Journal for ImmunoTherapy of Cancer

XCL1-secreting CEA CAR-T cells enhance endogenous CD8$^+$ T cell responses to tumor neoantigens to confer a long-term antitumor immunity

Xing-Ning Li,[1] Feifei Wang,[1] Kun Chen ,[2] Zhiyuan Wu,[2] Ruochan Zhang,[1] Chentong Xiao,[1] Fei Zhao,[1] Dongmei Wang,[2] Hong Zhao,[3] Yuliang Ran,[4] Chunfeng Qu [2]

To cite: Li X-N, Wang F, Chen K, *et al*. XCL1-secreting CEA CAR-T cells enhance endogenous CD8$^+$ T cell responses to tumor neoantigens to confer a long-term antitumor immunity. *Journal for ImmunoTherapy of Cancer* 2025;**13**:e010581. doi:10.1136/jitc-2024-010581

► Additional supplemental material is published online only. To view, please visit the journal online (https://doi.org/10.1136/jitc-2024-010581).

X-NL, FW and KC contributed equally.

X-NL, FW and KC are joint first authors.

Accepted 14 December 2024

Check for updates

© Author(s) (or their employer(s)) 2025. Re-use permitted under CC BY-NC. No commercial re-use. See rights and permissions. Published by BMJ Group.

For numbered affiliations see end of article.

Correspondence to
Dr Kun Chen;
chenkun@cicams.ac.cn

Professor Yuliang Ran;
ran_yuliang@126.com

Professor Chunfeng Qu;
quchf@cicams.ac.cn

ABSTRACT

Background Therapeutic efficacy of carcinoembryonic antigen (CEA)-specific chimeric antigen receptor (CAR) T cells against colorectal cancer (CRC) remains limited due to the unique characteristics and distinct microenvironments of tumor tissues. We modified CEA-specific CAR-T cells, aiming to stimulate endogenous CD8$^+$ T cell responses against neoantigens that were derived from CEA-positive tumors destroyed by the CAR T cells.

Methods In a conventional CEA CAR (reg-CAR), we modified it to express lymphotactin XCL1 and interleukin (IL)-7 genes, constructing a modified 7XCL1-CAR. By generating the CEA-specific 7XCL1-CAR T cells, we assessed their antitumor efficacy against CRC cells with varying levels of CEA expression, both in cell-cultures and in two strains of tumor-bearing syngeneic mice.

Results Following retroviral transduction, 7XCL1-CAR T cells and reg-CAR T cells exhibited similar positive proportions of CEA-CAR and CD4:CD8 ratios. In co-culture system with CEA-negative CT26 cells, no differences in cytotoxicity were observed between 7XCL1-CAR and reg-CAR T cells. However, in co-culture with CT26.CEA^{high} and CT26.CEA^{int} cells, 7XCL1-CAR T cells displayed higher cytotoxicity than that reg-CAR T cells after 60 hours. On interaction with CT26.CEA-positive cells, 7XCL1-CAR T cells secreted higher levels of XCL1 and IL-7, effectively recruited the most potent cross-presenting cDC1s (type-I conventional dendritic cells), and sustained the antitumor activity of CAR-T cells. In treating mice that carried tumors derived from universally CEA-positive cells, 7XCL1-CAR T cells exhibited no difference compared with reg-CAR T cells. However, in treating mice with tumors containing both CEA-positive and CEA-negative cells, 7XCL1-CAR T cells displayed greater inhibition than that of reg-CAR-T cells. After treatment of 7XCL1-CAR T cells, tumor-bearing mice exhibited enhanced infiltration of cDC1s, maintained CAR-T activity, and generation of endogenous neoantigen-specific T cells. Consequently, 7XCL1-CAR T cell-treated mice demonstrated resistance to challenge with CEA-negative CT26 cells.

Conclusion Treatment with CEA-specific, XCL1-secreting CAR-T cells for CEA-positive tumors promoted the generation of CD8$^+$ T cells against tumor neoantigens, mediating a long-term antitumor immunity against heterogeneous CRCs.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Safety was indicated in patients from clinical trials with the chimeric antigen receptor (CAR) T cell therapy targeting carcinoembryonic antigen (CEA). The antitumor efficacy remains limited due to unique characteristics and distinct microenvironments of the solid tumor tissues.

WHAT THIS STUDY ADDS

⇒ The modified CEA-specific, 7XCL1-CAR T cells secreted higher levels of lymphotactin XCL1 and IL-7 on interaction with CEA-positive tumor cells. Locally secreted XCL1 and IL-7 sustained the antitumor activity of CAR-T cells, effectively recruited the most potent cross-presenting cDC1s to tumor tissues, and supported generation of endogenous CD8$^+$ T cells to the neoantigens released from the damaged tumor cells. Promoting the generation of endogenous CD8$^+$ T cells by 7XCL1-CAR T cells improved the CEA-CAR T cell therapeutic efficacy against a heterogeneous tumor with different levels of CEA expression.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ XCL1-secreting CEA CAR-T cells can enhance endogenous CD8$^+$ T cell responses by mobilizing migratory cDC1s to the tumor tissues. The efficacy in treating patients with a heterogeneous tumor expressing CEA at varying levels might be improved with 7XCL1-CAR T cells.

BACKGROUND

The prognosis of metastatic colorectal cancer (CRC) remains poor, approximately 20% of newly diagnosed patients having metastatic CRC and 40% of patients with previously treated localized CRC experiencing recurrence.[1] Immunotherapy with immune checkpoint blockades (ICBs) has improved the survival of patients with microsatellite instability-high/mismatch repair-deficiency CRCs, most likely by driving the local

expansion of CD8+ T cells within tumor tissues, thereby mediating long-term antitumor responses.[1 2] However, CRCs with a mismatch repair-proficient/microsatellite instability-low profile resist ICB immunotherapy even with inflammatory cell infiltration.[1] Adoptive cell therapy employing genetically modified T cells expressing a chimeric antigen receptor (CAR)-targeting carcinoembryonic antigen (CEA), termed CEA CAR-T cells, has emerged as a promising approach for combating metastatic CRCs.[3 4] However, CAR-T cell therapy against solid tumors is less effective than that against hematological malignancies, primarily due to the unique tumor properties and distinct microenvironments of solid tumors. Multiple strategies are currently being explored to improve the therapeutic efficacy against solid tumors.[5–7]

Experimental evidence has indicated that strategies to enhance CAR-T cell infiltration, proliferation, and persistence within tumor tissues are important for treating the solid tumors.[5–10] Notably, clinical studies have indicated that driving the local expansion or generation of neoantigen-specific CD8+ T cells, particularly cytotoxic T lymphocytes (CTLs), confers effective antitumor immunity in patients with cancer.[11–13] During CRC progression, distinct cancer neoantigens arising from somatic mutations are expressed in different malignant clones.[1 13] Hypothetically, the therapeutic efficacy of CEA CAR T cells could be improved by promoting the generation of endogenous neoantigen-specific CD8+ T cells when CEA-positive cells are destroyed.

In metastatic CRC, the liver is the most affected organ, where the tissue presents an immunotolerant environment, forming a unique immunosuppressive tumor microenvironment.[14 15] It has been demonstrated that hepatically expressed interleukin-7 (IL-7) directly controls T-cell responses, including enhanced CD4+ and CD8+ T cell survival, and augmented CD8+ T cell cytotoxic activity.[16] To generate antigen-specific CD8+ T cells (CTLs), dendritic cells (DCs) are essential for cross-presenting tumor antigens to resting T cells.[17–19] However, DCs are functionally heterogeneous and display distinct capacities to elicit antitumor immunity. Certain DC types can even drive tolerance in the tumor microenvironment.[17] Type I conventional DCs (cDC1s) excel at cross-presenting cell-associated antigens to CD8+ T cells and are crucial for initiating CD8+ T cell-mediated antitumor immunity.[17 18] In mice, cDC1s include lymphoid tissue-resident CD8α+ DCs and migratory $CD103^{high}CD11b^{neg}$ DCs. Human cDC1s correspond to the $CD141^{high}$ ($BDCA3^{+}$) $CD11b^{neg/low}$ DCs. Both mouse and human cDC1s specifically express the chemokine receptor XCR1 that selectively responds to the chemokine lymphotactin XCL1. The newly arriving migratory cDC1s, chemoattracted by XCL1, form sustained conjugates with antigen-specific CD8+ T cells more efficiently.[19] In tumor tissues, the concurrence of PD1(+) T cells and XCR1(+) cDC1s predicts prognostically favorable human cancers.[17 20] In tumor-draining lymph nodes, a reservoir of tumor-antigen specific stem-like CD8+ T cells that preserve the ongoing antitumor immune response is efficiently maintained by cDC1s.[21 22] Stem-like CTLs phenotypically express PD1 at an intermediate level and proliferate vigorously in response to specific antigen stimulation.[23 24] PD1 expression of CAR-T cells maintains the activities of cell proliferation and differentiation into effector T cells, and mediates their potent antitumor activities.[25] Terminally exhausted T cells with high expression levels of Tim3 and Lag3 are prone to apoptosis.[23 24] Compared with terminally exhausted CTLs, stem-like CTLs express greater levels of *Xcl1* that selectively mobilizes migratory cDC1s to promote T-cell immunity against tumors.[18]

Our previous study indicated that the conjugation of *Xcl1* with the tumor antigen glypican-3 enhanced its cross-presentation by cDC1s and boosted antitumor immunity by generating potent CTL responses and activating NK and NKT cells in mouse livers.[26] Therefore, it is important to provide the chemokine XCL1 at the site where the tumor antigen is presented, to recruit cDC1s and promote the generation of CD8+ T cells against the tumor neoantigen. Consequently, we introduced lymphotactin XCL1 and IL-7 genes into a CEA-specific CAR and constructed 7XCL1-CAR. We aimed to maintain the presence of CEA CAR-T cells within tumor tissues and mobilize migratory cDC1s to tumor tissues to promote the generation of endogenous CD8+ T cells. This study examined the antitumor effectivity of 7XCL1-CAR T cells on CRC cells expressing different CEA levels in culture. The antitumor effectiveness of 7XCL1-CAR T cells was evaluated in two strains of syngeneic mice that carried tumors composed of universal CEA-positive cells and in mice carried tumors composed of CEA-positive and CEA-negative cell mixtures.

