difference was observed when different CAR-T cells cocultured with PAN02-A2 cells but greater amounts of IL2 were produced by mBBZ CAR-T cells than those of 7 × 21 and 7 × 19 CAR-T cells when cocultured with E0771-A2 and Hepa1-6-A2 cells (Fig. 2B).

The immune function of IL7 on CAR-T cells

IL7 is a cytokine essential for survival of naïve and memory T cell in the periphery (23–25). Therefore, we investigated the proliferation and apoptosis of different CAR-T cells. Following 2-day withdrawal of IL2, we found that the absolute number and divisions of CAR-T cells expressing IL7 were higher than mBBZ CAR-T cells (7 CAR vs. mBBZ CAR: P < 0.001; 7 × 21 CAR vs. mBBZ CAR: P < 0.001; Fig. 2C and D). And CAR-T cells expressing IL7 also showed a lower apoptosis level compared with mBBZ CAR-T cells (7 CAR vs. mBBZ CAR: P < 0.05; 7 × 21 CAR vs. mBBZ CAR: P < 0.05; Fig. 2E).

Immune checkpoint molecules play an important role in cancer immunotherapy (26), and previous reports have suggested that IL7 signaling could prevent T-cell exhaustion (27). Our results showed that expression of immune checkpoint molecules, including PD-1 and LAG-3 on CAR-T cells expressing IL7, was lower than those on mBBZ CAR-T cells at baseline and after stimulation with target tumor cells. Although the level of TIM-3 on different CAR-T cells had no significant difference, CAR-T cells expressing IL7 also had a trend toward lower TIM-3 expression than mBBZ CAR-T cells (Supplementary Fig. S2A). IL7 also contributes to regulate homeostasis of CD8+ T cells and has the important role in proliferation of memory T cells (28). We also investigated the memory phenotype of CAR-T cells in response to antigen stimulation. The results indicated that more CD8+ T cells had CD44+/CD62L- central memory phenotype and TCF-1 expression when coexpressing with IL7 (Supplementary Fig. S2B).

The chemotactic capacity of CAR-T cells improved by expressing CCL21

To evaluate the chemotactic function of CCL21, transwell migration assays were performed. DCs were isolated from mouse bone marrow (Supplementary Fig. S3A), and the mature DCs were identified by FACS (Supplementary Fig. S3B). Firstly, we engineered CAR-T cells expressing CCL19 (19 CAR) as control (Supplementary Fig. S4A and S4B), and the tumor-lytic activity of 19 CAR-T cells was also accessed (Supplementary Fig. S4C). T cells and DCs expressing CCR7 (Fig. 2F).
act as target cells in the upper chamber in transwell migration assay (Fig. 2G). The results indicated that supernatant from CAR-T cells with secretion of CCL21 or CCL19 could attract more T cells (21 CAR vs. mBBZ CAR: \( P < 0.05 \); 7/C21 CAR vs. mBBZ CAR: \( P < 0.05 \); 7/C19 CAR vs. mBBZ CAR: \( P < 0.05 \); 19 CAR vs. mBBZ CAR: \( P < 0.05 \)) or DCs (21 CAR vs. mBBZ CAR: \( P < 0.001 \); 7/C21 CAR vs. mBBZ CAR: \( P < 0.001 \); 7/C19 CAR vs. mBBZ CAR: \( P < 0.001 \); 19 CAR vs. mBBZ CAR: \( P < 0.001 \)) to the lower chambers than mBBZ CAR-T cells, and no significant difference was observed between the chemotactic capacity of CCL21 and CCL19 in vitro (Fig. 2H).

Regression of pancreatic cancer cell xenografts by treatment with 7 × 21 CAR-T cells under condition of lymphodepletion

To investigate antitumor effects of different CAR-T cells with lymphodepleting chemotherapy in vivo, the mouse PANC02-A2 tumor models were established (Fig. 3A). As shown in Fig. 3B, tumors in mice treated with 7 × 21 CAR-T or 7 × 19 CAR-T cells grew more slowly than those in mice treated with mBBZ CAR-T cells (7 × 21 CAR vs. mBBZ CAR: \( P < 0.001 \); 7 × 19 CAR vs. mBBZ CAR: \( P < 0.05 \) or DCs (21 CAR vs. mBBZ CAR: \( P < 0.001 \); 7 × 21 CAR vs. mBBZ CAR: \( P < 0.001 \); 7 × 19 CAR vs. mBBZ CAR: \( P < 0.001 \); 19 CAR vs. mBBZ CAR: \( P < 0.001 \)) to the lower chambers than mBBZ CAR-T cells, and no significant difference was observed between the chemotactic capacity of CCL21 and CCL19 in vitro (Fig. 2H).

