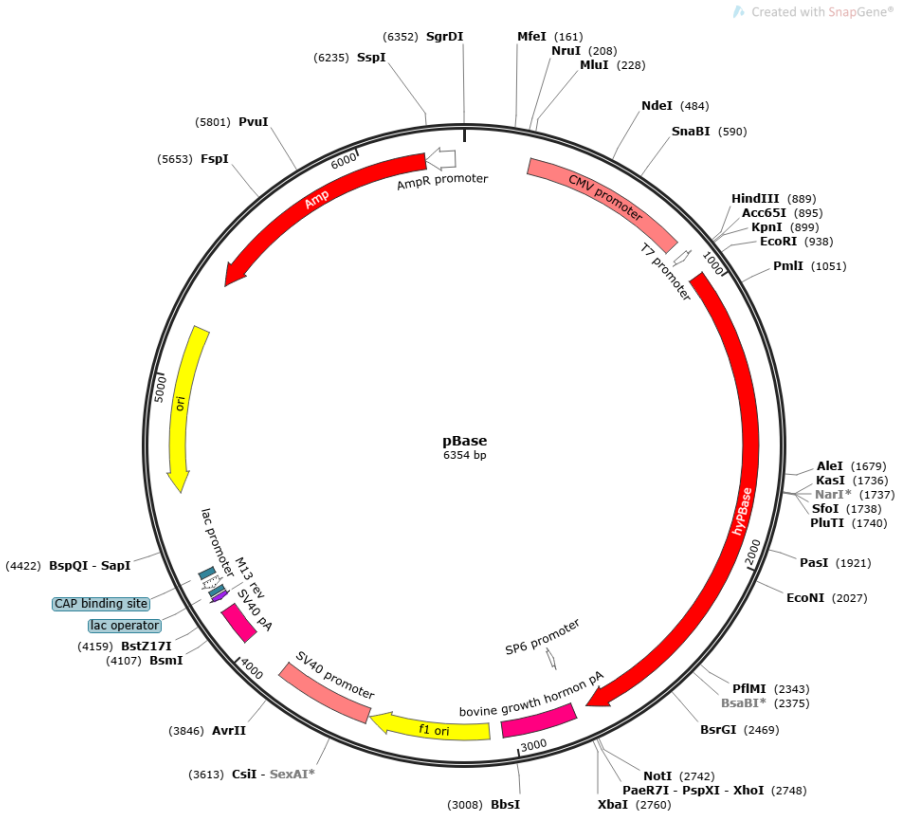
9 Přílohy

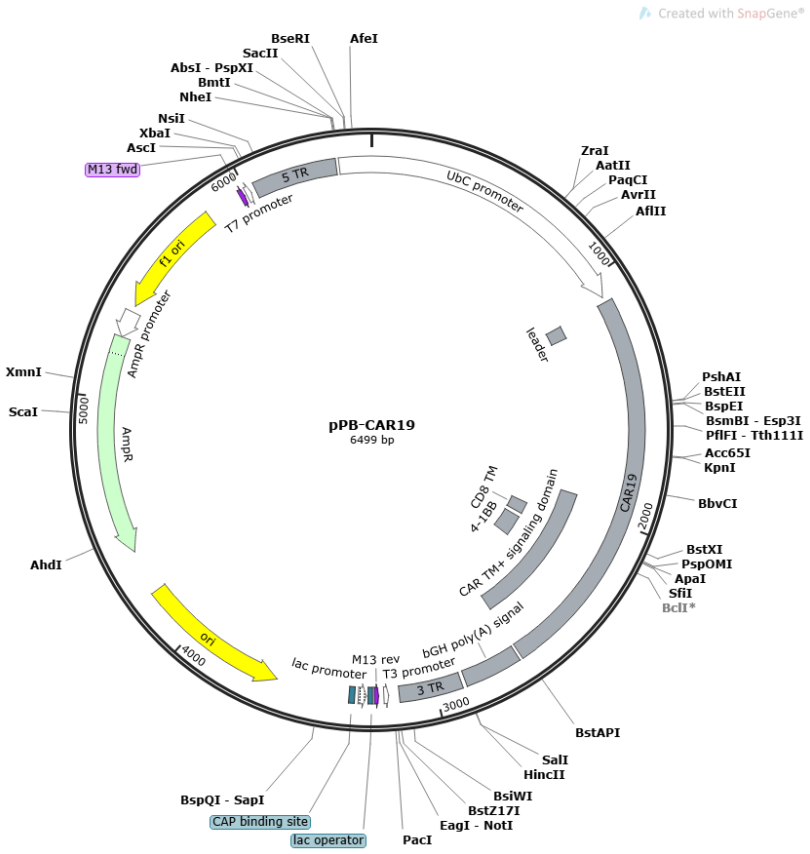
9.1 Plazmidy piggyBac vektorů

Ilustrační mapy vybraných vektorů použitých v Diplomové práci.