METHODS

Cell lines

BALB/c mouse-derived CT26 and CT26.CL25 cells were purchased from the American Type Culture Collection (ATCC), C57BL/6 mouse-derived MC38 cells were obtained from the National Infrastructure of Cell Line Resources (NICLR, Beijing, China). By transducing MC38 and CT26 cells with the gene encoding human CEACAM5 (Gene ID: 1048), we generated MC38.CEA cells, and two CT26 subclones, $CT26.CEA^{high}$ and $CT26.CEA^{int}$. MC38.OVA cells were generated by transducing the gene encoding ovalbumin (OVA, Gene ID: AUD54808.1). We transfected the $CT26.CEA^{high}$ cells with a pcDNA3.1(-) vector containing β-galactosidase C-terminal region, generating CT26.CEA/gal cells for transiently expressing the $H\text{-}2L^{d}$-restricted epitope (TPHPARIGL).[27] Human CRC LS180 cell line and human umbilical vein endothelial cells (HUVECs) were purchased from ATCC and NICLR, respectively.

Plasmids and gene construction

Two forms of CEA-scFv genes were synthesized and inserted into the pFUSE-hIgG1-Fc2 vector at GenScript

(Nanjing, China). To produce mouse CAR-T cells, we designed a CAR based on the retroviral vector of the MSGV1. From N-terminus to C-terminus, regular CEA CAR (reg-CAR) contain the domains that include CEA-scFv, the human CD8α hinge and transmembrane (CD8H/TM) area (amino acid 183–206 of Gene 925), and intracellular motifs of 4-1BB (214–255 of Gene 3604) and CD3ζ (52–164 of Gene 919). To construct 7XCL1-CAR, we added the cytokine domains IL-7 (1–177 of Gene 3574) and XCL1 (1–114 of Gene 6375) to the C-terminus of reg-CAR. The sequences encoding Thosea asigna virus 2A peptide (T2A, Gene 41332124) were inserted into the CD3ζ C-terminus, and equine rhinitis A virus 2A peptide (E2A, Gene 37627023) was added to the IL-7 C-terminus.

Generation of CEA CAR-T cells

To prepare the retroviruses for T-cell infection, 293 T cells were co-transfected with the plasmids MSGV1 and pCL-Eco using Lipofectamine 2000 reagent (ThermoFisher, USA). The viral supernatant was collected at 48 hours and at 72 hours, filtered using 0.45 µm Millipore filters, aliquoted, and stored in −80°C. $CD3^+$ T cells were purified from splenocytes using Pan T-Cell Isolation Kit-II (Miltenyi Biotec, Germany). In 1×10^6/mL purified T cell suspension, 25 µL anti-mouse CD3/28 Dynabeads were added into the complete RPMI-1640 medium that contains 10% fetal bovine serum, 1% sodium pyruvate, 1% non-essential amino acids, 55 µM 2-mercaptoethanol, and 40 IU/mL hIL-2. The Dynabeads were washed out after 48 hours stimulation, and the activated T cells were infected with retroviral supernatants in plates that were pre-coated with RetroNectin (Takara, Japan) by centrifugation at 2,000×*g* at 32°C for 2 hour as previously reported.[8] Activated T cells infected with empty viral vectors were termed as Un-T cells and used as a control. CEA scFv expression on the T-cell surface was examined using the CEA.CAM5-hIgG-Fc fusion protein (Sino biological, Beijing, China).

Animal models and antitumor treatment of CEA CAR-T cells

Mice were purchased from Beijing Huafukang Bioscience Company. The Institutional Animal Care and Use Committee of the National Cancer Center/Cancer Hospital of the Chinese Academy of Medical Sciences approved the study protocol. Due to mice having no CEACAM5 homologue, CEA-overexpressing tumor cells can be immunologically rejected in the host with normal immunity, concealing the antitumor effects mediated by CEA-CAR T cells. A 5 Gy X-ray dose for BALB/c mice, 0.5 Gy for C57BL/6 mice was administered 2 days before inoculation of tumor cells to facilitate the tumor growth. On day 0, 2×10^5 tumor cells per BALB/c mouse, and 4×10^5 tumor cells per C57BL/6 mouse were injected subcutaneously. On day 7, when tumor volume in all mice reached ≥100 mm^3, the mice were randomly divided into 4 groups, 5–7 mice per group. On day 7 and day 9, each group of mice was treated, respectively, with reg-CAR-T cells, or 7XCL1-CAR T cells, or Un-T cells via tail vein injection. Each injection contained 2×10^6 T cells in a 100 µL volume. As a control, one group of tumor-bearing mice received PBS. Tumor growth was measured every 2–3 days, and tumor volume was calculated as $width^2\times length\times0.5$.

Flow cytometry

To examine the expression of scFv on T cells, 0.5 µg CEA.CAM5-hIgG-Fc was mixed with 2×10^5 cell suspension, followed by the addition of PE-conjugated anti-hIgG-Fc (eBioscience). Purified human IgG was used as an isotype control. A standard laboratory protocol was used to stain the cell markers using the fluorescence-conjugated antibodies listed in online supplemental table 1. To determine the absolute numbers of specified cell populations, Precision Count Beads (Biolegend, USA) were used following the manufacturer's instructions. Data were acquired in BD LSR-II and analyzed using Flowjo software (Tree Star, USA).

Online supplemental materials and methods provide the other methods used in this study.

Statistical analysis

GraphPad Prism (V.8.0) was used for the statistical analysis. Continuous variables were compared using unpaired Student's t-test between two groups and one-way analysis of variance for more than two groups. A $p<0.05$ was statistically significant.

RESULTS

Constructs of two types of CEA CARs

To obtain the scFv for better affinity with CEA, we generated CEA-(V_L–V_H) and CEA-(V_H–V_L) using a linker (G_3S)×3 to conjugate the anti-CEA variable regions of the light chain (V_L) and heavy chain (V_H) of our previously identified antibody sequences.[28] We generated and purified two forms of hIgG1-Fc-fused CEA-scFv by using the pFUSE-hIgG1-Fc2 vector (figure 1A, online supplemental figure S1). Examination of 200 ng of purified CEA coated on the surface of the immunoassay plate revealed a higher binding capacity of CEA-(V_L–V_H) than that of CEA-(V_H–V_L). The relatively high binding capacity of CEA-(V_L–V_H) was confirmed in CEA-expressing LS180 cells, a human CRC cell line (figure 1B). However, CEA(V_L–V_H) did not bind to CEA-negative vascular endothelial cells (HUVECs) (online supplemental figure S1). Therefore, we used CEA-(V_L–V_H) as CEA-scFv to construct two types of CEA-specific CAR. In reg-CAR, the CEA-scFv was linked to the CD8H/TM area of CD8α, cytoplasm domains of 4-1BB and CD3ζ chain. In 7XCL1-CAR, IL-7 and XCL1 were added to the C-terminus of reg-CAR (figure 1C). CEA-CAR expression was confirmed on the T-cell surface after the cells were transduced with retroviral vectors containing the 7XCL1-CAR or reg-CAR genes (figure 1D). T cells transduced with an empty viral vector were labeled Un-T cells.

Cytotoxicity of two types of CAR-T cells on CRC cells expressing CEA at different levels

CEA-CAR density, reflected by CAR expression levels, was lower in 7XCL1-CAR T cells than in reg-CAR T cells

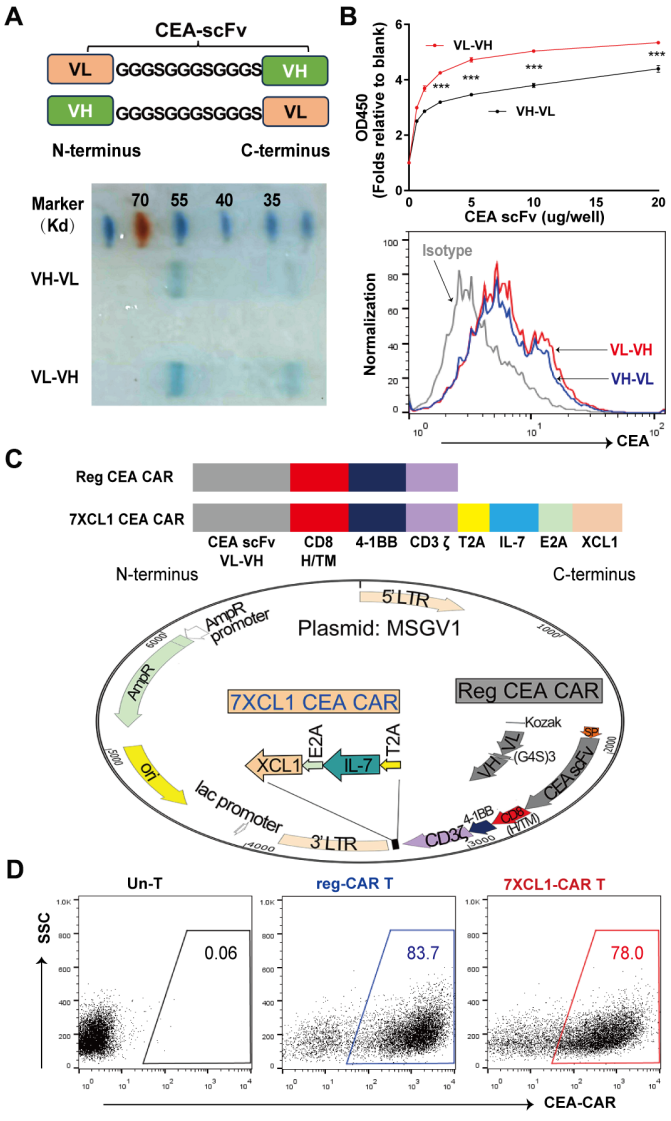

Figure 1 Constructs of two types of carcinoembryonic antigen (CEA)-specific chimeric antigen receptor (CAR) T cells. (A) Schematics of two forms of CEA-scFv, V_L–V_H and V_H–V_L. Two forms of hIgG1-CEA-scFv fusion proteins were purified as presented. (B) Graph presents the binding of V_L–V_H or V_H–V_L with 200 ng recombinant CEA coated on Costar Stripwell microplates at the specified concentration. ***p<0.001 compared with T-test. Flow cytometry profile presents the binding of 15 µg/mL of V_L–V_H or V_H–V_L with 2×10^5 LS180 cells. One of three independent experiments is shown. (C) Map of retroviral vectors used for generating murine CEA-specific 7XCL1-CAR T and reg-CAR T cells. (D) CEA-specific CAR expression on T cell surface determined with CEA-hIgG-Fc fusion protein.

(figure 2A). However, no significant difference in the CEA-CAR-positive proportion and CD4:CD8 ratio was observed between 7XCL1-CAR T cells and reg-CAR-T cells (figure 2B).