Antitumor effects of 7 × 21 CAR-T cells in three solid tumor models without preconditional lymphodepletion chemotherapy

Previous studies have shown a combined therapy with CPA and immune T cells could inhibit tumor growth in mice (29, 30), and a few clinical trials use the similar chemoimmunotherapeutic strategies to treat patients with cancer (31, 32). However, considering that some patients may be intolerant to traditional lymphodepletion chemotherapy and DC cells function may be affected by CPA treatment, we then tested the antitumor ability of 7 × 21 CAR-T cells against PANC02-A2 tumor xenografts without CPA pretreatment (Fig. 3E). As shown in Fig. 3F and G, administration of 7 × 21 CAR-T cells had an obvious inhibitory effect on tumor growth with a growth inhibition rate of 75.7% (Fig. 3H), whereas mBBZ CAR-T and 7 × 19 CAR-T cells showed only a limited growth suppression effect (7 × 21 CAR vs. mBBZ CAR: \( P < 0.001 \); 7 × 19 CAR vs. mBBZ CAR: \( P < 0.001 \)). Furthermore, the antitumor activities of anti-CLDN18.2 CAR-T were also tested in mice bearing established E0771-A2 or Hepa1-6-A2 xenografts.
tumor xenografts. In the E0771-A2 xenograft model (Fig. 3I), both $7/C2_{19}$ and $7/C2_{21}$ CAR-T cells induced tumor regression when compared with mBBZ CAR-T cell group ($7/C2_{21}$ CAR vs. mBBZ CAR: $P < 0.001$; $7/C2_{19}$ CAR vs. mBBZ CAR: $P < 0.001$; Fig. 3J). However, $7/C2_{21}$ CAR-T cells had a significantly better tumor-suppression effect than $7/C2_{19}$ CAR-T cells ($P < 0.05$) according to the tumor volume and weight (Fig. 3J and K). And the tumor growth inhibition of $7 \times 21$ CAR-T cell treatment was up to 98.8% in E0771-A2 tumor model (Fig. 3L). Surprisingly, in Hepa1-6-A2 tumor model (Fig. 3M), $7 \times 21$ but not $7 \times 19$ CAR-T cells had better antitumor activities than mBBZ CAR-T cells ($P < 0.05$; Fig. 3N and O). Note that $7 \times 21$ also had better antitumor activities than $7 \times 19$ CAR-T cells in this tumor model ($P < 0.01$; Fig. 3O).

Figure 2.
In vitro immune function of CAR-T cells. A, CAR-T cells were coincubated with the parental and CLDN18.2-overexpressed target cells at varying effector:target (E:T) ratios for 18 hours. Cell lysis was tested using a standard nonradioactive cytotoxicity assay. B, CAR-T cells were cocultured with different target cells at a 1:1 E:T ratio; after 24 hours, the production of IFNγ, granzyme B, TNFα, and IL-2 by T cells was determined by ELISA. C, Proliferation capacity of CAR-T cells. A total of $1 \times 10^7$ CAR-T cells were cultured in 96-well plates without IL-2 for 2 days. The numbers of viable T cells were counted. D, The division of CAR-T cells. Anti-CLDN18.2 CAR-T cells were labeled with celltrace violet and cultured in 24-well plates without IL-2 for 2 days. The dilution of celltrace violet was analyzed by flow cytometry. E, The apoptosis and survival of T cells. Annexin V and PI were used to determine the proportion of apoptotic T cells after a 2-day culture without IL-2. F, The expression levels of CCR7 were analyzed on naïve T cells and mature DCs. G and H, Transwell coculture of naïve T cells or mature DCs with the CAR-T cell culture supernatant (G). Different CAR-T cells were cocultured with Panc02-A2 cells at a 1:1 E:T ratio for 24 hours, and the cell culture supernatants were collected. Then, naïve T cells or mature DCs were added to the upper chamber, and the supernatants were added to the lower chamber. After 2, 4, and 6 hours, T cells or DCs in the lower chamber was counted (H). Each experiment was repeated independently at least 3 times with similar results, and representative data are shown. Assays were performed in triplicate ($n = 3$ biologically independent wells), and data represent mean ± SEM. Significance of findings was defined as follows: ns, not significant; $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. 