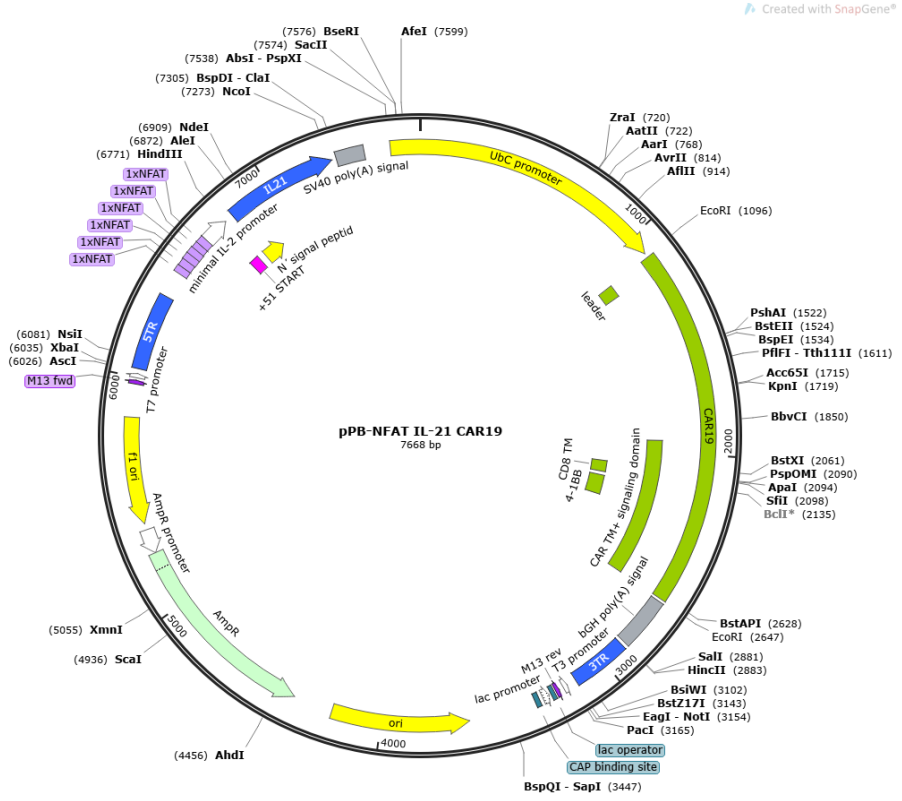
9.1.1 pBase



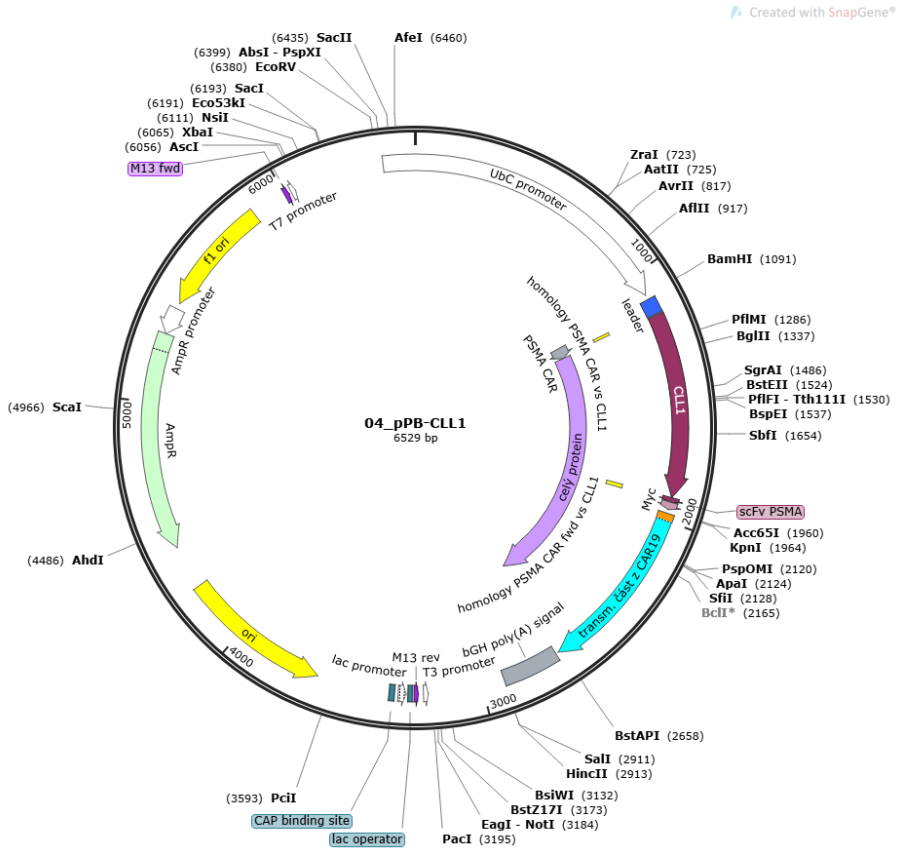
9.1.2 pPB-CAR19



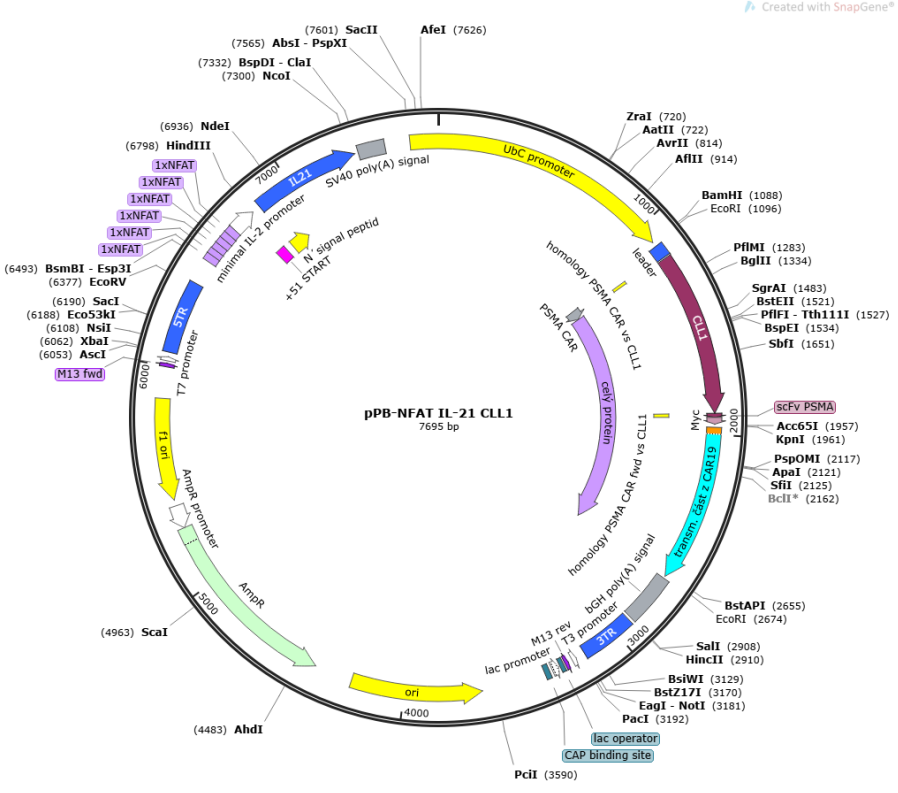
9.1.3 pPB-NFAT IL-21 CAR19



9.1.4 pPB-CLL1



9.1.5 pPB-NFAT IL-21 CLL1



9.2 DNA sekvence transpozonu pPB-NFAT IL-21 CAR19

Tripletová DNA sekvence transpozonu pPB-NFAT IL-21 CAR19

5 ITR

CTAGATTAACCCTAGAAAGATAGTCTGCGTAAAATTGACGCATGCATTCTTGAAATATTGCTCTCTCTTTCTAAATAGCGC
GAATCCGTCGCTGTGCATTTAGGACATCTCAGTCGCCGCTTGGAGCTCCCGTGAGGCGTGCTTGTCAATGCGGTAAGTG
TCACTGATTTTGAACTATAACGACCGCGTGAGTCAAAATGACGCATGATTATCTTTTACGTGACTTTTAAGATTTAACTC
ATACGATAATTATATTGTTATTTCATGTTCTACTTACGTGATAACTTATTATATATATATTTTCTTGTTATAGATATCAACTAGA
ATGCTAGCTGTCTCACAAAATAAAGTAAGCCCGGACTGAGTGCGGAAAGGCGGGCCTGGCGGGTCTGGTCTCCCCATG
CGGGCCACCAGAGGCCCTGCAGCCTTCAGTCT

6X NFAT

CGAGACGCCTTCTGTATGAAACAGTTTTTCCTCC**ACGCCTTCTGTATGAAACAGTTTTTCCTCC**ACGCCTTCTGTATG
AAACAGTTTTTCCTCC**ACGCCTTCTGTATGAAACAGTTTTTCCTCC**ACGCCTTCTGTATGAAA

minimalní IL-2 promotor

CAGTTTTTCCTCC**ACGCCTTCTGTATGAAACAGTTTTTCCTCC**CATTTTGACACCCCCATAATATTTTTCCAGAATTAA
CAGTATAAATTGCATCTCTTGTTCAAGAGTTCCCTATCACTCTCTTTAATCACTACTCACAGTAACCTCAACTCCTGCCCA
AGCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGAGGTCAAGTCCTGGCAAC

IL21

atggaacgcattgtgatttgcctgatggtgattttctgggcaccctggtgcataaaagcagccagggccaggatcgccatatgattcgcatgcgccagctgattgatattgtggatcagctga
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SV40 polyA

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AAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTACGCTTGAAGGGGTAATG
GCGCTTCCACTCACAAACATGGCGGACAGAGCGTGTGAACGAGATGAACAGCCCC

UBC promotor

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TGACTGGAGAACTCGGTTTGTCGTCTGTTGCGGGGGCGGCAGTTATGGCGGTGCCGTTGGGCAGTGCACCCGTACCTT
TGGGAGCGCGCGCCCTCGTCGTGTCGTGACGTCACCCGTTCTGTTGGCTTATAATGCAGGGTGGGGCCACCTGCCGGT
AGGTGTGCGGTAGGCTTTTCTCCGTCGCAGGACGCAGGGTTCGGGCCTAGGGTAGGCTCTCCTGAATCGACAGGCGCC
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ATTTGGGTCAATATGTAATTTTCAGTGTTAGACTAGTAAATTGTCCGCTAAATTCTGGCCGTTTTTGGCTTTTTTGTTAGA
CGGATCCATGAATTCGCCAGC

CAR19

ATGGCCCTGCCTGTGACAGCCCTGCTGCTGCCTCTGGCTCTGCTGCTGCATGCCGCTAGACCCACGCGTGAAGTGCAG
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ACTAGCTATGTTATGCACTGGGTGAAGCAGAAGCCTGGGCAGGGCCTTGAGTGGATTGGATATGTTAATCCTTACAATG
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CTGAGCCTCGTGATCACCCTGTACTGCAACCACCGGAACCGGCGGAGAGTGAAGCGGGGCAGAAAGAAGCTGCTGTA
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GCAC

bGH polyA

CGCCACCAAGGACACCTATGACGCCCTGCACATGCAGGCCCTGCCCCCCAGATGATGAGAATTCCGACTGTGCCTTCTA
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TAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAG
GGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTC

3 ITR

TATGGGTCGACATACTAGTTAAAAGTTTTGTTACTTTATAGAAGAAATTTTGAGTTTTTGTTTTTTTTTAATAAATAAATAA
ACATAAATAAATTGTTTGTTGAATTTATTATTAGTATGTAAGTGTAAATATAATAAAACTTAATATCTATTCAAATTAATAAA
TAAACCTCGATATACAGACCGATAAAACACATGCGTCAATTTTACGCATGATTATCTTTAACGTACGTCACAATATGATTA
TCTTTCTAGGGTTAATCTAGTATAC

9.3 Aminokyselinová sekvence CAR19

Leader (CD8) **Ig-kappa light chain**
MALPVTALLLPLALLLHAARPTREVQLQQSGPELVKPGASVKMSCKASGYTFT
SYVMHWVKQKPGQGLEWIGYVNPYNDGTKYNEKFKGKATLTSDKSSSTAY
MELSSLTSEDSAVYYCARGPYYYGSSPFDYWGQGTTVTV
linker **Ig heavy chain**
SGGGGSGGGGSGGGGSGGGGSDIVMTQSPLSLPVSLGDQASISCRSSQSLE
NSNGNTYLNWYLQKPGQSPQLLIYRVSNRFSGVLDRFSGSGSGT
CD8
DFTLKISRVEAEDLGVYFCLQVTHVPPTFGAGTKLEIKRTSPTTTPAPRP

transmembrane domain
PTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD**IYIWAPLAGTC**
4-1BB intracellular domain
GVLLLSLVITLYCNHRNRRRVKRGRKKLLYIFKQPFMRPVQTTQEEDGC
TCR zeta intracellular domain
SCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKR
RGRDPEMGGKPQRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGH
DGLYQGLSTATKDTYDALHMQALPPR*

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FULL-LENGTH ARTICLE

Translational Research

Inducible secretion of IL-21 augments anti-tumor activity of piggyBac-manufactured chimeric antigen receptor T cells

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ABSTRACT

Background: The efficiency of chimeric antigen receptor (CAR) T-cell-based therapies depends on a sufficient expansion of CAR T cells in vivo and can be weakened by intra-tumoral suppression of CAR T cell functions, leading to a failure of therapy. For example, certain B-cell malignancies such as chronic lymphocytic leukemia are weakly sensitive to treatment with CAR T cells. Co-expression of proinflamatory cytokines such as IL-12 and IL-18 by CAR T cells have been shown to enhance their antitumor function. We similarly engineered CAR T cell to co-express IL-21 and studied the effects of IL-21 on CAR T cells specific to CD19 and prostate-specific membrane antigens using an in vitro co-culture model and NSG mice transplanted with B-cell tumors.

Results: IL-21 enhanced the expansion of CAR T cells after antigenic stimulation, reduced the level of apoptosis of CAR T cells during co-culture with tumor cells and prevented differentiation of CAR T cells toward late memory phenotypes. In addition, induced secretion of IL-21 by CAR T cells promoted tumor infiltration by CD19-specific CAR (CAR19) T cells in NSG mice, resulting in reduced tumor growth. By co-culturing CAR19 T cells with bone-marrow fragments infiltrated with CLL cells we demonstrate that IL-21 reduces the immunosupressive activity of CLL cells against CAR19 T cells.

Conclusions: CAR19 T cells armed with IL-21 exhibited enhanced antitumor functions. IL-21 promoted their proliferation and cytotoxicity against chronic lymphocytic leukemia (CLL). The results suggest that arming CAR T cells with IL-21 could boost the effectiveness of CAR T-mediated therapies.

Introduction

Various strategies are currently being explored to improve the functionality of chimeric antigen receptor (CAR) T cells and enhance their survival in vivo. In addition to T-cell receptor engagement and co-stimulatory signaling, cytokines play a critical role in modulating T-cell function. Therefore, an appealing strategy is to engineer CAR T cells to produce cytokines that enhance anti-tumor responses by acting on tumor stroma or influence CAR T cells directly in a paracrine/autocrine manner. Several pro-inflammatory cytokines, including IL-12, IL-15 and IL-18, that are co-expressed by CAR T cells have been shown to modulate their anti-tumor functions [1 3], supporting this strategy. The authors studied another relevant cytokine as a potential enhancer of CAR T cells, IL-21. IL-21 is a pleiotropic cytokine that acts on many lymphocyte subsets but in natural T cells and CAR T cells promotes expansion and the maintenance of the early memory phenotype [4]. Interestingly, the effects of IL-21 are counteracted by high concentrations of IL-2, which directs CAR T cells toward terminal memory phenotypes and, eventually, activation-induced cell death (AICD) [5]. It is thought that such early memory CAR T cells (CD62L+/CCR7+/CD45RA+/CD28+/CD27+/CD95+/PD-1) have enhanced in vivo persistence and provide greater anti-tumor effects than the more differentiated T-cell subsets [6,7]. These experimental observations were further verified by the outcomes of clinical studies with CD19-specific CAR (CAR19) T cells in patients with B-cell acute lymphoblastic leukemia (B-ALL), showing that more efficient treatment correlated with an increased number of less differentiated CAR T cells with early memory phenotype [8]. Although CAR19 T cells have been shown to be highly effective against B-ALL, their efficiency against indolent B-cell lymphoproliferative diseases, such as chronic lymphocytic leukemia (CLL), is significantly weaker [9]. Recently, CLL cells were shown to directly inhibit proliferation of CAR19 T cells [10] and impede their functions. The efficiency of CAR T cells against solid tumors is even worse, as solid tumors induce

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abortive activation and apoptosis of infiltrating lymphocytes, which prevents their long-term expansion [11].