To determine the tumor lysis effects of the two types of CAR-T cells, we generated CT26.CEA^{high} and CT26.CEA^{int} subclones that expressed CEA on the CT26 cell surface at high and intermediate levels, respectively (online supplemental figure S2A). For several CEA-negative tumor cell (Tu) targets, both 7XCL1-CAR T cells and reg-CAR T cells that serve as effectors (Effs) exhibited negligible cytotoxicity. Notably, after 4 hour of co-culture, 7XCL1-CAR T cells exhibited a relatively lower level of cytotoxicity than that of reg-CAR-T cells on both CT26.CEA^{high} and CT26.CEA^{int} cells at the same Eff:Tu ratios (figure 2C). We prolonged the duration of co-culture of tumor cells with the two types of CAR-T cells. When examined with an optical microscope, the growth of CT26.CEA^{high} cells co-cultured with 7XCL1 CAR-T cells exhibited no obvious difference from that of reg-CAR-T cells after 30 hours co-culture. However, at 60 hours, growth inhibition of CT26.CEA^{high} cells was primarily observed in the wells co-cultured with 7XCL1-CAR T cells. The 7XCL1-CAR T cells mediated greater 60 hours cytotoxicity than that of reg-CAR T cells on CT26.CEA^{high} cells. 7XCL1-CAR T cells also caused greater cytotoxicity than that of reg-CAR T cells on CT26.CEA^{int} cells (figure 2D). CAR-T cells were harvested after being co-cultured with CT26.CEA^{high} cells for 60 hours. Flow cytometry (FCM) analysis indicated that a smaller percentage of 7XCL1-CAR T cells underwent apoptosis than that of reg-CAR-T cells. Meanwhile, 7XCL1-CAR T cells maintained the surface expression of PD1 but did not elevate the expression of Tim3 and Lag3 (figure 2E).

7XCL1-CAR T cells secreted XCL1 and IL-7 in recognition of CEA-positive cells

The results of cell counting indicated that T cell numbers increased persistently within 72 hours postinfection with the 7XCL1-CAR gene but stopped increasing at this time point with the reg-CAR gene (figure 3A). The 7XCL1-CAR T cells displayed higher proliferative capacity than that of reg-CAR-T cells, incorporating more BrdU (figure 3B). Granzyme B production by 7XCL1-CAR T cells did not differ from that of reg-CAR T cells. However, 7XCL1-CAR T cells expressed higher levels of TNF-α and IFN-γ and maintained a greater proportion of TNF-α$^+$IFN-γ$^+$ cells than that of reg-CAR T cells (figure 3C). When examined within the duration of 72 hours post viral infection, 7XCL1-CAR T cells began secreting XCL1 and IL-7 proteins, and this increased gradually. However, neither IL-7 nor XCL1 were detectable in the supernatants of reg-CAR T or Un-T cells at the same time points (figure 3D). After co-culture with CT26.CEA^{high} cells, 7XCL1-CAR T cells exhibited increased generation of XCL1 and IL-7, regardless of the Eff:Tu ratio. Reg-CAR-T cells generated trace amounts of XCL1 and non-detectable levels of IL-7 after being co-cultured with CT26.CEA^{high} cells, even in the ratio of 20 CAR-T cells and one CEA-positive tumor cell. When CEA-negative CT26 cells were used as targets, 7XCL1-CAR T cells generated limited amounts of XCL1 and IL-7 (figure 3E, online supplemental figure S3).

7XCL1-CAR T cells efficiently recruited cDC1s to promote antigen-specific T cell activation after destroying CEA-positive tumor cells in culture

After eradication of targeted CEA-positive tumor cells, promotion of endogenous tumor-antigen-specific CD8$^+$ T cells by potent antigen-presenting dendritic cells (DCs) could control tumors that expressed no target

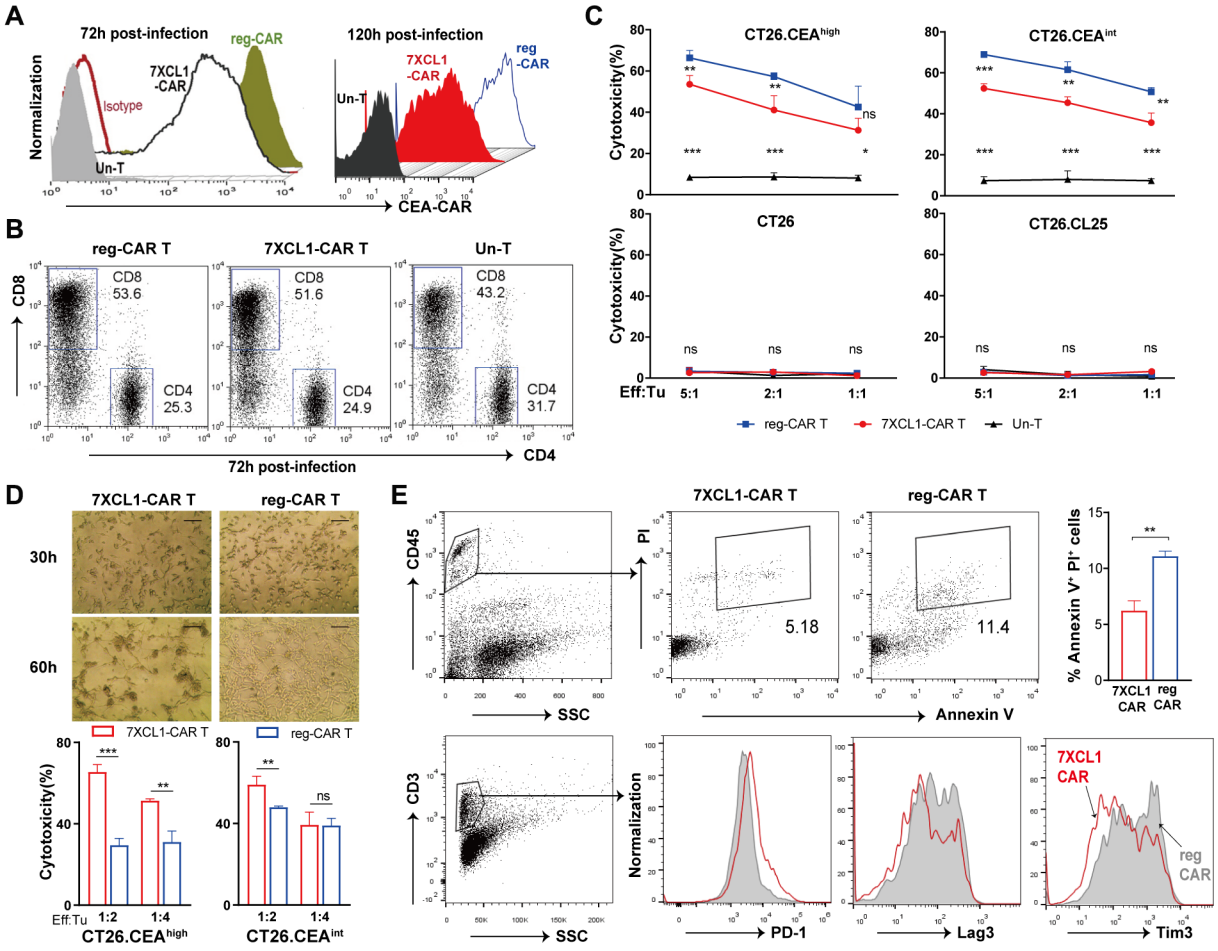

Figure 2 Tumor cytotoxicity of two types of chimeric antigen receptor (CAR) T cells. (A) Expression levels of CEA-CAR in two types of CAR-T cells examined at 72 hours (left) and 120 hours (right) post-transduction with the CAR gene, repeated at least three times. (B) Proportion of $CD4^+$ and $CD8^+$ T cells of infected T cells, repeated at least three times. (C) The 4 hours cytotoxicity of different effector T cells (Eff) on indicated tumor cells (Tu). At the specified Eff:Tu ratios, 7XCL1-CAR T cells (red lines), reg-CAR T cells (blue lines), or Un-T cells (black lines) were co-cultured respectively with 1×10^4 CFSE-labeled tumor cells in each tube for 4 hours. The 4 hours cytotoxicity of CAR-T cells was calculated as [(Eff:Tu–Tu spontaneous)/(100–Tu spontaneous)]×100%. Graphs present one of three independent experiments. (D) The 60 hours cytotoxicity of two types of CAR-T cells on indicated tumor cells. In an independent tube, 3×10^4 CT26.CEA^{high} or CT26.CEA^{int} cells were mixed with 7XCL1-CAR T cells or with reg-CAR T cells at various Eff:Tu ratios for 20 min. The mixed cells were then added into each well of a 48-well plate. By 60 hours, dead tumor cells and T cells were gently washed out using warmed PBS. Tumor cell viability was determined using a Cell Counting Kit-8, expressed as OD450 value. The 60 hours cytotoxicity (%) of CAR-T cells was calculated as [(Un-T:Tu–Eff:Tu)/Un-T:Tu]×100%. Graphs one of three independent experiments. Representative optical microscope images indicate the growth of CT26.CEA^{high} cells that were co-cultured with 7XCL1 CAR-T or with reg-CAR T cells at the Eff:Tu=1:2 for 30 hours and 60 hours, respectively. Scale bars, 100 µm. (E) At Eff:Tu=1:2, two types of CAR-T cells were co-cultured with CT26.CEA^{high} cells for 60 hours and analyzed by FCM. The profiles present one of three independent experiments. Bar graph indicates the average frequency of Annexin-V^+PI^+ cells in each group. Data in graphs represent mean+SD of triplicate and analyzed with one-way ANOVA (C) or t-test (D, E). *p<0.05; **p<0.01; ***p<0.001; ns, no statistically significant. ANOVA, analysis of variance; CEA, carcinoembryonic antigen; FCM, flow cytometry.

antigen. We collected the supernatant of 7XCL1-CAR T cells or reg-CAR-T cells after co-culturing with CT26.CEA^{high} cells. Human lymph node cells were added into the upper chambers of 5 µm pore size Transwell inserts (depicted in figure 4A). When all cells that migrated into the lower chambers were collected, FCM analysis indicated that the co-culture supernatant from 7XCL1-CAR T cells recruited more $CD11c^+HLA\text{-}DR^+$ DCs than that of the supernatant from reg-CAR T cells. Notably, the total number and proportion of migratory cDC1s that were defined as $CD141^+HLA\text{-}DR^{high}$ cells, increased greater by the 7XCL1-CAR T cell supernatant (figure 4B). We then harvested the migrated cells in the lower chambers and pulsed them with a model antigen (influenza A virus M1 protein) to test their activity on autologous PBMCs (depicted in figure 4A). In same numbers of PBMCs, the DCs chemoattracted by 7XCL1-CAR T cell supernatant stimulated more cells to secrete IFN-γ than that by reg-CAR T cell supernatant in the presence of M1 protein (figure 4C). LEGENDplex analysis indicated that the DCs