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0.01), and the tumor growth inhibition of 7 × 21 CAR-T cell treatment was 86.7% (Fig. 3P). These results suggested that in absence of preconditional lymphodepletion treatment, 7 × 21 CAR-T cells had better therapeutic potential than both mBBZ and 7 × 19 CAR-T cells in solid tumors.

The infiltration of T cells/DCs increased and the tumor angiogenesis inhibited by the treatment with 7 × 21 CAR-T cells in solid tumors.

To elucidate the mechanisms of enhanced antitumor ability of 7 × 21 CAR-T cells, we next investigated the infiltration of T cell in tumor...
Figure 4.
Treatment of solid tumors with 7 × 21 CAR-T cells increases the infiltration of T cells and DCs and inhibits tumor angiogenesis. A–E, C57BL/6 mice were injected s.c. with PANC02-A2 cells and treated with CAR-T cells on day 10 after tumor inoculation without CPA pretreatment as in Fig. 3E. On day 32, tumor tissues were resected from the mice, and each tissue was divided into three parts. CAR copy number in genomic DNA from one part of tumor tissues was measured by real-time PCR (A). The second part of tumor tissues was fixed with formalin, embedded in paraffin, and used for IHC to detect the infiltration of CD8⁺ T cells and CD4⁺ T cells (B). The last part of tumor tissues was immersed in Tissue-Tek O.C.T. compound and snap-frozen to produce cryosections and used for IHC to detect the infiltration of DCs (B). The images were obtained under original magnifications of ×200. Scale bar, 100 μm. C–E, The histograms show the quantification of T cells and DCs’ infiltration in tumor tissues. F, Tumor tissues were resected from the mice in three tumor models as described in Fig. 3. The sections of formalin-fixed, paraffin-embedded tumor tissue were used for IHC to detect the vascular marker CD31. The images were obtained under original magnifications of ×200. Scale bar, 100 μm. G, The histograms show the number of blood vessels in tumor tissues. In A, C, D, E, and G, data represent mean ± SEM (n = 3). Significance of findings was defined as follows: ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001; and ****P < 0.0001.
tissues of treated mice. Significantly higher CAR copy numbers (Fig. 4A), as well as more CD4 and CD8 T cells could be observed in the tumor tissues in 7 × 21 CAR-T treatment group than those of both mBBZ and 7 × 19 CAR-T treatment groups in PANC02-A2 xenografts without CPA pretreatment (Fig. 4B-D). Similar results were also observed in the E0771-A2 and Hepa1-6-A2 models (Supplementary Fig. S5). In addition, IHC staining of the tumor tissues also revealed that 7 × 21 CAR-T cells induced more DC infiltration in the tumor tissues than those of both mBBZ and 7 × 19 CAR-T treatment groups (Fig. 4B and E). These results indicated that the chemotactic ability of 7 × 21 CAR-T cells was better than 7 × 19 CAR-T cells. In addition, we also found that the number of MDSCs in tumor tissues was reduced in 7 × 21 and 7 × 19 CAR-T cell treatment groups (Supplementary Fig. S6).

Besides CCR7, CCL21 can also bind to the receptor CXCR3 that could inhibit the tumor angiogenesis (9, 10). We found that different tumor cells used in our study expressed CXCR3 by FACS and qPCR could inhibit the tumor angiogenesis (9, 10). We found that different engineered CAR-T cells had different ability of 7 × 21 CAR-T cell could also be enhanced by the angiostatic activity of CCL21.