These facts led the authors to further study the effects of IL-21 on memory differentiation of CAR T cells. The authors hypothesized that the efficiency of CAR T cells can be augmented by modulating their memory differentiation by inducing the expression of IL-21. The authors constructed an artificial nuclear factor of activated T cells (NFAT) promoter, as described for IL-12 or IL-18 [1,3,12], which becomes activated upon anti-genic stimulation of CAR T cells. The authors prepared human CAR T cells to react to prostate-specific membrane antigen (PSMA) and CD19 antigen co-expressing NFAT-regulated IL-21 (NFAT IL-21). CAR T cells were manufactured via electroporation of piggyBac transposon vector, followed by in vitro expansion in a cocktail of cytokines containing IL-4, IL-7 and IL-21, as the authors described previously [6], resulting in a homogeneous population of >90% CAR+ T cells with an early memory phenotype. CAR T cells were then repeatedly restimulated with tumor cells in vitro or analyzed in NSG mice transplanted with B-cell lymphoma cells. To study the effects of CAR19 T cells against CLL, the authors developed an in vitro model based on co-cultivation of CAR19 T cells with bone marrow (BM) fragments obtained from patients with CLL that were infiltrated by CLL cells.

The authors found that IL-21 inhibited terminal differentiation of CAR T cells after anti-genic restimulation with tumor cells and promoted their expansion and the development of effector functions. By contrast, IL-2 enhanced the expansion of CAR T cells to lower levels, with opposite effects on memory differentiation, producing terminally differentiated CAR T cells expressing significantly more PD-1. CAR T cells engineered with induced secretion of IL-21 had increased anti-tumor effects against B-cell tumors transplanted in NSG mice and enhanced activity against CLL cells compared with non-armed CAR19 T cells. In summary, the authors' results show that IL-21 is an important regulator of T-cell memory development and suggest that modification of CAR19 T cells to secrete IL-21 upon their activation could be another way to enhance their therapeutic potency—for example, against weakly sensitive B-cell malignancies, such as CLL.

Methods

Cell source

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from blood donors. The use of all human materials was approved by the institutional review board, and all donors signed informed consent for the use of biological materials. Ramos is a CD19+ Burkitt lymphoma cell line (American Type Culture Collection CRL-1596). LNCaP is a prostate carcinoma cell line positive for PSMA (American Type Culture Collection CRL-1740). BM fragments were obtained by trephine biopsy from donors with newly diagnosed CLL, and 20 90% infiltration of the BM was verified by fluorescence-activated cell sorting (FACS) and immunohistochemistry.

DNA plasmids and sequences

CAR19 and PSMA CAR constructs have been described previously [6,13]. Both CARs have the same design and contain a 4-1BB co-stimulatory domain and T-cell receptor zeta domain. The hyperactive piggyBac transposase is driven by a cytomegalovirus promoter [14]. To construct the NFAT IL-21 vector, the promoter was synthesized containing £6 NFAT binding motif, minimal IL-2 promoter, human full-length IL-21 and SV40 polyA. This segment was cloned behind the CAR sequence in the same direction as the CAR gene into CAR19 and PSMA CAR vectors via XhoI-SalI. All CAR constructs were cloned into the piggyBac vector behind the ubiquitin C promoter. For fluorescent labeling of T cells, green fluorescent protein (GFP) was cloned into a piggyBac vector in a manner similar to that used for the CAR constructs. Plasmids were purified by standard techniques using EndoFree kits (Qiagen, Germany). The sequences of PSMA CAR and NFAT promoter are provided in the supplementary material. The sequence of CAR19 has been published previously [13].

Cell culture, electroporation and antibody stimulation

For all experiments, the authors used CellGro media (CellGenix, Germany) supplemented with 10% heat-inactivated fetal calf serum (Gibco, USA) and the antibiotics penicillin and streptomycin (Gibco, USA). PBMCs were isolated from buffy coats or fresh blood samples by gradient centrifugation using Ficoll-Paque premium (GE Healthcare). After separation, cells were transfected with a Neon electroporator (Thermo Fisher Scientific, USA); 1 £ 10e7 cells were resuspended in 100 mL buffer T and mixed with 5 mg plasmid DNA (2:1 mixture of piggyBac vector expressing CAR and a plasmid expressing a hyperactive piggyBac transposase) and electroporated for 20 ms at 2300 V using 100-mL tips. Cells then rested overnight in cell media with cytokines. The next day, the cells were stimulated with immobilized antibodies or left unstimulated. To manufacture GFP+ CAR T cells, a 2-mg GFP pPB vector was added to a mixture of plasmids. Virtually all GFP+ cells were positive for CAR and GFP cells negative for CAR (data not shown).

To stimulate T cells with antibodies, both anti-CD3 and anti-CD28 mouse monoclonal antibodies (Miltenyi Biotec, Germany) and mouse anti-myc-tag antibody clone 9E10 (Exbio, Czech Republic) were diluted in phosphate-buffered saline at a concentration of 1 mg/mL and adhered onto a cell culture plate for 1 h at 37°C. The cells were washed with phosphate-buffered saline and used for cell activation. After 3 days of stimulation, cells were replated onto new culture plates.

Cell media were supplemented with cytokines as follows: 1000 U/mL IL-2 (Proleukin; Roche), 20 ng/mL IL-4 (Miltenyi Biotec), 10 ng/mL IL-7 (Miltenyi Biotec) and 40 ng/mL IL-21 (Miltenyi Biotec). Cells were fed with fresh media containing cytokines every 3 4 days according to growth rate. To sort cells by FACS, the authors used fluorescently labeled antibodies and isolated on a FACSAria instrument (BD Biosciences, USA).

Antibodies, FACS and enzyme-linked immunosorbent assay

Mouse anti-human CD3 APC-Cy7, CD45RA Alexa Fluor 488, CCR7 BV605, CD45RA-BUV737 (clone HI100), CD62L-BV650 (clone DREG-56), CD56-BUV563 (clone NCAM16.2), CD244-APC (clone 2-69), BTLA-BV421 (clone J168-540), CD3-BV786 (clone UCHT1), Tim3-BV480 (clone 7D3), CD160-PE (clone B455), PD1-BB700 (clone EH12.1), TIGIT-BV421 (clone 741182) and LAG3-PE (clone T47-530) were purchased from BD Biosciences (USA). LAG3-PE-eFluor610 (clone 3DS223H) and LAG3-APC (clone 3DS223H) were purchased from eBioscience (USA). TIGIT-PE-Cy7 (clone A151536), CD4-qDOT605 (clone S3.5), CD28-PE-Cy7 (clone CD28.2) and PD1-PE (clone EH12.2H7) were purchased from BioLegend (USA). CD8-AF700 (clone MEM-31), CD27-PE-Dy590 (clone LT27), Myc-FITC (clone 9E10), CD107a-PE (clone H4A3) and Annexin V-Phycoerythrin were purchased from Exbio. CD57-APC-Vio7701 (130-104-197), Myc-FITC (120-003-159), CD28 (clone 15E8) and CD3 (130-093-387) were purchased from Miltenyi Biotec. CD4-PB (clone RPA-T4) was obtained from Sony (USA). Live cells were identified using a fixable blue dead cell stain kit (Thermo Fisher Scientific, USA).

To detect the CAR transgene, cells were stained with Alexa-647- or Alexa-488-labeled Fab2 fragment from goat anti-mouse IgG (Jackson ImmunoResearch, UK). This polyclonal antibody reacts with mouse anti-CD19 scFv in CAR19. To detect PSMA CAR, cells were labeled with anti-myc-tag antibody clone 9E10 (Exbio). Cells were labeled with goat anti-mouse antibody, washed twice and blocked with 10% mouse serum and then stained with fluorescently labeled

mouse monoclonal antibodies. The specificity of staining was extensively tested to rule out possible binding of mouse antibodies to cells due to the goat anti-mouse antibodies used to detect CAR.

To visualize in situ proliferation of CAR19 T cells, Ramos cells were adhered onto polylysine-coated (Merck, USA) chambered glass coverslips (Labtek, USA), and adherent LNCaP cells were directly grown on glass slides. Next, cells were briefly co-incubated with CAR T cells at a low E:T ratio and then overlaid with 0.5% agarose prepared in cell media. Cells were imaged using a fluorescent microscope with a digital camera (Olympus, Japan).

To determine the degranulation of CAR T cells, cells were incubated with or without Ramos B cells at a 1:1 ratio for 4 h. Phycoerythrin-labeled anti-CD107a antibody (Exbio) was added to the cell media at the beginning of the co-incubation. After 4 h, cells were harvested and labeled with anti-CD8 and anti-CD4 antibodies and analyzed by FACS on a FACS LSRFortessa instrument (BD Biosciences). To detect apoptotic cells, fluorescent caspase-3/7 substrate (Life Sciences, USA) was added to the cells for 30 min before FACS analysis.

To detect IL-21, CAR T NFAT 21 cells were stimulated overnight with anti-CD3 antibody or Ramos cells. After incubation, cells were freeze-thawed twice and the supernatants centrifuged to remove cell debris. The samples were then analyzed using an IL-21 enzyme-linked immunosorbent assay kit (BioLegend, USA). Absorbances were determined on an enzyme-linked immunosorbent assay plate reader and concentrations of IL-21 calculated against IL-21 standards provided with the kit using an Excel spreadsheet.

The production of interferon gamma was determined using a cytokine capture kit (Miltenyi Biotec) according to the manufacturer's instructions. Briefly, T cells cultured in the presence of CLL cells for 7 days were restimulated with Ramos B cells for 4 h in the presence of an interferon gamma labeling reagent. The cells were then washed and labeled with an interferon gamma detection reagent and monoclonal antibodies. FACS samples were analyzed using the LSRFortessa instrument (BD Biosciences) and FACS data processed using FlowJo software. Statistical analysis was performed using Prism software (GraphPad). The gating strategy for the phenotype analysis is provided in supplementary Figure 1.