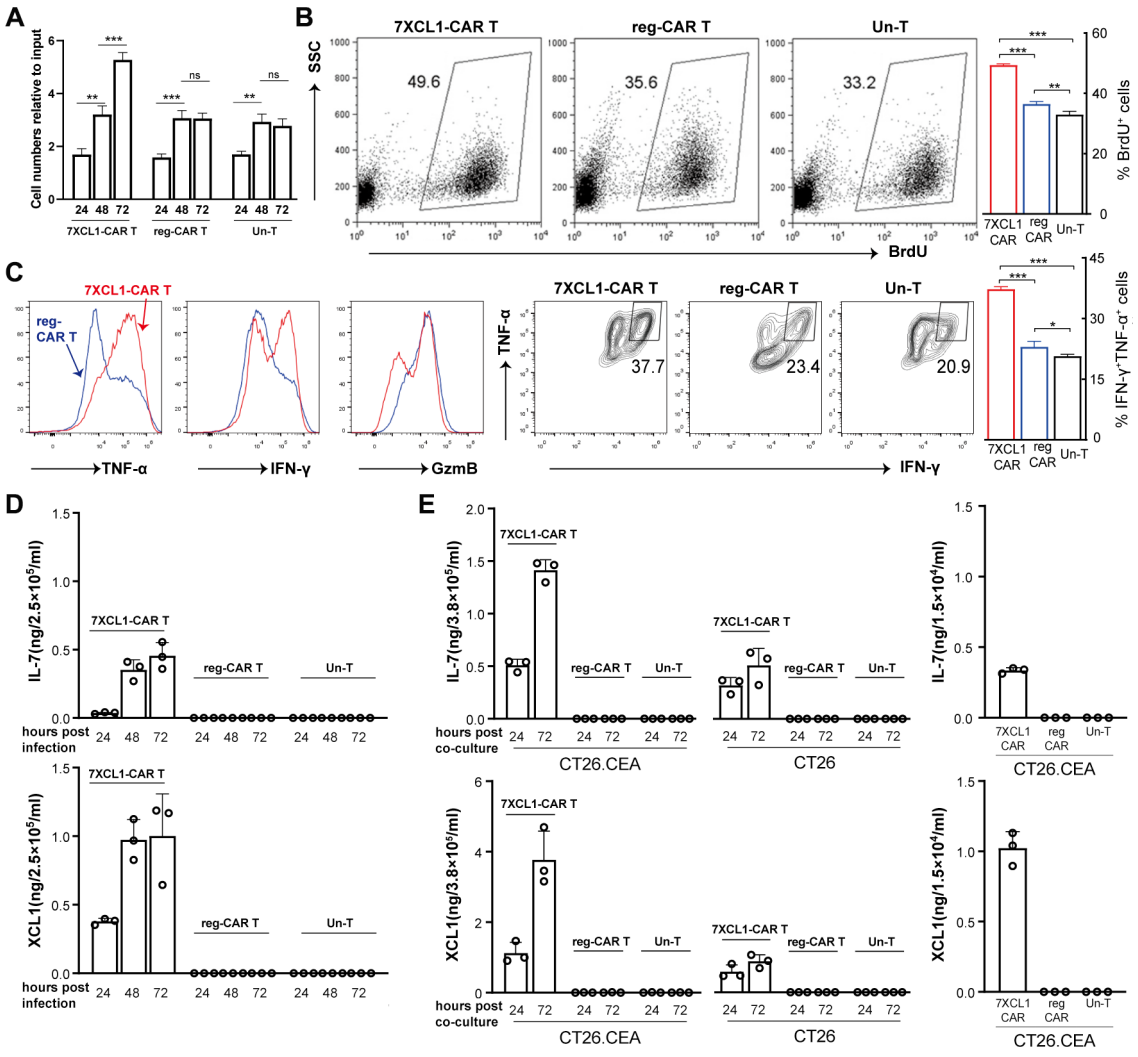

Figure 3 CEA-specific CAR T cell proliferation and generation of IL-7 and XCL1 after tumor cell stimulation (A.) T cell numbers were counted daily post-transduction with the indicated CAR gene. Bar graph indicates (mean+SEM) cell numbers at the specified time points relative to the initial cell numbers. Three independent experiments were conducted in triplicate. (B.) T cells were collected at 96 hours postinfection, and 20 µM BrdU was supplemented into the culture medium for 2 hours. Dot plots present one of three independent experiments. The average frequency of BrdU-positive cells for each group is presented in the bar graph. (C.) Flow cytometry profiles indicate intracellular staining of TNF-α, IFN-γ, and granzyme B (GzmB) in the indicated T cells that were examined at 120 hours postinfection. Bar graph indicates the average frequency of TNF-α^+IFN-γ^+ cells of each group. One of three independent experiments is presented. (D.) Accumulated amounts of XCL1 and IL-7 generated by two types of CAR-T cells at the indicated time points post-transduction with indicated CAR gene. Each dot represents one experiment. (E.) Accumulated amounts of XCL1 and IL-7 produced by two types of CAR-T cells after stimulation with the indicated tumor cells (Tu). At the Eff:Tu ratio of 10:1 (left panels) or 1:4 (right panels), specified T cells (Eff) were co-cultured with CEA-negative CT26 cells or CT26.CEA cells for indicated duration. The supernatant of co-cultured cells was collected, and the levels of XCL1 and IL-7 were determined using commercialized ELISA kits. Each dot represents one repeat. Data in the bar graphs represent mean+SD of triplicate wells, and analyzed with one-way ANOVA. *p<0.05; **p<0.01; ***p<0.001. ANOVA, analysis of variance; CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen.

chemoattracted by the 7XCL1-CAR T cell supernatant stimulated their autologous PBMCs to generate smaller amounts of IL-10 than that of the DCs recruited by the reg-CAR-T cell supernatant (figure 4D).

Transfer of 7XCL1-CAR T cells and reg-CAR T cells equally controlled the tumors of universal CEA-positive cells

The antitumor effect of 7XCL1-CAR T cells was examined in syngeneic BALB/c mice subcutaneously injected with CT26.CEA^{high} cells. When tumor volume in each of them reached ≥100 mm^3, the mice were randomly divided into four groups and administrated with same numbers of Un-T, or reg-CAR T, or 7XCL1-CAR T cells via tail vein injection twice for a total of 2×10^6 T cells per mice. Sham-treated mice were injected with the same volume of PBS (figure 5A). Tumor growth was significantly inhibited in mice that received either 7XCL1-CAR T or reg-CAR-T cells, with no significant difference between the two types of CAR-T cells (figure 5B). No inflammation

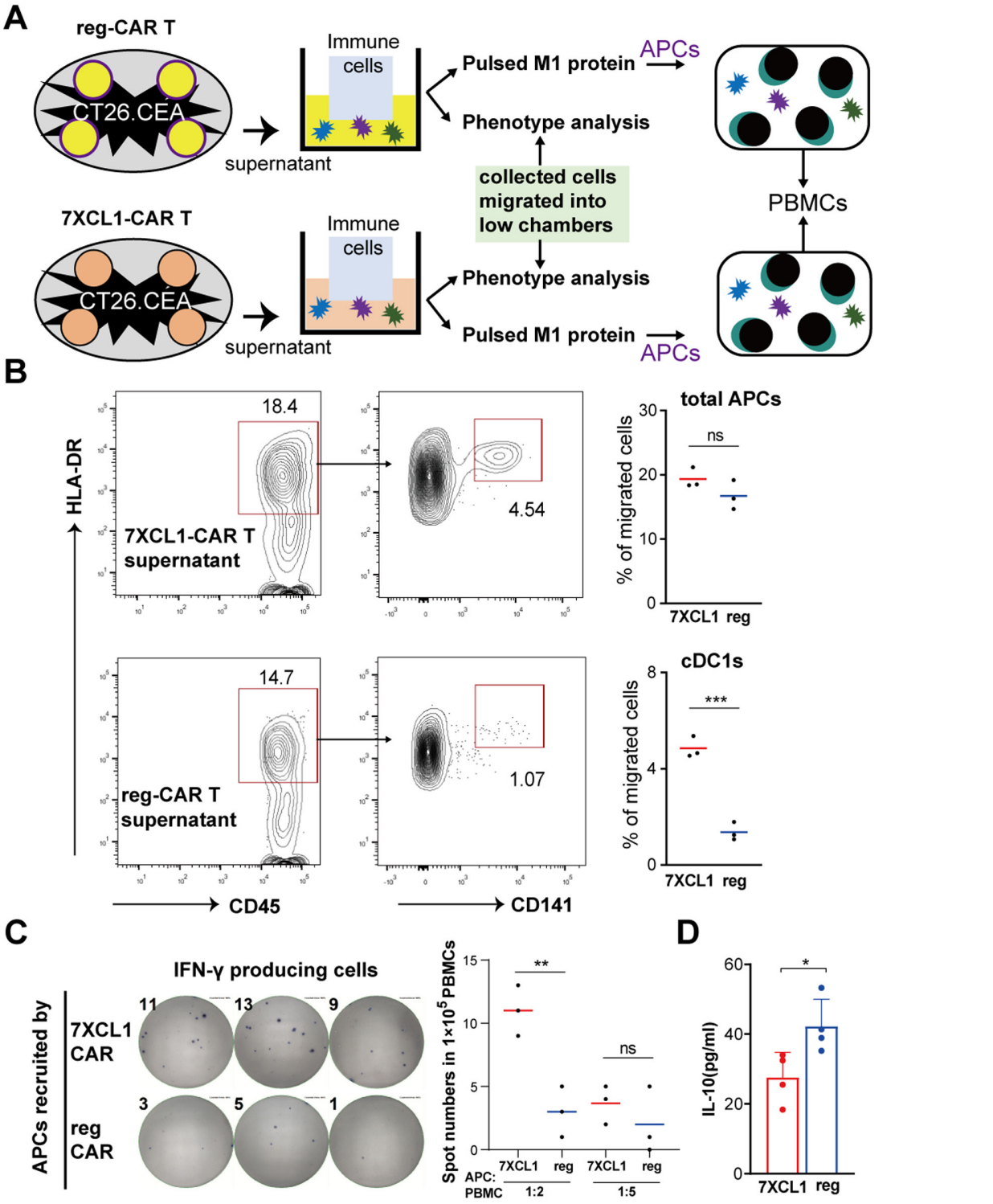

Figure 4 Effects of the supernatant derived from two types of CAR-T cells after their co-culture with CT26.CEA^{high} tumor cells. (A) Schematic of the experiments. (B) Representative flow cytometry plots indicate the total antigen presenting cells (total APC, HLA-DR$^+$) and the type I conventional dendritic cells (cDC1s, HLA-DR$^+$CD141$^+$) that were recruited by the supernatants from 7XCL1-CAR T or reg-CAR T cells after their co-culture with CT26.CEA^{high} tumor cells for 72 hours. Bar graphs present the frequency of indicated cells in the lower chamber, and each dot represents one independent experiment. (C) and D) Autologous 1×10^5 peripheral blood mononuclear cell (PBMCs) were stimulated with the same amounts of recruited cells using the supernatants of 7XCL1-CAR T cells or reg-CAR T cells after stimulation with CT26.CEA^{high} tumor cells for 72 hours at an APC:PBMC ratio of 1:2 or 1:5. (C). Image presenting M1-specific IFN-γ-producing cells in 1×10^5 PBMCs from one of three independent experiments. Average spot numbers are presented in the bar graph, each dot represents one independent experiment. (D) The level of IL-10 in the co-cultured cells at the APC:PBMC ratio of 1:2 was determined using the LEGENDplex Human B Cell Panel. Data in the bar graphs represent mean+SD of triplicate wells and analyzed with unpaired t-test. *p<0.05. **p<0.01; ***p<0.001, ns, no statistically significant. APC, antigen presenting cell; CAR, chimeric antigen receptor.