Development of memory phenotype in T cells by the treatment with 7 × 21 CAR-T cells

Effective induction of tumor-specific memory T cells is essential for the persistent control of tumors in immunotherapy. In vivo, we had found that IL7 expression contributed to increased central memory phenotype of CD4+ T cells (Supplementary Fig. S2B). Then we examined the T-cell phenotypes in the spleen of mice 10 days after CAR-T cells treatment. As shown in Fig. 5A, there were more CD62L−CD44+ central memory T cells in the spleen of mice treated with 7 × 21 and 7 × 19 CAR-T cells than treated with BBZ CAR-T cells in PANC02-A2 mouse model without CPA pretreatment, whereas the percentage of CD8+ T cells had no difference (Fig. 5B and C). And the same results were found in Hepa1-6-A2 tumor model (Supplementary Fig. S8A and S8B). Furthermore, the spleen CD3+ T cells of treated mice were isolated and restimulated with CLDN18.2-negative and -positive tumor cells for 24 hours (Fig. 5D). T cells from 7 × 21 CAR-T cells group produced more IFNγ than mBBZ CAR-T cells group in both of PANC02-A2 and Hepa1-6-A2 tumor models (Fig. 5E; Supplementary Fig. S8C). In addition, 7 × 21 CAR-T cell–treated mice with tumors completely regression under lymphodepletion were rechallenged with PANC02-A2 tumor cells. The results showed long-lasting resistance to PANC02-A2 tumor cell growth (Fig. 5F), indicating immunological memory formation could provide long-term protection from tumor recurrence.

Antitumor effects of 7 × 21 CAR-T cells in heterogeneous solid tumor without preconditional lymphodepletion chemotherapy

Solid tumors have a high degree of heterogeneity. The conventional CAR-T cells only recognize the specific target tumor cells and might lead to tumor escape. Our results indicated CCL21 could recruit more DCs to the tumor tissues, which may increase the ability of the antigen cross-presentation. Because cross-presentation was supposed to be the dominant priming mechanism to activate T cells for antitumor immunity (33, 34), whether the cross-presentation of DCs could activate the T cells inside tumor and kill the antigen negative tumors need to be explored. Because both 7 × 19 and 7 × 21 CAR-T cells have potent antitumor activities in Hepa1-6A2 model, we mixed Hepa1-6 and Hepa1-6-A2 tumor cells at the ratio of 1:1 and established the heterogeneous subcutaneous tumors (Fig. 6A). The CLDN18.2 expression on the mixture tumor cells was about 50% detected by FACS analysis (Fig. 6B), and different engineered CAR-T cells could lyse the mixture tumor cells effectively in vitro (Fig. 6C). As shown in Fig. 6D, 7 × 21 CAR-T cells still had the better antitumor ability, and the tumor in 1 of 6 had already completely disappeared. Besides, 7 × 21 CAR-T cells prolonged mice survival, whereas mBBZ and 7 × 19 CAR-T cells showed no obvious therapeutic efficacy (Fig. 6E).

Systemic toxicities in the mouse models treated with 7 × 21 CAR T cells

The expression of IL7 and CCL21 by CAR-T cells is antigen independent. So, one possible concern would be the induction of systemic toxicity of IL7 or CCL21. As shown in Supplementary Fig. S9A—S9D, in all mouse models used in our study, there was no significant body weight loss in mice treated with 7 × 21 CAR-T cells compared with other groups. Furthermore, no obvious pathologic damage could be observed in the vital organs from the mice treated with 7 × 21 CAR-T cells (Supplementary Fig. S9E). In order to future explore the toxicity of 7 × 21 CAR-T cells, we also analyzed long-term persistence of CAR-T cells in nontumor bearing hosts. As shown in Supplementary Fig. 9F, no more CAR-T cells were detected in blood, spleen, and lymph node from all treated mice at 20 and 50 days after CAR-T cells administration. These results suggested that 7 × 21 CAR-T cells could be safe to treat solid tumors.