In vitro co-cultivation assays

CAR T cells were cultured together with their tumor target cells at a 1:5 E:T ratio without cytokines or with the addition of IL-2 or IL-21. Ramos cells were used for CAR19 and LNCaP cells for PSMA CAR. The CAR T cells were restimulated with cells two to three times every 3 4 days depending on the experiment. To restimulate CAR T cells with BM fragments, a BM specimen obtained by trephine biopsy was cut with a blade into several pieces approximately 2 mm in size. BM fragments were cultured in a suspension of CAR19 T cells (1 million/mL) for 24 h and then gently washed with fresh media and cultured for 12 days in media without cytokines. Microscopic images of GFP+ cells were taken during the culture using a confocal microscope to asses infiltration of the BM. At the end of the experiments, BM fragments were washed with media and dissociated by pipetting and then used for functional assays and FACS analysis. The differences between groups were analyzed by a two-tailed unpaired t-test using Prism software (GraphPad).

In vivo experiments and statistical analysis

In vivo studies were approved by the Institutional Animal Care and Use Committee. Immunodeficient NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, or NSG, mice (Jackson Laboratory) were maintained in individually ventilated cages under specific pathogen-free conditions. Groups of five or six mice aged 6 12 weeks and of the same sex were injected subcutaneously with 5 £ 10e6 Ramos B cells. After 12 days, when large macroscopic tumors had formed, the mice received one intravenous dose of 5 £ 10e6 CAR T cells. Both CAR19 NFAT IL-21 and control CAR19 T cells were grown in IL-4, IL-7 and IL-21. After another 18 days, the mice were killed, tumors excised and weighed and tumor tissues mechanically dissociated by pipetting and analyzed by flow cytometry for the presence of human CD4 and CD8 T cells. The differences between groups were analyzed by a two-tailed unpaired t-test. Mice were also analyzed by one-way analysis of variance with Dunnett's multiple comparison post-test using Prism software (GraphPad). All animal work was performed under approved ethical guidelines.

Results

To characterize the prepared cells, the authors determined the percentage of CAR19 T cells with early memory phenotype or a stem cell memory-like (Tscm-like) phenotype, characterized by expression of antigens CD45RA, CD62L, CD27 and CD28 [7]. The data in Figure 1A show that the majority of CAR19 T cells cultivated in the presence of IL-4, IL-7 and IL-21 were mostly of the early memory phenotype. Both cultivation methods produced CAR19 T cells with an activated phenotype, as the authors detected upregulation of antigen CD95 (Figure 1F). The expansion of CAR19 T cells did not require anti-CD3/CD28 stimulation because the transduced T cells were activated by B cells, which were present in the mixture of electroporated PBMCs. Omitting anti-CD3/CD28 activation enabled selective expansion of the modified T cells, yielding >90% CAR+ T cells [6], and did not significantly influence the phenotype of CAR19 T cells. By contrast, cultivation in the presence of IL-2 plus activation with anti-CD3/CD28 antibodies yielded a population with a very low number of Tscm-like CAR19 T cells (Figure 1A).

CAR19 T cells grown in IL-4, IL-7 and IL-21 had significantly better viability than CAR19 T cells grown in the presence of IL-2 with anti-CD3/CD28 after anti-genic restimulation with Ramos B cells (Figure 1B). The addition of IL-2 or IL-21 during co-cultivation with Ramos B cells improved their expansion, but the effects of IL-21 were stronger (Figure 1C). The authors noted minimal expansion of CAR19 T cells grown in the presence of IL-2 with anti-CD3/CD28 in the absence of exogenously added cytokines. Subsequently, the authors determined the memory phenotype (Figure 1D) and expression of exhaustion marker PD-1 (Figure 1E) on CAR19 T cells after restimulation with Ramos B cells. The addition of IL-21 maintained the Tscm-like phenotype of proliferating cells, whereas the addition of IL-2 induced the expansion of more differentiated CAR19 T cells expressing high levels of PD-1 compared with non-restimulated CAR19 T cells (Figure 1E). These results suggest that IL-21 promotes not only the expansion of CAR19 T cells but also the maintenance of early memory phenotype and improves their survival after anti-genic restimulation with tumor cells.

In the next experiment, the authors sorted CD8+ CAR19 T cells expressing CD62L+ and CD45RA+ (Figure 2A) and restimulated them twice with Ramos B cells in the presence of IL-2 or IL-21. Similar to previous experiments, the addition of IL-21 significantly enhanced expansion more than the addition of IL-2 (Figure 2B), and the CAR19 T cells restimulated in the presence of IL-21 retained their immature phenotype (>90% of cells were CD27+CD28+CD62L+) (Figure 2C). By contrast, the addition of IL-2 during restimulation resulted in a significant loss of expression of CD62L, CD27 and CD28, indicating that although IL-2 promotes the expansion of CAR19 T cells, it induces their differentiation toward effector CD8+ T cells.

Next, the authors assessed the effects of IL-21 on CAR19 T cells grown in the presence of IL-2 and vice versa. Unsorted CAR19 T cells grown in the presence of IL-2 with anti-CD3/CD28 were co-cultured with Ramos B cells in the presence of IL-21, and CAR19 T cells grown in the presence of IL-4, IL-7 and IL-21 were co-cultured with Ramos B cells in the presence of IL-2 (Figure 2D). IL-21 enhanced the expansion of Tscm-like CAR T cells during co-culture, but the upregulation

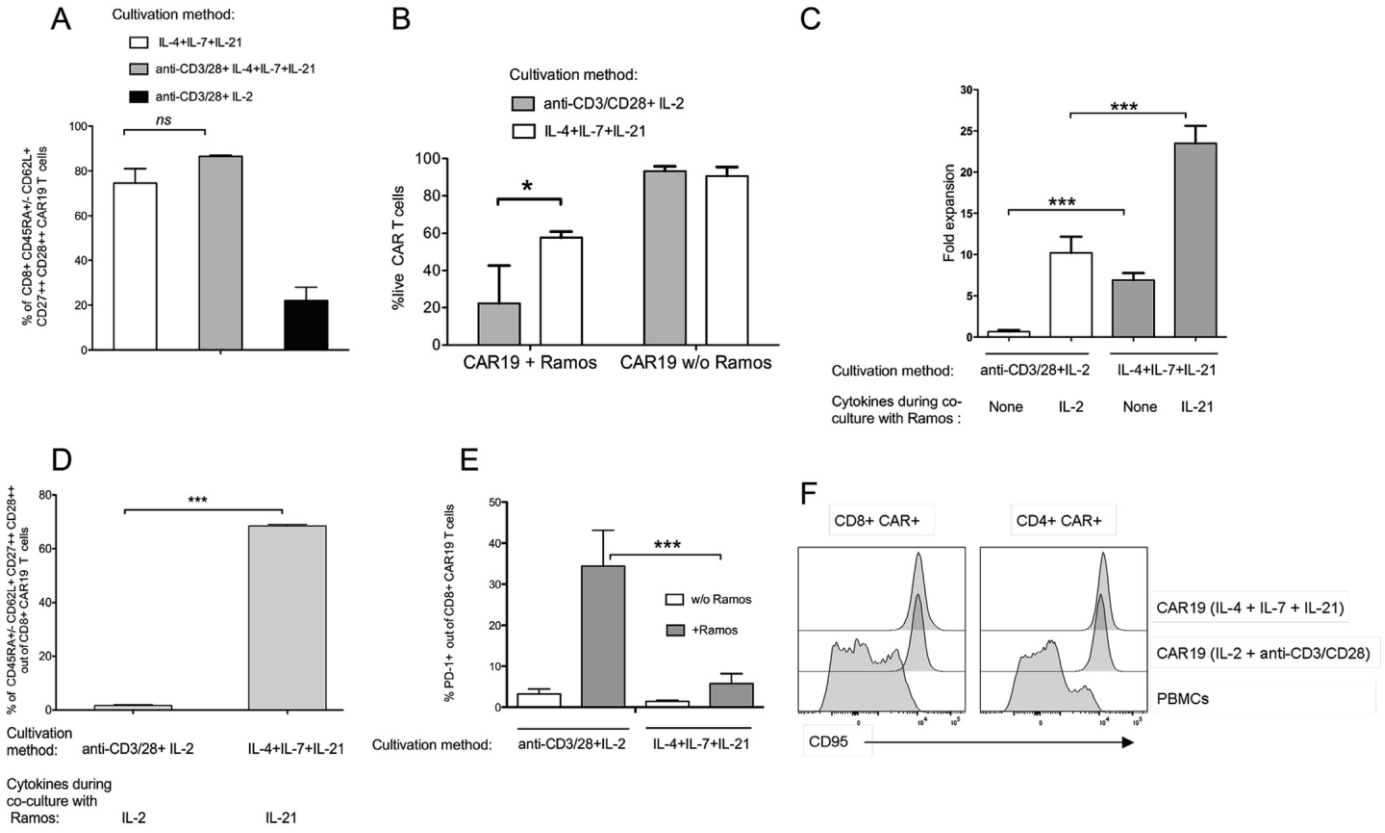

Figure 1. IL-21 promotes expansion of CAR19 T cells and maintenance of early memory phenotype after anti-genic restimulation. (A) Percentage of Tscm-like (CD45RA+/ CD62L+ CD27++ CD28++) CAR19 T cells in the product obtained by cultivating in a mixture of IL-4, IL-7 and IL-21 or a mixture of IL-4, IL-7 and IL-21 plus activation with anti-CD3/28 antibodies or CAR19 T cells manufactured by cultivation in IL-2 activated with anti-CD3/28 antibodies. (B) Percentage of viable (non-apoptotic) CAR T cells after co-cultivation with Ramos cells for 5 days. No exogenous cytokines were added to the cells in this experiment. The viability was determined with fluorescent caspase-3/7 substrate and a DNA dye (DAPI). (C) The fold expansion after two rounds of restimulation with Ramos cells in the presence or absence of IL-2 or IL-21. (D) Percentage of Tscm-like CD8+ CAR T cells after two rounds of restimulation with Ramos cells in the presence of IL-2 or IL-21. (E) Percentage of PD-1+CD8+ CAR T cells after two rounds of restimulation with Ramos cells in the presence or absence of IL-2 or IL-21. The white bar shows the expression of PD-1 on the product without restimulation with Ramos cells. N = 3; error bars indicate SEM. Significance was determined by unpaired t-test. (F) Histogram of the expression of CD95 on non-co-cultivated CD4+ and CD8+ CAR T cells in one representative donor compared with CD4+ and CD8+ T cells from a healthy donor. ns (not significant) P > 0.05, * P 0.05, *** P 0.001. DAPI, 4',6-diamidino-2-phenylindole; ns, not significant; SEM, standard error of the mean; w/o, without.

of PD-1 depended on the presence of IL-2. If IL-2 was added during the co-culture of CAR T cells prepared in IL-4+IL-7+IL-21, it induced the downregulation of CD62L, CD27 and CD28 but did not induce the upregulation of PD-1 (Figure 2D, middle panel). By contrast, the addition of IL-21 during the co-culture of CAR T cells prepared in IL-2 did not prevent upregulation of PD-1 but enabled expansion and maintenance of the early memory phenotype (CD62L+CD27+CD28+) (Figure 2D, bottom panel). Thus, the upregulation of PD-1 depends on previous exposure of CAR T cells to IL-2.