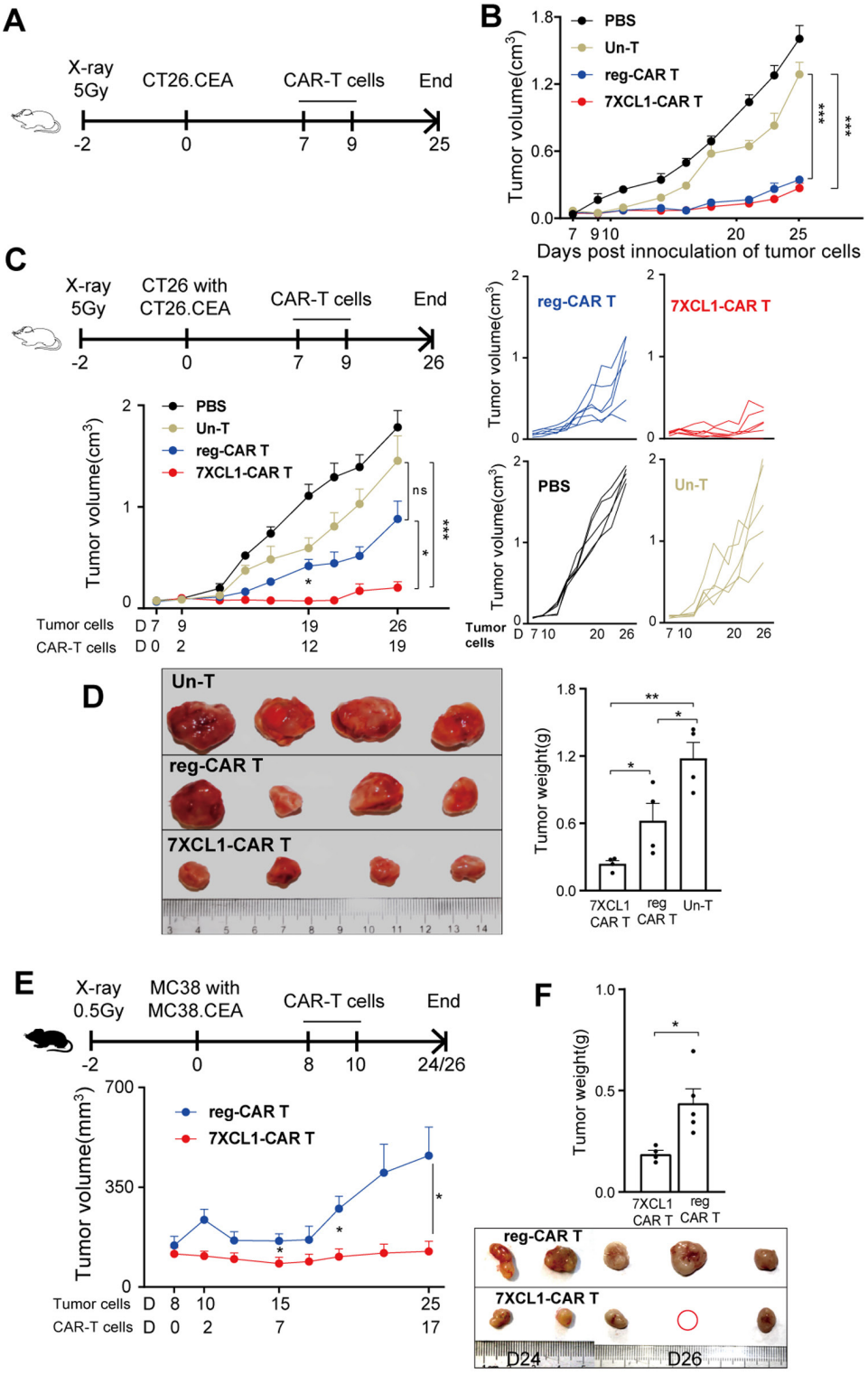

Figure 5 Treatment of two types of CEA-specific CAR-T cells in mice carrying tumors of CEA-positive cells and a mixture of CEA-positive and CEA-negative cells. (A) Experimental scheme in BALB/c mice carried tumors of universal CT26.CEA^{high} cells, with at least five mice per group. (B) Tumor growth with universal CT26.CEA^{high} cells as indicated. (C) Experimental scheme in BALB/c mice carrying tumors composed of a mixture of CT26.CEA^{high} cells (75%) and CT26 cells (25%). Graphs indicate the tumor growth of a group (left) and that of individuals (right). N=5–8 in each group. (D) Image presenting tumors derived from a mixture of CT26.CEA^{high} and CT26 cells removed on D26. The bar graph (mean+SD) indicates the tumor weight of each group, and each dot represents one mouse. (E) Experimental scheme in C57BL/6 mice carrying tumors with a mixture of MC38.CEA cells (75%) and MC38 cells (25%). Graph presents the tumor growth as indicated (mean+SD). N=5–8 in each group. (F) Images present tumors derived from a mixture of MC38.CEA and MC38 cells removed on D24 and D26. The bar graph (mean+SD) presents the tumor weight of each group, and each dot represents one mouse. Differences were compared with one-way ANOVA. *p<0.05; **p<0.01; ***p<0.001. CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen.

in esophagus, stomach, small intestine and colon was observed in differently treated mice after histology examination (online supplemental figure S4).

Transfer of 7XCL1-CAR T cells better controlled the tumors with the mixture of CEA-positive and CEA-negative cells

Due to the heterogeneity of human cancers that express distinct tumor antigens, we mixed CT26.CEA^{high} and CEA-negative CT26 cells to develop a tumor comprising approximately 75% CEA-positive cells and 25% CEA-negative cells. Tumor-bearing mice with a mixture of tumor cells were treated as described in figure 5A. The mice that received 7XCL1-CAR T cells displayed greater inhibition of tumor growth than those that received reg-CAR-T cells. This difference became significant 10–12 days postinjection of the first dose of CAR-T cells (figure 5C). Tumors were smaller in the mice treated with 7XCL1-CAR T cells than in those treated with reg-CAR-T cells (figure 5D).

To confirm the better antitumor effect of 7XCL1-CAR T cells for treating heterogeneous tumors in BALB/c mice, we generated an MC38.CEA subclone expressing CEA on the MC38 cell surface (online supplemental figure S2B). We mixed MC38.CEA with CEA-negative MC38 cells and injected subcutaneously into C57BL/6 mice to develop a tumor comprising approximately 75% CEA-positive cells and 25% CEA-negative cells. As treated in BALB/c mice, the tumor-bearing C57BL/6 mice were treated with 7XCL1-CAR T or reg-CAR T cells, and those treated with 7XCL1-CAR T cells also displayed greater tumor inhibition than those treated with reg-CAR-T cells. In one of the five mice, the established tumor was diminished after treatment of 7XCL1-CAR T cells (figure 5E). When a tumor was developed from 50% MC38.CEA cells and 50% MC38 cells, the tumor-bearing C57BL/6 mice remained to exhibit better tumor control after treatment with 7XCL1-CAR T cells than those with reg-CAR T cells (online supplemental figure S5).

The 7XCL1-CAR T cell-treated mice exhibited greater infiltration of cDC1s and T cells in tumor tissues

Neither IL-7 nor XCL1 were detected in the blood of treated mice. Intercellular fluid was prepared from tumors composed of CT26.CEA^{high} and CT26 cells. Higher amounts of IL-7 and XCL1, as well as IFN-γ and IL-2 were detected in the intercellular fluid of mice treated with 7XCL1-CAR T cells than that of mice treated with reg-CAR-T cells (figure 6A). FCM analysis of tumor-infiltrating lymphocytes revealed a higher number and proportion of MHC-II-positive cells. Migratory cDC1s, MHC-II^{high} CD11c^{high} CD103^{high}, were more profound in mice treated with 7XCL1-CAR T cells than in those treated with reg-CAR-T cells (figure 6B). Paraffin-embedded tumor tissues were examined using multiplex immunohistochemistry (mIHC) staining. Significantly greater infiltration of CD3$^+$ and CD8$^+$ T cells, particularly CD8$^+$ T cells, was detected in the tumor tissues of 7XCL1-CAR T-treated mice compared with that in reg-CAR-T cell-treated mice. Notably, the tumors of 7XCL1-CAR T-treated mice exhibited a higher density of proliferative Ki67$^+$CD3$^+$ and Ki67$^+$CD8$^+$ T cells than those of the reg-CAR-T cell-treated mice (figure 6C).

To examine the origin of the increased infiltration of CD3$^+$ and CD8$^+$ T cells in tumor tissues, we prepared 7XCL1-CAR T cells or reg-CAR-T cells from congenic CD45.1 mice and transferred them into CD45.2 mice carrying tumors composed of MC38.CEA cells and CEA-negative MC38.OVA cells (figure 6D). On day 11 (3 days post-cell transfer), the numbers of CAR-T cells in the peripheral blood and tumor tissues of mice that received 7XCL1-CAR T cells exhibited no significant difference from those of mice that received reg-CAR-T cells. On day 25 (17 days post-cell transfer), few CAR T cells were detected in the blood and tumor tissues of mice that received reg-CAR T cells. However, CAR-T cells remained abundant in both the peripheral blood and tumor tissues of mice that received the 7XCL1-CAR T cells (figure 6D,E). Additionally, the number of host-derived endogenous cells (CD4$^+$ and CD8$^+$ T cells) was also elevated in tumor tissues of mice treated with 7XCL1-CAR T cells compared with that in mice treated with reg-CAR-T cells (figure 6E).