Discussion

Antitumor immunotherapy is designed to take advantage of the immune system to reject and destroy cancers. However, immunosuppressive microenvironment has been regarded as the major issue for the limited efficacy of CAR-T therapy on solid tumors. In order to improve the infiltration and survival of CAR-T cells, previous study had engineered CAR-T cells to express IL7 and CCL19 (7 × 19 CAR-T) which are essential for the maintenance of T-cell zones in lymphoid organs. In this study, we exploited a new combination of CCL21 and IL7. CCL21 shares the same receptor CCR7 with CCL19. But the binding of CCL19 and CCR7 can effectively promote the phosphorylation and internalization of CCR7 and desensitize the receptor to CCL19, whereas CCL21 has no such effect (35). CCL21 could induce a strong temporal signal, whereas CCL21 could induce a weaker, but more persistent signal. Intratumoral administration of recombinant CCL21 (11) or DCs overexpressing CCL21 (15, 36) could generate potent antitumor responses, and the clinical study on patient with cancer is undergoing. These differences between CCL19 and CCL21 promoted us to express CCL21 in CAR-T cells.

We firstly investigated and compared in vivo antitumor effects of 7 × 21 and 7 × 19 CAR-T cells in PANC02-A2 tumor model with CPA pretreatment. The results showed that 7 × 21 CAR-T cells had a slighter better antitumor activity than 7 × 19 CAR-T cells, and some mice treated with 7 × 21 CAR-T cells induced complete regression of tumor. CPA pretreatment before CAR-T treatment is not only a chemotherapy for tumors, but also a common tool for lymphodepletion in clinical (31, 32). However, conditioning chemotherapy is associated with many complications, principally susceptibility to infection. And lymphodepletion of the recipient T cells may influence their interaction with DCs inside tumor tissues. Thus, no prior lymphodepletion could more reflect the interaction between CAR-T cells and tumor or immune cells in tumor environment and exclude the side effects of lymphodepletion. We then investigated the ability of
7 \times 21 \text{CAR-T cells in PANC02-A2 tumor model without CPA pretreatment. Intriguingly, we observed that without prior chemotherapy, 7 \times 21 \text{CAR-T cells could potently suppress tumor growth and displayed significantly better antitumor activities in three different solid tumor models than conventional and 7 \times 19 \text{CAR-T cells.}}

The hostile tumor environment induces T-cell anergy and dysfunction (37). IL7 is a potent immune regulatory protein (23) and assists in the development of lymphocytes (24). Recently, IL7 was also shown to be required for survival and proliferation of mature and naive peripheral T cells (38, 39). As expected, our results demonstrated that IL7 could not only enhance the proliferation of CAR-T cells but also inhibit the apoptosis of CAR-T cells. Previous researches had reported that IL7 signaling could prevent T-cell exhaustion (27). In this study, we also found that some exhaustion markers’ level on CAR-T cells expressing IL7 was lower than those of mBBZ CAR-T cells. However, it was noted that the CAR expression level of mBBZ CAR-T cells was

Figure 5.

Treatment of solid tumors with 7 \times 21 \text{CAR-T cells develops memory phenotype in T cells. A–E, C57BL/6 mice were injected s.c. with PANC02-A2 cells and treated with CAR-T cells as described in Fig. 3E. Ten days after CAR T cells treatment, the spleens were harvested. Single-cell suspensions of spleens were analyzed for immune cells by flow cytometry. A, Representative flow cytometric profiles showing the percentage of CD8^+ T cells in splenocytes and the expression of memory T cell markers (CD44 and CD62L) on CD8^+ T cells. The histogram shows the percentage of CD8^+ (B) and Tcm cells (C) in spleen. D, Experimental schema of the spleen CD3^+ T cells isolated and restimulated with tumor cells ex vivo. The spleen CD3^+ T cells from mice was isolated by magnetic beads and then cocultured with negative parental tumor cells or positive tumor cells. After 24 hours, the production of IFN\gamma by T cells was determined by ELISA. E, The histogram shows the secretion of IFN\gamma. F, Inoculation with PANC02-A2, pretreatment with CPA on C57BL/6, and administration of anti-CLDN18.2 CAR-T cells were conducted as described in Fig. 3A. Forty-six days after tumor inoculation, the tumor-rejected mice were rechallenged with PANC02-A2 on the left flanks. As control, naive mice were inoculated with the tumors in the same way (n = 3 mice per group). The volumes of the tumors were assessed. Representative flow cytometric data from one animal of each group are shown and in B, C, and E, and data represent mean \pm SEM (n = 3). Significance of findings was defined as follows: ns, not significant; P > 0.05; \*, P < 0.05; and ***, P < 0.001.
higher than other CAR-T cells. Thus, the level of CAR expression may also influence the exhaustion of CAR-T cells.