The authors decided to investigate whether the effects of IL-21 were also observed using CAR T cells targeting tumor cells of non-hematological origin. The authors tested CAR T cells specific for antigen PSMA, which specifically recognizes the LNCaP carcinoma cell line. The PSMA CAR construct contains a myc-tag epitope between scFv and a transmembrane domain, and activation of transduced T cells with immobilized myc-tag-specific antibody (clone 9E10) leads to selective expansion of PSMA CAR T cells. Total T-cell expansion was equal regardless of whether cells were activated via anti-myc antibody or anti-CD3/CD28 antibodies or whether IL-2 or a cocktail of IL-4, IL-7 and IL-21 was used during in vitro culture (Figure 3A). However, anti-myc activation produced >90% CAR+ cells, whereas polyclonal activation with anti-CD3/CD28 antibodies produced only about 15% CAR+ T cells (Figure 3C). The phenotype of PSMA CAR T cells also depended on the cytokines used for the expansion, as the authors have shown in previous experiments [6]. Cultivation in IL-2 yielded approximately 5% Tscm-like CD8+ PSMA CAR T cells (CD45RA +/ CD62L+CD27++CD28++), whereas cultivation in the presence of IL-4, IL-7 and IL-21 yielded approximately 60 70% Tscm-like PSMA CAR T cells after anti-myc activation and approximately 85% Tscm-like CD8+ PSMA CAR T cells after anti-CD3/CD28 activation (Figure 3B). The authors noted a slightly higher number of Tscm-like cells after anti-CD3/CD28 activation in the presence of IL-4, IL-7 and IL-21, similar to what was observed with CAR19 T cells (Figure 1A).

Next, the authors hypothesized that IL-21 may enable the expansion of Tscm-like PSMA CAR T cells after restimulation with LNCaP cells, similar to what has been observed in previous experiments. Instead of using PSMA CAR T cells expanded in IL-4, IL-7 and IL-21, the authors tested whether the small number of Tscm-like cells detected after expansion in IL-2+ anti-myc (Figure 3B) could be "rescued" by IL-21. Thus, the authors determined the phenotype (Figure 3D) and level of expansion (Figure 3E) of PSMA CAR T cells (prepared in IL-2) after two rounds of restimulation with LNCaP in the presence of IL-21 or IL-2 or without any exogenous cytokines. PSMA CAR T cells expanded roughly 2.5 times more in the presence of IL-21 than in the presence of IL-2 (Figure 3E), whereas no expansion was observed in the absence of exogenous cytokines; therefore, it was not possible to reliably determine the phenotype of remaining cells. FACS analysis showed (Figure 3D) that CD8+ PSMA CAR T cells expanded in the presence of IL-2 were late-stage T cells, the majority of which were Temra (CD62L CD45RA+CD27 CD28) cells. By contrast, co-cultivation in the presence of IL-21 led to a stronger expansion of PSMA CAR T cells, and a significant proportion of

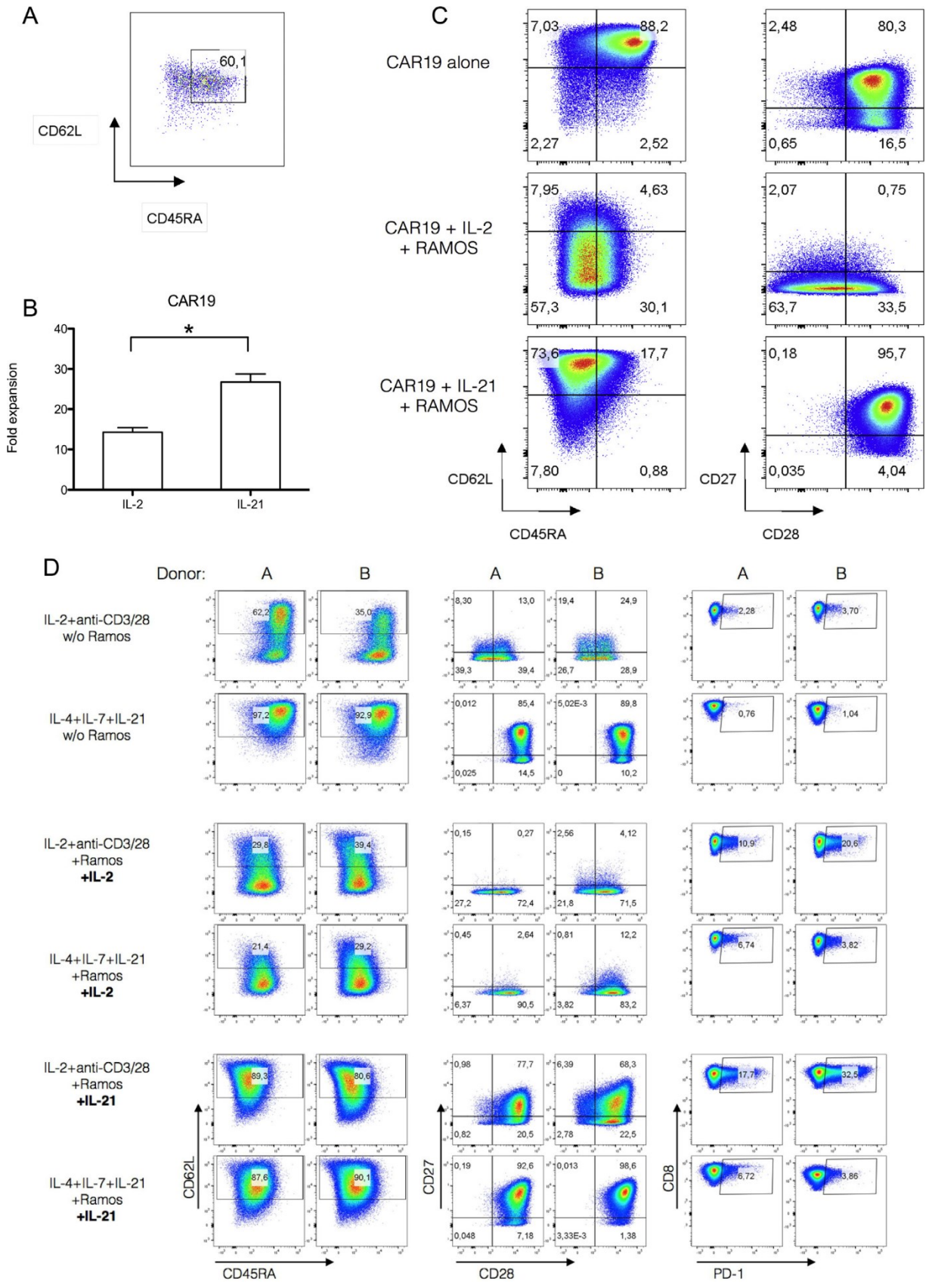

Figure 2. IL-2 supports proliferation of CAR19 T cells but does not promote maintenance of early memory phenotype. (A) CAR19 T cells cultivated in IL-4, IL-7 and IL-21 were sorted by FACS to isolate early memory T cells (CD62L+CD45RA+) and then restimulated with Ramos B cells in the presence of exogenous IL-2 or IL-21 or left in media with cytokines without Ramos cells. After 7 days, CAR19 T cells were counted and their developmental status determined by FACS. (B) Fold expansion of sorted CAR19 T cells after co-cultivation with Ramos cells in the presence of IL-2 or IL-21. N = 2; error bars indicate SEM. Significance was determined by unpaired t-test. (C) Dot plot showing the phenotype of CD8+ CAR T cells after restimulation for one representative donor out of two. Both donors had almost identical results. (D) CAR19 T cells were obtained by cultivation in a mixture of IL-4, IL-7 and IL-

these expanded cells were CD62L+CD28+; some were also positive for CD27. Notably, the initial population contained very low numbers of Tscm-like CD8+ T cells because PSMA CAR T cells were cultured in IL-2.

Next, the authors analyzed the sensitivity of differently expanded PSMA CAR T cells to apoptosis after restimulation with LNCaP cells (Figure 3F). Apoptotic cells were identified by staining cells with a fluorescent caspase-3/7 substrate after 1 day, 2 days or 3 days of restimulation. The data show that the least sensitive cells to apoptosis were PSMA CAR T cells expanded in IL-4, IL-7 and IL-21 and restimulated with LNCaP cells in the presence of exogenous IL-21. Thus, PSMA CAR T cells with Tscm-like phenotype are significantly more resistant to AICD than the more differentiated cells, and exogenous IL-21 can improve their survival. These findings are in agreement with similar data presented in Figure 1B,C.

As the authors' data suggested a positive effect of IL-21 on the viability and expansion of CAR T cells, CAR T cells were engineered to secrete IL-21 after T-cell activation using an NFAT-regulated artificial promoter [3] to drive the expression of IL-21. In T cells, the antigen receptor stimulation activates the Ca^{2++} -calmodulin pathway, resulting in dephosphorylation of NFAT family transcription factors via phosphatase calcineurin, inducing them to translocate to the nucleus, where they bind NFAT response elements and modulate gene expression [15]. Artificial promoters containing NFAT response elements activate transcription similar to endogenous NFAT promoters and are commonly used to regulate gene expression in an activation-dependent manner [3]. The promoter DNA sequence is provided in supplementary Figure 1C,D.

To characterize the functionality of the NFAT IL-21 gene, the authors determined the production of IL-21 by CAR19 NFAT IL-21 T cells after activation via anti-CD3 antibody or Ramos B cells (Figure 4A). IL-21 was produced after anti-CD3 activation and at lower amounts after activation with Ramos cells. The functionality of PSMA CAR NFAT IL-21 T cells was similarly verified by measuring the production of IL-21 after activation (data not shown). To determine whether transgenic production of IL-21 enhances the expansion of CAR T cells, the authors prepared GFP-labeled CAR19 T cells and restimulated them with Ramos cells in a soft agar matrix. The growth of the CAR19 T cells was then monitored under a fluorescent microscope by measuring the size of proliferating cell clusters. The images in Figure 4B show an example of representative GFP+ clusters after 7 days. Estimation of the cell number per cluster shows that the majority of clusters in the CAR19 NFAT IL-21 sample were composed of more than five cells, whereas the clusters in the CAR19 T cell sample were small, with one or two cells per cluster (Figure 4C). The authors performed a similar experiment with GFP-labeled PSMA CAR NFAT IL-21 T cells (see supplementary Figure 2A,B), which similarly showed that expansion was stimulated by endogenously secreted IL-21.