7XCL1-CAR T-treated mice promoted tumor neoantigen-specific CD8$^+$ T cell generation to resist CEA-negative tumor growth

In addition to CEA expression, CRC cells can generate cancer neoantigens due to various somatic mutations.[1 13] Treatment outcomes in tumor-bearing mice indicated the generation of endogenous T cells against non-targeted neoantigens following the transfer of CEA-targeted 7XCL1-CAR T cells. To validate the results, we generated CT26.CEA/gal cells that transiently expressed H-2L^d-restricted β-gal epitope (TPHPARIGL) in the CT26.CEA^{high} subclone and developed the subcutaneous primary tumors composed of CT26.CEA^{high} cells (~80%) and CT26.CEA/gal cells (~20%). After being treated as in figure 5A, the BALB/c mice were challenged with the β-galactosidase-positive but CEA-negative CT26.CL25 cells, 10 days post-transfer of CAR T cells for developing the secondary tumors (figure 7A). Primary tumor growth was mostly inhibited in mice treated with 7XCL1-CAR T cells as observed in previous experiments. Additionally, the growth of secondary tumors was significantly suppressed in 7XCL1-CAR T-treated mice, where 1/5 mice acquired long-term protection against challenge by CEA-negative CRC cells (figure 7B). Infiltrating lymphocytes in challenged tumor tissues were analyzed using FCM. Higher numbers of β-gal-specific CD8$^+$ T cells were detected in the mice treated with 7XCL1-CAR T cells than in those treated reg-CAR T-treated mice (figure 7C).

To confirm the 7XCL1-CAR T cell-mediated effects in promoting the generation of endogenous CD8$^+$ T cells against non-targeted tumor cells, we prepared CAR-T cells from congenic CD45.1 mice and transferred them into CD45.2 mice that carried tumors composed of a mixture of MC38.CEA cells and CEA-negative MC38.

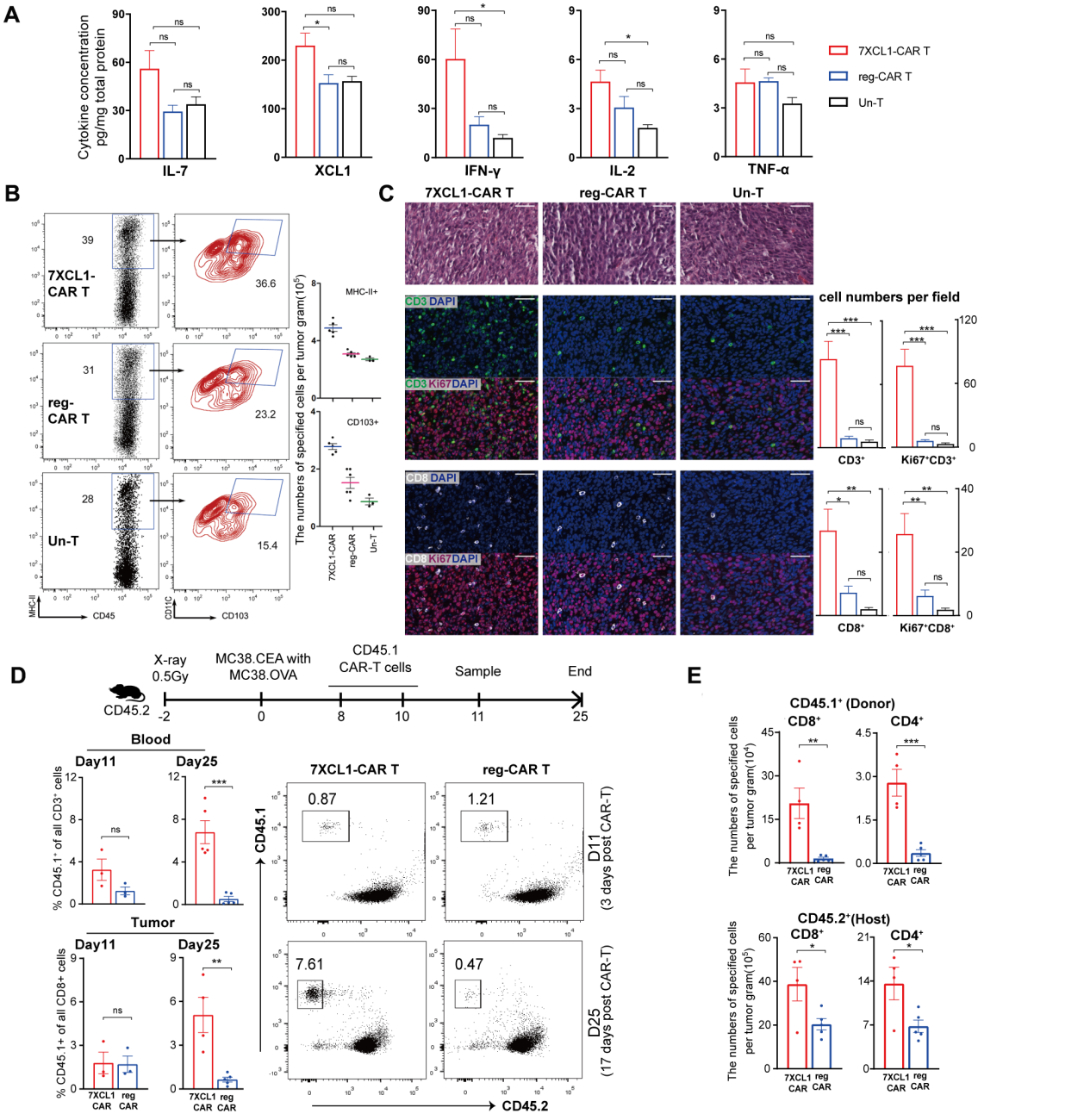

Figure 6 Detection of cDC1s and T cells in tumor tissues of differently treated mice. (A) Indicated cytokines (mean+SD) in the intercellular fluid of tumors consisted of the mixture of CT26.CEA^{high} and CT26 cells from differently treated mice. In each group, three mice were sampled. (B) Flow cytometry (FCM) profiles indicate the total MHC-II$^+$ cells and the CD11c$^+$CD103$^+$ cDC1s in the tumors composed of CT26.CEA^{high} and CT26 cells from differently treated mice. Bar graphs (mean+SD) present the cell numbers per gram of tumor tissue of treated-mice as indicated. Each dots represents one mouse. (C) Representative images of tumors composed of CT26.CEA^{high} and CT26 cells from differently treated mice. Top, HE staining; middle, CD3 (green) and Ki67 (red) double staining; bottom, CD8 (white) and Ki67 (red) double staining. Nuclei were stained with DAPI (blue). Scale bars, 50 µm. In each group, three mice were sampled. In each tumor section, five fields were counted, and the bar graphs (mean+SEM) indicate the cell density (numbers per field) of each group of mice. (D) Experimental scheme in C57BL/6 mice (CD45.2) carried tumors composed of MC38.CEA (75%) and MC38.OVA (25%). Two types of CAR-T cells were prepared from congenic CD45.1 mice. The transferred CAR-T cells were determined on D11 and D25, respectively, corresponding to 3 days and 17 days post-transfer of the first dose of CAR-T cells. Representative dot plots of FCM analysis indicate the frequency of transferred CAR-T cells examined in the tumor tissues based on gate of total CD8$^+$ T cells. The frequency of transferred CAR-T cells determined in their peripheral blood and tumor tissues of the indicated group are presented in the bar graphs, and each dot represents one mouse. (E) After staining with florescence-conjugated antibodies, a 20 µL of precision count beads (1030 Beads/µL) was added into 300 µL of stained cell suspension for FCM analysis. Absolute cell number (cells/µL) was calculated as (cell count×20 µL)/(bead count×300 µL)×1030. The absolute numbers (mean±SD) presented in the bar graphs were calculated as (cells/µL)×300 µL/tumor weight, and each dot represents one mouse. Differences were compared with one-way ANOVA (A, C), or unpaired Student's t-test (D, E). *p<0.05; **p<0.01; ***p<0.001; ns, no statistically significant. ANOVA, analysis of variance.

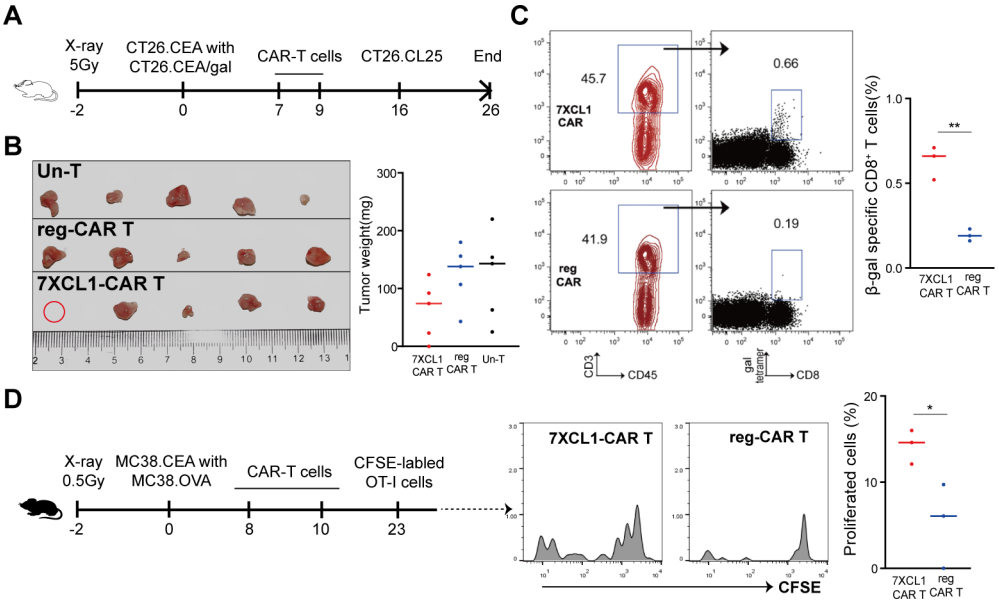

Figure 7 Generation of endogenous tumor neoantigen-specific CD8$^+$ T cells in mice after CAR-T cell treatment. (A) Experimental scheme in BALB/c mice, with five mice per group. Primary tumors were developed by subcutaneous injection of a mixture of CT26.CEA cells (approximately 80%) and CT26.CEA/gal cells (approximately 20%). The mice were treated with different T cells and challenged with CT26.CL25 cells to form the secondary tumors. (B) Images present the secondary tumors from differently treated mice removed on D26. Tumor weights of each group are presented in the bar graph, and each dot represents one mouse. (C) Representative flow cytometry profiles indicate the β-gal-specific CD8$^+$ T cells in the secondary tumor tissues of differently treated mice. One of three experiments is presented. Bar graph indicates the percentage of β-gal-specific CD8$^+$ T cells, and each dot represents one mouse. (D) OVA-specific CD8$^+$ T cells were labeled with 5 µM CFSE, and 2×10^6 cells per mouse were injected via tail vein into differently treated C57BL/6 mice that carried tumors developed from the cell mixture of MC38.CEA (75%) and MC38.OVA (25%). The proliferation of OVA-specific T cells was determined 6 days later, and all CFSE-positive CD8$^+$ T cells in peripheral blood were gated for analysis. Representative histograms present one of three independent experiments. The proliferated cell percentage of each group is presented in the bar graph, and each dot represents one mouse. Differences were compared with unpaired Student's t-test. *p<0.05; **p<0.01. CAR, chimeric antigen receptor.