Previous studies have been suggested that CCL21 may promote recruitment of APC and T lymphocytes to a tumor mass to facilitate antigen recognition and enhance tumor-specific immune responses through the chemokine receptor CCR7 (40). In consistent with previous findings, we observed that CCL21 could recruit T cells and DCs effectively in vitro, and more T and DCs infiltrated into the tumor tissues with 7C21 CAR-T cell treatment in vivo. In addition to CCR7, mouse CCL21 also binds to the chemokine receptor CXCR3. It had been shown that CCL21 binding to CXCR3 had antitumor effects, and this inhibition was associated with a reduction in tumor vascularity with no change in leukocyte infiltration (10, 41). Tumor angiogenesis is crucial for tumor growth and metastasis, thus blocking or inhibiting tumor angiogenesis could potentiate antitumor activities (42). In this study, we observed that 7C21 CAR-T cell treatment could decrease the numbers of blood vessels in different solid tumor models. Our previous study had demonstrated that sorafenib, an antiangiogenesis inhibitor, could increase the antitumor activities of GPC3-targeted CAR-T cells (43). Thus, the property of angiogenesis inhibition may enhance the antitumor activities of 7C21 CAR-T cells. However, some previous studies reported that tumor vessel number showed a positive correlation with tumor size (44, 45). Thus, considering the marked differences in tumor volumes between the groups at the time of tumor resection, it is hard to determine whether the differences are due to differences in tumor size or due to alterations in vasculature mediated by CCL21. As we know, CXCR3 is highly expressed on activated T cells and mediates chemotaxis in response to its ligands CXCL9, CXCL10, and CXCL11 (46). In this study, we also assessed CXCR3 expression on different CAR-T cells and found that all CAR-T cells expressed similar level of CXCR3 (Supplementary Fig. S10). However, whether CCL21 plays a role in CCL21/CXCR3 signal on chemotaxis is unknown. It needs to do more studies to address the function of CCL21/CXCR3 signal on chemotaxis and its contribution on the increased antitumor activities.

One of the important immune functions of DCs is antigen cross-presentation (47). Many tumors express tumor-specific antigens capable of being presented to CD8+ T cells by MHC class I molecules on professional APCs. DCs can package, delivery, process, and present tumor antigen by MHC class I molecules and then initiate cytotoxic T lymphocyte responses (48). Although direct cross-presentation and priming of tumor-associated antigens were not checked directly, we did note that 7C21 CAR-T cells also had better antitumor activities in heterogeneous Hepa1-6-A2 tumor, and complete remission was observed in one mouse. What is more, splenic CD3+ T cells isolated from mice treated with 7C21 CAR-T cells produced more IFNγ ex vivo when restimulated with tumor cells.

Tumor-specific memory T cells are essential for the persistent control of tumors in immunotherapy. It is known that CD8+ memory T cells are heterogeneous with respect to phenotypic markers, effector function, and homing capabilities. Central memory T cells (Tcm) are antigen-experienced cells that constitutively express two surface
molecules, CD62L and CD44. Tcm cells provide protection from the systemic challenge and can generate a second wave of effector cells and preferentially migrate to secondary lymphoid organs which are super mediators of therapeutic antitumor immunity to an established cancer (14, 49, 50). We found that more T cells in the spleen of 7 × 21 CAR-treated mice differentiated into tumor-specific memory T cells. Rechallenging experiments in tumor-rejecting mice further proved the immunologic mechanism of IL7/CCL21-mediated tumor rejection and acquisition of protective immunologic memory against pancreatic cancer. These results could be attributed to the immunogenic effect of tumor eradication caused by the 7 × 21 CAR treatment.

Taken together, these results suggested that the potent antitumor effects of 7 × 21 CAR-T cells were mediated by infiltration of T cells and their interaction with DCs inside tumor tissues, and the inhibition of tumor angiogenesis. However, there is no doubt that more detailed mechanism should be further explored to elucidate the increase antitumor activities of 7 × 21 CAR-T cells, especially in heterogeneous antigen situation. In summary, our findings suggest that 7 × 21 CAR-T cells are promising for the treatment of solid tumors, and clinical study on this new therapeutic modality is warranted.

**References**


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