Next, the authors determined the effect of induced IL-21 secretion on the phenotype of CAR19 T cells after restimulation with Ramos B cells (Figure 4D). FACS analysis showed that majority of CAR19 NFAT IL-21 T cells were CD62L+CD27++CD28++ compared with non-armed CAR19 T cells (~56% versus 13%). Repeated anti-genic restimulation may result in inhibition of effector functions; therefore, the authors tested the functionality of co-cultivated CAR19 NFAT IL21 cells via degranulation assay (see supplementary Figure 3B). This experiment revealed that CAR19 NFAT-21 cells were functional. In addition, the authors tested the production of pro-inflammatory cytokines by CAR19 NFAT IL-21 T cells and non-armed CAR19 T cells before and after co-cultivation with Ramos cells using a Bio-Plex assay (see supplementary Figure 4). These results indicate that CAR19 NFAT IL-21 remained functional and retained the ability to secrete pro-inflammatory cytokines upon repeated anti-genic challenge. Thus, the authors demonstrated that induced secretion of IL-21 is biologically active and enhances the expansion of functional early memory CAR T cells.

Next, the authors determined whether CAR19 NFAT IL-21 T cells have enhanced anti-tumor functions using NSG mice transplanted with Ramos B cells (Figure 5A). Transgenic expression of IL-21 enhanced the anti-tumor activity of CAR19 NFAT IL-21 cells (Figure 5B) and significantly enhanced the infiltration of tumors by CD8+ CAR19 NFAT IL-21 (Figure 5D) compared with non-armed CAR19 T cells. The infiltration by CD4+ CAR T cells was approximately 10-fold lower than the infiltration by CD8+ CAR T cells (Figure 5C,E). Analysis of the immunophenotype of T cells recovered from tumors revealed that CAR19 NFAT IL-21 maintained the early memory phenotype (see supplementary Figure 5). These results further demonstrate the enhancement of CAR T-cell functions by induced secretion of IL-21.

The authors' next goal was to try to assess CAR19 NFAT IL-21 T-cell functions in a more physiological manner against directly isolated malignant human B cells. The microscopy images in Figure 6A show the representative samples after 3 days and 10 days of cultivation. The graph in Figure 6B shows the percentage of GFP+ cells in paired samples (the same PBMC donors tested against the same CLL patients). Both the microscopic images and the FACS results show that CAR19 NFAT IL-21 T cells infiltrated the BM to much higher levels than non-armed CAR19 T cells. The FACS analysis of cells recovered from BM fragments showed that induced secretion of IL-21 promoted maintenance of the Tscm-like phenotype (Figure 6C). However, no changes in the CD8:CD4 ratio between CAR19 and CAR19 NFAT IL-21 T cells were detected in these samples (Figure 6F). Interestingly, when testing the functionality of infiltrating CAR19 T cells in degranulation assays, the authors found that the non-armed CAR19 T cells had reduced degranulation, whereas CAR19 NFAT IL-21 T cells efficiently degranulated after challenge with Ramos cells (Figure 6E). In addition, the cytotoxic test against Ramos cells revealed that NFAT IL-21 CAR T cells recovered from BM fragments were functional (Figure 6D), similar to control CAR19 NFAT IL-21 T cells cultivated in media without BM fragments. To further demonstrate the ability of IL-21 to counteract the immunosuppressive effects of CLL cells, the authors determined the production of interferon gamma by CAR T cells co-cultured with CLL cells. CAR19 NFAT IL-21 T cells produced significantly more interferon gamma than CAR19 T cells not armed with IL-21 after anti-genic restimulation (0% versus 53%) (Figure 6G). The basis of this inhibition is not yet known, but the suppressed CAR19 T cells did not express inhibitory receptors PD-1, TIM-3 or LAG-3, and they were not senescent, as they did not express CD57 (data not shown). These data suggest that IL-21 enhances the activity of CAR19 T cells against CLL by stimulating their proliferation.

In conclusion, the data show that IL-21 augments the anti-tumor functions of CAR T cells via modification of their memory differentiation. IL-21 promotes maintenance of the early memory phenotype (CD62L+CD27++CD28++PD-1) after anti-genic restimulation, which supports the survival and expansion of functional CAR T cells. These findings suggest that arming CAR T cells with inducible secretion of IL-21 could be a reasonable strategy for boosting the effectiveness of CAR T cell-mediated therapies.

21 or in IL-2 plus activation with anti-CD3/28 antibodies. The dot plots show the memory phenotype and expression of PD-1 antigen by CD8+ CAR19 T cells before co-culture with Ramos B cells (top two rows), after co-culture with Ramos B cells in the presence of IL-2 (middle two rows) or in the presence of IL-21 (bottom two rows). Two representative donors are shown. The experiment was repeated once with similar results. *$P < 0.05$. SEM, standard error of the mean; w/o, without. (Color version of figure is available online).

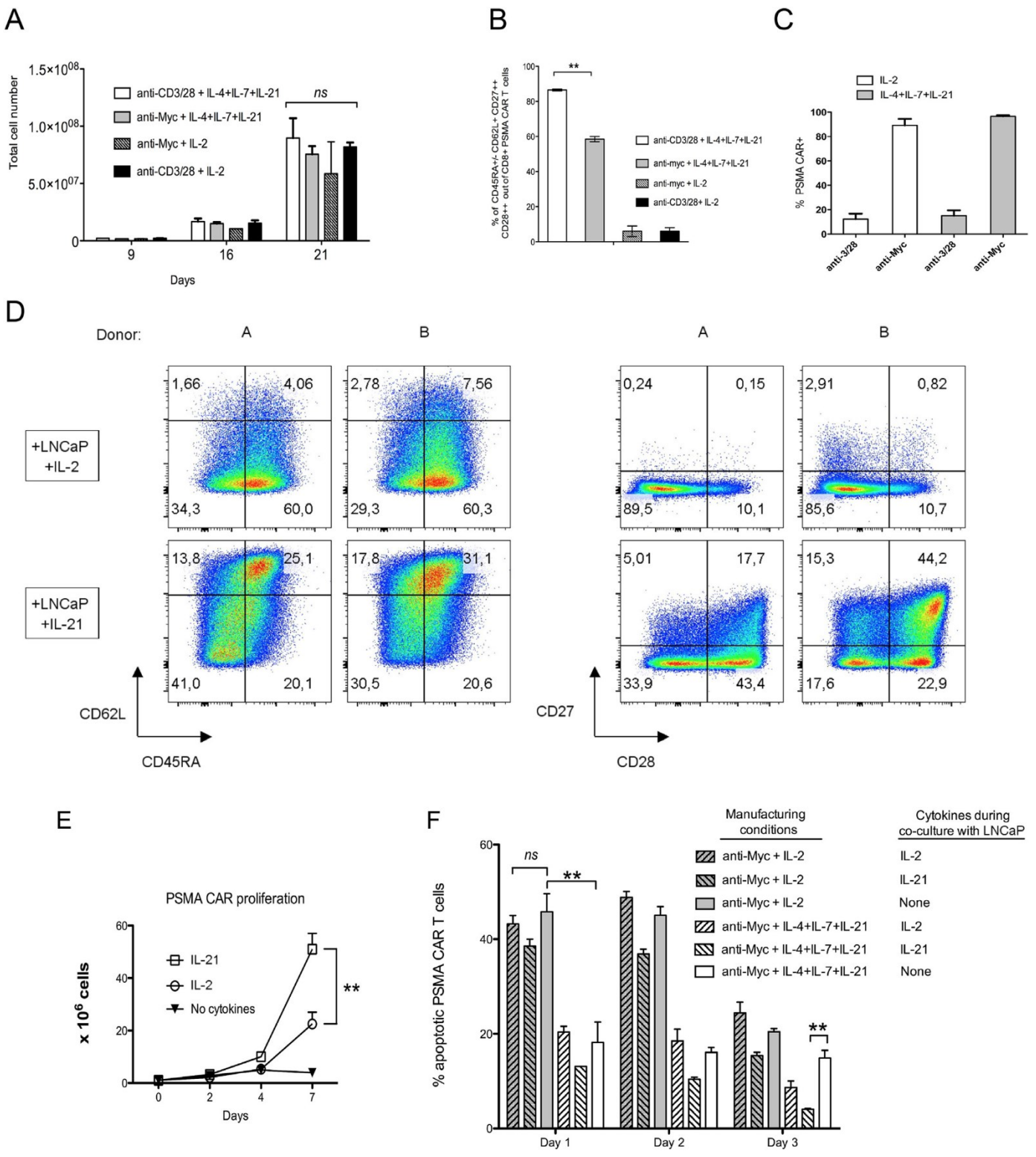

Figure 3. IL-21 enhances expansion of Tscm-like PSMA CAR T cells and reduces their apoptosis after anti-genic restimulation. (A C) Characterization of manufactured PSMA CAR T cells. PBMCs were electroporated with DNA plasmids and activated with anti-CD3/CD28 antibodies or anti-Myc antibody, followed by expansion in the presence of IL-4, IL-7, IL-21 or IL-2. The expansion of all cells (A), percentage of CD8+ PSMA CAR T cells with Tscm-like phenotype after 21 days (B) and percentage of transduced T cells after 21 days (C) are shown. N = 3. (D) PSMA CAR T cells generated with anti-CD3/28 and IL-2 were co-cultured for 7 days with LNCaP cells in the presence of exogenous IL-2 or IL-21. Two representative donors are shown. The experiment was performed twice with similar outcomes. Importantly, PSMA CAR T cells before co-cultivation were manufactured in IL-2 and contained <10% Tscm-like T cells, as shown in (B). The image shows the memory phenotype. (E) Expansion of PSMA CAR T cells in the presence of IL-2 or IL-21 compared with PSMA CAR T cells co-cultivated with LNCaP without any exogenously added cytokines. N = 3; error bars indicate SEM. Significance was determined by unpaired t-test. (F) PSMA CAR T cells were manufactured under various indicated conditions and then restimulated with LNCaP cells in the presence of IL-2, IL-21 or no cytokines. The graph shows the percentage of apoptotic PSMA CAR T cells at the indicated time points. N = 3; error bars indicate SEM. Significance was determined by unpaired t-test. ns (not significant) P > 0.05, * P 0.05, ** P 0.01; SEM, standard error of the mean. (Color version of figure is available online).