OVA cells. Naïve OVA-specific CD8$^+$T cells were injected into mice that were, respectively, treated with two types of CAR-T cells. Notably, the proliferation of OVA-specific CD8$^+$T cells was primarily detected in mice treated with 7XCL1-CAR T cells (figure 7D).

DISCUSSION

We modified conventional/regular CEA-specific CAR T cells (reg-CAR T) by inserting the genes encoding IL-7 and XCL1, generating CEA-specific 7XCL1-CAR T cells. In the cell co-culture system, the 7XCL1-CAR T cells exhibited cytotoxicity, secreted both IL-7 and XCL1 on interaction with CEA-positive CRC cells. The XCL1 secreted from 7XCL1-CAR T cells was confirmed to recruit the migratory cDC1s, which promoted the generation of antigen-specific CD8$^+$ T cells, and IL-7 was confirmed to maintain the activity of CAR-T cells and support the generation of neoantigen-specific T cells. When treating mice that carried tumors developed from universal CEA-positive cells, the 7XCL1-CAR T cells exhibited no difference from the reg-CAR-T cells. However, when treating mice that carried tumors composed of a mixture of CEA-positive and CEA-negative cells, 7XCL1-CAR T cells conferred better inhibition of tumor growth compared with that of reg-CAR-T cells. After treatment with 7XCL1-CAR T cells, the mice were counterattacked by CEA-negative tumor cells. The improved antitumor efficacy mediated by 7XCL1-CAR T cells was associated with their sustained antitumor effectivity to CEA-positive cells, and their capability to enhance tumor infiltration of cDC1s for promoting the generation of CD8$^+$ T cells against cancer neoantigens released from CEA-positive cells. Our current results from cell-culture experiments and tumor-bearing mice indicated that XCL1-secreting 7XCL1-CAR T cells could mediate better therapeutic efficacy and confer long-term antitumor immunity against heterogeneous CRCs.

In the conventional CEA-targeting CAR-T (reg-CAR T) cells, the scFv sequence of CEA-specific antibody is linked to the 4-1BB-CD3ζ signaling domain. Clinical trials by transfusion of the conventional CEA-CAR T cells have demonstrated the evidence suggesting effectiveness in controlling progression in a subset of metastatic CRCs.[3 4 10] However, the anticipated therapeutic efficacy has not been achieved due to the unique characteristics and distinct microenvironments of solid tumors.[5–7] Metastatic CRCs primarily affect the liver that presents a particularly immunosuppressive tumor microenvironment.[14] To address this challenge, we inserted IL-7 at the C-terminus of CD3ζ, and XCL1 at the C-terminus of IL-7, separating

them with T2A and E2A, respectively. Following retroviral transduction, the 7XCL1-CAR T cells exhibited relative lower levels of CEA-CAR, likely due to the additional genetic burden imposed by the inserted IL-7 and XCL1 genes at the C-terminus of reg-CAR, along with the limited cleavage efficiency of the viral 2A peptides during protein synthesis in the infected T cells.[29] A previous study, that investigated the utility of CAR-T cells targeting anaplastic lymphoma kinase (ALK), demonstrated that the activity of ALK CAR-T cells within 24 hours was positively regulated by CAR density on interaction with same levels of target antigen.[30] In our cell culture system with CEA-positive cells, 7XCL1-CAR T cells displayed relatively low cytotoxicity within 4 hours co-culture due to the relatively reduced CEA-CAR density. However, on interaction with CEA-positive cancer cells, the 7XCL1-CAR T cells were activated and secreted both IL-7 and XCL1. In the presence of IL-7, the viability of transferred CAR-T cells was maintained,[16] forming a positive feedback loop after exposure to CEA-positive tumor cells. The 7XCL1-CAR T cells thus exhibited sustained cytotoxicity on both CT26.CEA^{high} and CT26.CEA^{int} cells in a prolonged duration (60 hours co-culture). The secreted XCL1 directly mobilized the migratory cDC1s which potently cross-present the tumor-associated antigens.[17–19] Certainly, in tumor-bearing mice that carried a mixture of tumor cells, 7XCL1-CAR T cells displayed greater inhibition of tumor growth than those treated with reg-CAR-T cells. In the tumor bed, the 7XCL1-CAR T cells maintained the tumorlytic capability of CAR-T cells and persistently recruited migratory cDC1s to the site, where the tumor antigen was released, for efficiently promoting generation of endogenous neoantigen-specific T cells to mediate protection against CEA-negative tumors. Therefore, 7XCL1-CAR T cells are promising for treating heterogeneous CRCs in which the CEA antigen is only expressed in some of the malignant clones, particularly in cases with liver metastasis where the tissue presents an immunotolerant environment.[14 16]

The heterogeneity of human cancers that express distinct tumor antigens constrains the therapeutic efficacy of conventional CAR-T cells. Inducing the generation of endogenous CD8$^+$ T cells is a promising strategy to improve the therapeutic efficacy of CAR-T cell therapy. In experimental models, engineering CAR-T cells to generate chemokines for the recruitment of DCs via CCR7 to stimulate endogenous T cells can improve the therapeutic efficacy of CAR-T cells, including 7×19 CAR-T cells secreting IL-7 and CCL19[8 31] and 7×21 CAR-T cells secreting IL-7 and CC21.[32] Due to the functional heterogeneity of different types of DCs in antitumor immunity, migratory cDC1s (XCR1$^+$CD141$^+$ DCs in humans and XCR1$^+$CD103^{high} in mice) have been recognized as the most important professional inducers of antigen-specific CD8$^+$ T cells.[17] One previous study demonstrated that engineered T cells secreting Fms-like tyrosine kinase 3 ligand (Flt3L), which is a growth factor of cDC1s, expanded intratumoral cDC1s, and the combination of poly(I:C) and anti-4.1BB induces epitope spreading toward antigens beyond those recognized by the transferred CAR-T cells.[33] However, in human subjects, Flt3L was observed to expand CD4$^+$FoxP3$^+$ regulatory T (Treg) cells that exert suppressive activity on tumor-specific effector cells in the microenvironment.[34] In animal cancer models, Flt3L therapy increases all conventional DC subsets but results in no reduction in tumor growth associated with the induction of CD81$^+$migcDC1.[35] Having the tumor-antigen-specific CD8$^+$ T cells to transiently secrete IL-12 and injecting them into tumors but not blood, the tumor-bearing mice completely reject the distant concomitant tumors. Treatment effectivity depends on endogenous T cells and cDC1s.[36] IL-12 is generally secreted by activated DCs and phagocytes in response to infection through toll-like receptors.[37] A combination of dual-specific TCR-T cells, that are tumor reactive CD8$^+$ T cells with an additional TCR recognized a bacterial antigen, robustly eradicate tumors following intratumoral injection of bacterial antigen. Antitumor effects also depend on endogenous T cells and cDC1s.[38] XCR1 is selectively expressed in cDC1 subtypes and responds to XCL1 chemokine.[17] Given that the population of cDC1s is rare in the tumor tissue beds, we developed an approach in the current study to mobilize the specific type of cDC1s directly from the peripheral blood. Our current study, conducted in two strains of syngeneic mice, indicated that 7XCL1-CAR T cells with lymphotactin XCL1 and IL-7 genes proved effective for recruiting cDC1s to promote the de novo generation of CD8$^+$ T cells against cancer neoantigens, enhancing the endogenous antitumor immunity.

The tumor lysis functionality of CAR-T cells is significantly regulated by target antigen.[30] Staining with anti-CEA antibody in 52 colon cancer tissues, more than 28 tissue sections exhibited a high level of CEA expression, exceeding 75% CEA positive cells of total tumor cells.[39] Generation of endogenous CD8$^+$ T cells has proven to improve the antitumor efficacy of CAR-T cell therapy.[36 38] Our current results in the tumor-bearing mice suggested that the 7XCL1-CAR T cells could confer better antitumor efficacy for the patients with a tumor heterogeneously composed of CEA-positive cells less than 75%.

CEACAM5 (CEA) is considered as the major player in tumor progression and metastasis. However, certain amount of CEA is expressed in normal columnar epithelial and goblet cells of the colon.[40] In our mice models, we did not observe the tissue damage of examined esophagus, stomach, small intestine and colon after treatment of CEA-specific 7XCL1-CAR T cells. Due to mice having no CEACAM5 homologue, the observation of non-toxicity of the CEA CAR-T cell therapy on normal tissues in the mice models is limited. However, a previous study conducted in immune-competent mice that expressed the CEA transgene (CEAtg) in the intestinal and pulmonary tracts indicates that conventional CEA CAR-T cells via single intravenous injections efficiently eradicate CEA-positive carcinoma cells. The CEAtg mice developed

some noninflammatory infiltrations of CAR-T cells in intestine and lung, but there was no evidence of destruction of CEA+healthy tissues.[41] Several clinical trials have also demonstrated that the CEA CAR-T cell therapy in patients with liver metastatic CRCs either intravenous infusion or hepatic artery infusion was well tolerated, not associated with any serious adverse events above grade 3 even in high doses.[3 4]

Given that metastatic CRCs primarily affect the liver—an immunologically tolerant organ where adaptive immune cells readily become tolerogenic[14 15]—further studies are required to validate the antitumor effects of 7XCL1-CAR T cells in orthotopic tumor models and in patient-derived organoids. The therapeutic efficacy of 7XCL1-CAR T cells for patients with metastatic CRCs requires clinical evaluation to confirm the effects observed in this study without impairing the healthy tissues.

Author affiliations
[1]Immunology Department, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China
[2]Immunology Department, State Key Lab of Molecular Oncology, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China
[3]Department of Hepatobiliary Surgery, State Key Lab of Molecular Oncology, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China
[4]State Key Lab of Molecular Oncology, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

Contributors CQ concepted, designed and supervised the study; analyzed and interpreted data; drafted and critically revised the manuscript; and acts as the guarantor of the study. KC participated in the conception of CAR-T design. YR provided the sequence of CEA scFv and participated in study design. X-NL, FW and KC designed and performed most of the experiments; acquired, analyzed and interpreted data; and drafted the manuscript. ZW, RZ, CX, FZ, DW, and HZ provided technical support. All authors read and approved the final manuscript.