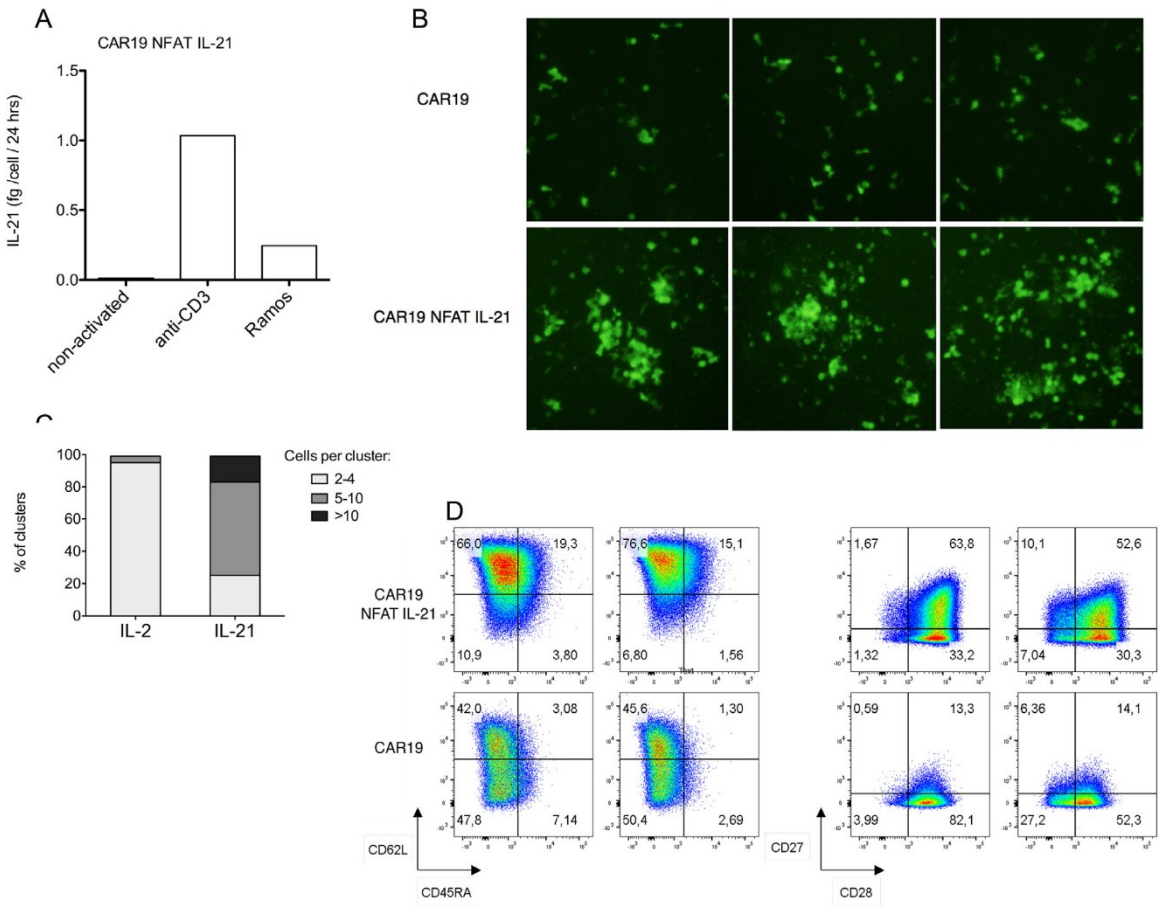

Figure 4. Inducible secretion of IL-21 stimulates the proliferation of CAR T cells after co-culture with tumor cells. (A) CAR19 T cells expressing IL-21 as a transgene under an artificial NFAT promoter produce IL-21 upon T-cell activation by anti-CD3 antibody or after co-cultivation with Ramos B cells. (B) CAR19 T cells and CAR19 NFAT IL-21 T cells were stably transduced with GFP-expressing transposon. CAR T cells were then co-incubated with Ramos B cells immobilized on the coverslip via polylysine and overlaid with soft agarose to immobilize cell clusters. After 7 days, the cell clusters were visualized by microscopy and their size determined by calculating the number of cells per cluster. The image shows examples of three areas containing the largest clusters. (C) The number of clusters containing two to four, five to 10 or more than 10 cells was calculated per bright field, and approximately 50 clusters were analyzed for each CAR19 construct. One representative donor is shown. The experiment was performed twice with similar results. (D) CAR19 T cells were restimulated twice at day 0 and day 4) with Ramos B cells to determine the effect of induced IL-21 on the phenotype of CAR19 T cells. The dot plots show the immunophenotype of CAR19 T cells or CAR19 NFAT IL-21 T cells at day 7. No exogenous cytokines were added to the cells in this experiment. Two representative donors are shown. The experiment was repeated twice with similar results. w/o, without. (Color version of figure is available online).

Discussion

Except for B-cell tumors treated with CD19 CAR T cells, a major limitation of current CAR T-cell approaches is insufficient expansion and persistence of infused T cells [11,16]. IL-21 has been shown to regulate T-cell homeostasis by promoting the proliferation and maintenance of the early memory phenotype of T cells. The rationale for the authors' study is to enhance CAR T-cell proliferation and utilize IL-21 as a growth-promoting cytokine to boost anti-tumor effects toward cancer cells that suppress the activity of CAR T cells.

The significance of the memory phenotype of CAR T cells in therapeutic efficiency is demonstrated by the results of recent clinical studies showing that an increased number of CAR T cells with a Tscm phenotype (CCR7+, CD62L+, CD27+, CD28+, CD45RA+ and PD-1) correlates with greater persistence and expansion in vivo and better efficiency [8]. Various cytokine cocktails containing IL-2, IL-7, IL-15 and IL-21, or the addition of signaling pathway modulators, such as PI3K inhibitor or GSK3-beta inhibitor [7,17], have been shown to increase the number of Tscm CAR T cells during culture. By contrast, cultivation of CAR T cells in a high concentration of IL-2 promotes terminal memory differentiation of CAR T cells [18]. These findings led the authors to optimize the composition of the cytokine cocktail used during in vitro expansion, and it was found that a mixture of cytokines (i.e., IL-4, IL-7 and IL-21) enables efficient expansion of early memory CAR T cells [6]. The authors also found that activation of transduced T cells with antigen (or anti-CAR antibody) instead of polyclonal activation with anti-CD3/CD28 antibodies selectively expands only the transduced T cells, yielding almost a pure population of CAR T cells. A similar approach has been described previously [19].

Because the authors used alternative methods of T-cell activation (via natural antigen or anti-CAR antibodies), as opposed to the commonly used anti-CD3/CD28 Dynabeads, the nature of CAR T-cell activation may influence their function and yield different results than activation via anti-CD3/CD28. The authors' data show that stimulation of PSMA CAR T cells via anti-myc or anti-CD3/CD28 antibodies produced functional CAR T cells with similar immunophenotypes and growth rates (Figure 3B,E). The authors did not detect any significant differences in regard to their cytotoxic functions, and similar findings were observed for CAR19 T cells (Figure 1). However, the authors found that CAR T cells cultivated in the presence of IL-2 performed poorly after antigenic restimulation compared with cells prepared in IL-4, IL-7 and IL-21, most likely because they had a more differentiated memory phenotype (Figures 1A, 3B).

Several published studies have shown similar effects of IL-21. For example, Singh et al.[20] showed that IL-21 enhances the development of less differentiated CAR T cells in vitro, but the effects were much weaker than those seen in the current study because Singh

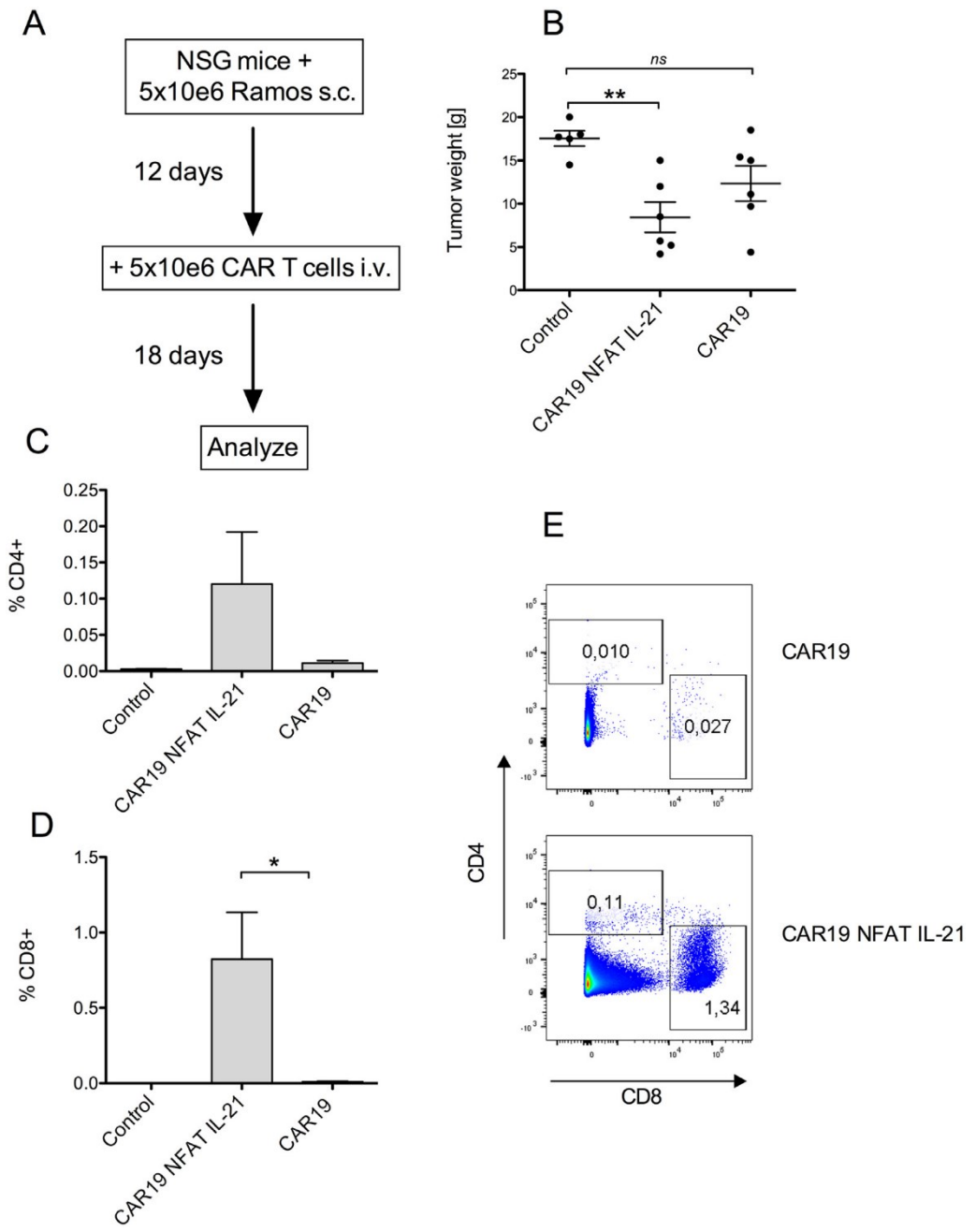

Figure 5. CAR19 T cells armed with IL-21 have enhanced anti-tumor efficacy in vivo. (A) Immunodeficient NSG mice were transplanted subcutaneously with 5 million Ramos cells. After 12 days, when macroscopic tumors had formed, mice received 5 million CAR T cells intravenously. (B) The treatment response was assessed by measuring the tumor weight at the end of the experiment and comparing it with a control group. N = 6; error bars indicate SEM. Significance was determined by unpaired t-test. (C D) Level of infiltration of tumors by CD4+ T cells and CD8+ T cells. (E) Representative dot plot of tumors stained for CD4 and CD8. N = 6; error bars indicate SEM. Significance was determined by unpaired t-test. *P < 0.05, **P < 0.01, ns (not significant) P > 0.05 i.v., intravenously; s.c., subcutaneously; SEM, standard error of the mean. (Color version of figure is available online).

et al.cultured CAR T cells in IL-21 and IL-2, which may have had antagonistic effects. Furthermore, IL-2 and IL-21 have been shown to confer opposing differentiation programs on CD8+ T cells for adoptive immunotherapy, and IL-21 plays a key role in the development and maintenance of central memory T cells by inducing an early differentiation phenotype [5]. Markley et al. [21] found that CAR19 T cells co-expressing IL-21 have an enhanced anti-tumor effect in mice compared with CAR T cells co-expressing IL-2, but they accumulated very poorly in vitro upon repeated anti-genic stimulation. The major difference between this study and the authors' observations is that Markley et al. used CAR19 T cells with a CD28 zeta signaling motif that secreted IL-21 constitutively, whereas the authors' CAR T cells contained the 4-1BB zeta signaling motif, and IL-21 was inducible after anti-genic stimulation.

The authors compared the effects of IL-2 and IL-21 on sorted CD8+ CAR19 T cells with the Tscm phenotype (CD45RA+CD62L+) after stimulation with CD19+ cells. IL-2 induced downregulation of CD62L, CD27 and CD28, which is consistent with their differentiation toward effector memory T cells. Conversely, IL-21 enabled their expansion, as well as maintenance of the Tscm phenotype, which suggests that IL-21 can block the transition from early to late memory subtypes.

Next, the authors hypothesized that the results of anti-genic stimulation of CAR T cells may depend on the phenotype and origin of the tumor cells. Therefore, several experiments were performed with PSMA CAR T cells challenged with LNCaP cells because this is a

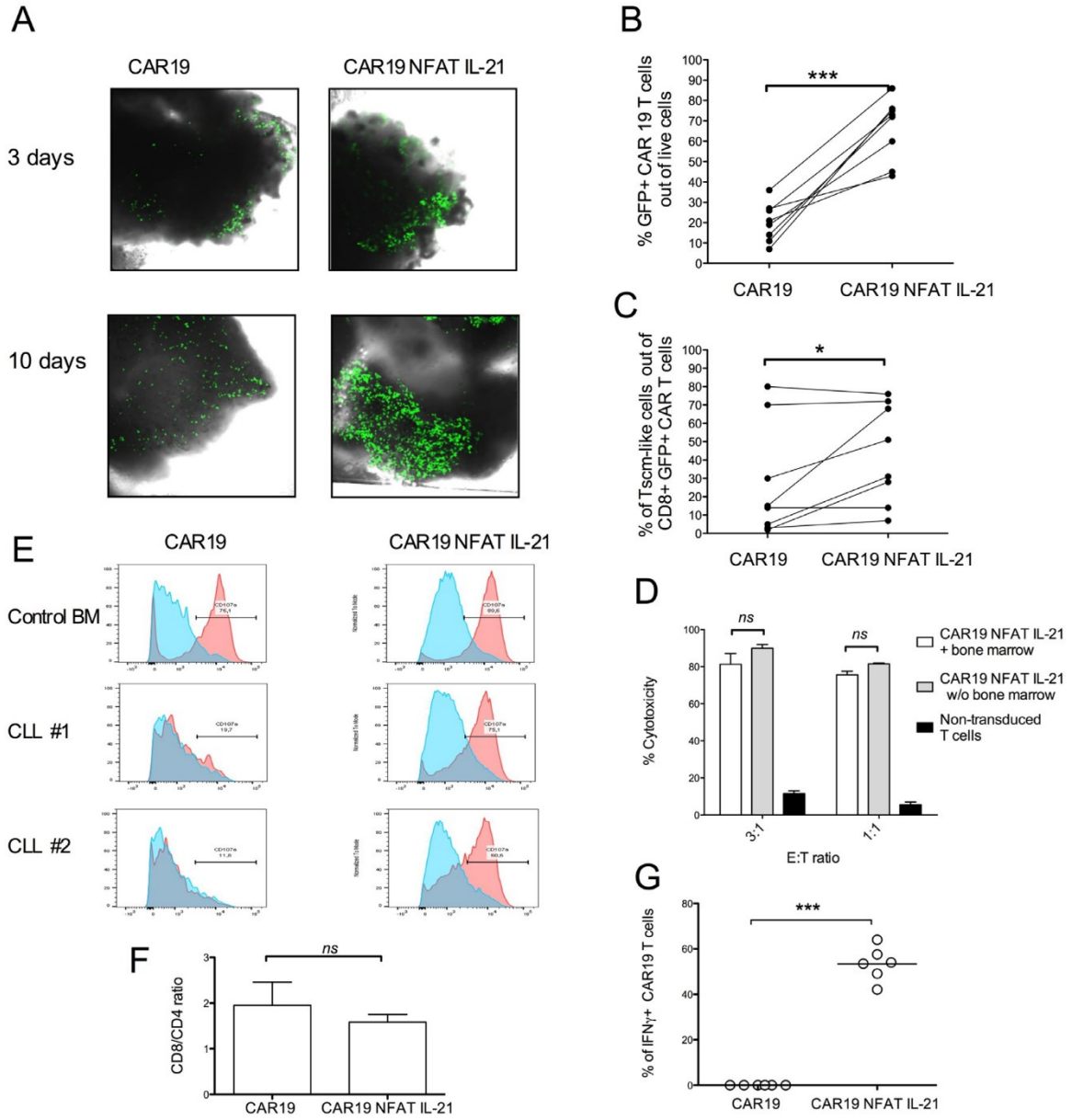

Figure 6. CAR19 T cells armed with IL-21 are resistant to immunosuppression induced by CLL cells. (A) GFP-expressing CAR19 T cells were co-cultivated with BM fragments obtained from CLL patients. The images show infiltration by GFP+ cells after 3 days and 10 days in one representative sample. The images are taken from different areas of the bone fragments £200 magnification. (B) The level of infiltration by GFP+ CAR T cells in each sample was analyzed by FACS. N = 8; error bars indicate SEM. Significance was determined by paired t-test. (C) Percentage of CD8+ CAR T cells with Tscm-like phenotype (CD45RA+/ CD62L+CD27++CD28++). N = 8; error bars indicate SEM. Significance was determined by paired t-test. (D) Cytotoxicity of CAR T cells against Ramos cells and comparison of CAR T cells co-cultivated with BM to control CAR T cells grown in vitro in the presence of cytokines without BM. N = 3. (E) Histograms showing the level of degranulation of CAR T cells recovered from BM fragments after 10 days. Control BM is a sample obtained from an otherwise healthy patient without significant pathology in the BM. Red histograms indicate results after challenging with Ramos cells. Blue histograms represent non-challenged CAR T cells (negative control). This experiment was repeated once with similar results. (F) CD8:CD4 ratio among CAR19 T cells infiltrating BM fragments. N = 8. (G) CAR19 T cells were co-cultivated with CLL cells and analyzed for the production of IFNg by cytokine capture assay after anti-genic restimulation with Ramos B cells. The graph shows the percentage of IFNg+ CAR19 T cells. N = 6; error bars indicate SEM. Significance was determined by paired t-test. ns (not significant) P > 0.05 , * P 0.05, *** P 0.001. IFNg, interferon gamma; ns, not significant; SEM, standard error of the mean; w/o, without. (Color version of figure is available online).

common CAR T cell model [22]. Furthermore, LNCaP is an adherent carcinoma cell line that does not express any co-stimulatory molecules [23]. Gargett et al. [11] similarly studied GD2-specific CAR T cells co-cultured with GD2-positive neuroblastoma cells and observed significant AICD of CAR T cells after repeated antigen stimulation with tumor cells. Natural antigen-specific effector T cells are more prone to apoptosis than memory T cells because of elevated levels of caspase 3, which indicates that sensitivity to apoptosis after anti-genic stimulation differs among various T-cell memory subsets [21]. Accordingly, the authors found that PSMA CAR T cells with effector phenotype (i.e., cultivated in the presence of IL-2) were significantly more sensitive to apoptosis after anti-genic restimulation than PSMA CAR T cells with a Tscm-like immunophenotype grown in the presence of IL-4, IL-7 and IL-21 (Figure 3F). The beneficial effects of IL-21 on CAR T-cell survival and expansion are further demonstrated by the findings that CAR19 NFAT IL-21 T cells were superior to non-armed CAR19 T cells in NSG mice bearing established human lymphoma tumors.

The authors developed an in vitro model of CLL, which was selected mainly because of good availability of tumor tissues and because all patients with lymphocytosis in the blood have highly infiltrated BM. In addition, CLL is far more resistant to CAR19-based therapies than B-ALL. Recent studies have shown that CLL cells can

directly inhibit the proliferation and effector functions of CAR19 T cells [10] and initiate abortive activation of CAR T cells, resulting in very low proliferation upon in vitro restimulation with CLL cells [23]. However, the molecular mechanism has not been clearly determined and is currently being studied by several groups, including the authors'. This in vitro co-cultivation model using directly isolated human primary CLL cells is a useful biological model for studying CAR19 T-cell functions. The authors' opinion is that it may provide interesting data that are useful for better understanding tumor-induced immunosuppression of CAR T cells, especially when targeting solid tumors, which are mostly refractory to CAR T cells. Moreover, IL-21 potentiated the activity of CAR19 T cells against CLL B cells and significantly enhanced their expansion compared with non-armed CAR19 T cells. In conclusion, the authors' data suggest that arming CAR T cells with inducible IL-21 could be another possible strategy to counteract tolerance mechanisms by which tumor cells evade CAR T-cell therapies.

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Declaration of Competing Interest

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Author Contributions

Conception and design of the study: PO, JM, PK. Acquisition of data: MS, PP, MM. Analysis and interpretation of data: PO, MS. Drafting or revising the manuscript: PO, MS. All authors have approved the final article.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2020.08.005.

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