Funding This work was supported by National Key Research and Development Program of China (2023YFC3403800), National Natural Science Foundation of China (32100756), and the Chinese Academy of Medical Sciences Innovation Fund for Medical Sciences (2023-I2M-2-004, 2016-I2M-1-007). The sponsors of the study have no role in study design, data collection, data analysis, data interpretation, or writing of the manuscript.

Disclaimer The sponsors of the study have no role in study design, data collection, data analysis, data interpretation, or writing of the manuscript.

Competing interests CQ, KC, FW, and HZ have filed a patent application (ZL 2023 1 1089771.8) to China National Intellectual Property Administration based on the data generated from this work. Other authors declare no competing interests.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and the usage of lymph nodes and peripheral blood from patients was approved by the Ethics Committee of NCC/CH, CAMS following the guidelines issued by the Ministry of Science and Technology of China, the number is NCC2021C-152. Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

Supplemental material This content has been supplied by the author(s). It has not been vetted by BMJ Publishing Group Limited (BMJ) and may not have been peer-reviewed. Any opinions or recommendations discussed are solely those of the author(s) and are not endorsed by BMJ. BMJ disclaims all liability and responsibility arising from any reliance placed on the content. Where the content includes any translated material, BMJ does not warrant the accuracy and reliability of the translations (including but not limited to local regulations, clinical guidelines, terminology, drug names and drug dosages), and is not responsible for any error and/or omissions arising from translation and adaptation or otherwise.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited, appropriate credit is given, any changes made indicated, and the use is non-commercial. See http://creativecommons.org/licenses/by-nc/4.0/.

ORCID iDs
Kun Chen http://orcid.org/0000-0003-3146-2577
Chunfeng Qu http://orcid.org/0000-0001-9973-0887

REFERENCES

1 Biller LH, Schrag D. Diagnosis and Treatment of Metastatic Colorectal Cancer: A Review. *JAMA* 2021;325:669–85.
2 Li F, Li C, Cai X, *et al*. The association between CD8+ tumor-infiltrating lymphocytes and the clinical outcome of cancer immunotherapy: A systematic review and meta-analysis. *EClinMed* 2021;41:101134.
3 Katz SC, Moody AE, Guha P, *et al*. HITM-SURE: Hepatic immunotherapy for metastases phase Ib anti-CEA CAR-T study utilizing pressure enabled drug delivery. *J Immunother Cancer* 2020;8:e001097.
4 Zhang C, Wang Z, Yang Z, *et al*. Phase I Escalating-Dose Trial of CAR-T Therapy Targeting CEA$^+$ Metastatic Colorectal Cancers. *Mol Ther* 2017;25:1248–58.
5 Tian Y, Li Y, Shao Y, *et al*. Gene modification strategies for next-generation CAR T cells against solid cancers. *J Hematol Oncol* 2020;13:54.
6 Qian S, Villarejo-Campos P, Guijo I, *et al*. Update for Advance CAR-T Therapy in Solid Tumors, Clinical Application in Peritoneal Carcinomatosis From Colorectal Cancer and Future Prospects. *Front Immunol* 2022;13:841425.
7 Maalej KM, Merhi M, Inchakalody VP, *et al*. CAR-cell therapy in the era of solid tumor treatment: current challenges and emerging therapeutic advances. *Mol Cancer* 2023;22:20.
8 Adachi K, Kano Y, Nagai T, *et al*. IL-7 and CCL19 expression in CAR-T cells improves immune cell infiltration and CAR-T cell survival in the tumor. *Nat Biotechnol* 2018;36:346–51.
9 Anurathapan U, Chan RC, Hindi HF, *et al*. Kinetics of tumor destruction by chimeric antigen receptor-modified T cells. *Mol Ther* 2014;22:623–33.
10 Cherkassky L, Hou Z, Amador-Molina A, *et al*. Regional CAR T cell therapy: An ignition key for systemic immunity in solid tumors. *Cancer Cell* 2022;40:569–74.
11 Ott PA, Hu-Lieskovan S, Chmielowski B, *et al*. A Phase Ib Trial of Personalized Neoantigen Therapy Plus Anti-PD-1 in Patients with Advanced Melanoma, Non-small Cell Lung Cancer, or Bladder Cancer. *Cell* 2020;183:347–62.
12 Rojas LA, Sethna Z, Soares KC, *et al*. Personalized RNA neoantigen vaccines stimulate T cells in pancreatic cancer. *Nature New Biol* 2023;618:144–50.
13 Schumacher TN, Scheper W, Kvistborg P. Cancer Neoantigens. *Annu Rev Immunol* 2019;37:173–200.
14 Kubes P, Jenne C. Immune Responses in the Liver. *Annu Rev Immunol* 2018;36:247–77.
15 Liu Y, Zhang Q, Xing B, *et al*. Immune phenotypic linkage between colorectal cancer and liver metastasis. *Cancer Cell* 2022;40:424–37.
16 Sawa Y, Arima Y, Ogura H, *et al*. Hepatic interleukin-7 expression regulates T cell responses. *Immunity* 2009;30:447–57.
17 Wculek SK, Cueto FJ, Mujal AM, *et al*. Dendritic cells in cancer immunology and immunotherapy. *Nat Rev Immunol* 2020;20:7–24.
18 Roberts EW, Broz ML, Binnewies M, *et al*. Critical Role for CD103(+)/CD141(+) Dendritic Cells Bearing CCR7 for Tumor Antigen Trafficking and Priming of T Cell Immunity in Melanoma. *Cancer Cell* 2016;30:324–36.
19 Kitano M, Yamazaki C, Takumi A, *et al*. Imaging of the cross-presenting dendritic cell subsets in the skin-draining lymph node. *Proc Natl Acad Sci U S A* 2016;113:1044–9.

20 Spranger S, Dai D, Horton B, *et al*. Tumor-Residing Batf3 Dendritic Cells Are Required for Effector T Cell Trafficking and Adoptive T Cell Therapy. *Cancer Cell* 2017;31:711–23.
21 Connolly KA, Kuchroo M, Venkat A, *et al*. A reservoir of stem-like CD8$^+$ T cells in the tumor-draining lymph node preserves the ongoing antitumor immune response. *Sci Immunol* 2021;6:eabg7836.
22 Schenkel JM, Herbst RH, Canner D, *et al*. Conventional type I dendritic cells maintain a reservoir of proliferative tumor-antigen specific TCF-1$^+$ CD8$^+$ T cells in tumor-draining lymph nodes. *Immunity* 2021;54:2338–53.
23 Siddiqui I, Schaeuble K, Chennupati V, *et al*. Intratumoral Tcf1+PD-1+CD8+ T Cells with Stem-like Properties Promote Tumor Control in Response to Vaccination and Checkpoint Blockade Immunotherapy. *Immunity* 2019;50:195–211.
24 Miller BC, Sen DR, Al Abosy R, *et al*. Subsets of exhausted CD8$^+$ T cells differentially mediate tumor control and respond to checkpoint blockade. *Nat Immunol* 2019;20:326–36.
25 Wei J, Luo C, Wang Y, *et al*. PD-1 silencing impairs the anti-tumor function of chimeric antigen receptor modified T cells by inhibiting proliferation activity. *J Immunother Cancer* 2019;7:209.
26 Chen K, Wu Z, Zhao H, *et al*. *XCL1/Glypican-3* Fusion Gene Immunization Generates Potent Antitumor Cellular Immunity and Enhances Anti-PD-1 Efficacy. *Cancer Immunol Res* 2020;8:81–93.
27 Irvine KR, Rao JB, Rosenberg SA, *et al*. Cytokine enhancement of DNA immunization leads to effective treatment of established pulmonary metastases. *J Immunol* 1996;156:238–45.
28 Lu Q, Bian G, Chen Y, *et al*. Radioimmunotherapy of carcinoma of colon with [131I]-labeled recombinant chimeric monoclonal antibodies to carcinoembryonic antigen. *Acta Pharmacol Sin* 2005;26:1259–64.
29 Chng J, Wang T, Nian R, *et al*. Cleavage efficient 2A peptides for high level monoclonal antibody expression in CHO cells. *MAbs* 2015;7:403–12.
30 Walker AJ, Majzner RG, Zhang L, *et al*. Tumor Antigen and Receptor Densities Regulate Efficacy of a Chimeric Antigen Receptor Targeting Anaplastic Lymphoma Kinase. *Mol Ther* 2017;25:2189–201.
31 Goto S, Sakoda Y, Adachi K, *et al*. Enhanced anti-tumor efficacy of IL-7/CCL19-producing human CAR-T cells in orthotopic and patient-derived xenograft tumor models. *Cancer Immunol Immunother* 2021;70:2503–15.
32 Luo H, Su J, Sun R, *et al*. Coexpression of IL7 and CCL21 Increases Efficacy of CAR-T Cells in Solid Tumors without Requiring Preconditioned Lymphodepletion. *Clin Cancer Res* 2020;26:5494–505.
33 Lai J, Mardiana S, House IG, *et al*. Adoptive cellular therapy with T cells expressing the dendritic cell growth factor Flt3L drives epitope spreading and antitumor immunity. *Nat Immunol* 2020;21:914–26.
34 Klein O, Ebert LM, Zanker D, *et al*. Flt3 ligand expands CD4+ FoxP3+ regulatory T cells in human subjects. *Eur J Immunol* 2013;43:533–9.
35 Clappaert EJ, Kancheva D, Brughmans J, *et al*. Flt3L therapy increases the abundance of Treg-promoting CCR7$^+$ cDCs in preclinical cancer models. *Front Immunol* 2023;14:1166180.
36 Etxeberria I, Bolaños E, Quetglas JI, *et al*. Intratumor Adoptive Transfer of IL-12 mRNA Transiently Engineered Antitumor CD8$^+$ T Cells. *Cancer Cell* 2019;36:613–29.
37 Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 2003;3:133–46.
38 Xin G, Khatun A, Topchyan P, *et al*. Pathogen-Boosted Adoptive Cell Transfer Therapy Induces Endogenous Antitumor Immunity through Antigen Spreading. *Cancer Immunol Res* 2020;8:7–18.
39 Davidson BR, Sams VR, Styles J, *et al*. Comparative study of carcinoembryonic antigen and epithelial membrane antigen expression in normal colon, adenomas and adenocarcinomas of the colon and rectum. *Gut* 1989;30:1260–5.
40 Beauchemin N, Arabzadeh A. Carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) in cancer progression and metastasis. *Cancer Metastasis Rev* 2013;32:643–71.
41 Chmielewski M, Hahn O, Rappl G, *et al*. T cells that target carcinoembryonic antigen eradicate orthotopic pancreatic carcinomas without inducing autoimmune colitis in mice. *Gastroenterology* 2012;143:1095–107.