Original Article

IL-21 is not necessary for anti-lymphoma effects of T cells expressing second-generation CD19-specific chimeric antigen receptors in a xenograft mouse model.

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Summary

Immuno-gene therapy using T cells with a second-generation anti-CD19 chimeric antigen receptor（CD19-CAR）, which contains CD28 as part of its construct, has been reported to be effective in the treatment of advanced B cell malignancies. We investigated whether co-expression of human interleukin-21（hIL-21）improved the anti-tumor effects of CD19-CAR T cells in a xenograft mouse model. Human peripheral blood lymphocytes from healthy donors were activated, and transduced with retroviral vectors that encoded CD19-CAR and hIL-21, or CD19-CAR alone. Both types of transduced T cells exhibited similar expansion properties *ex vivo* and transgene expression patterns. Compared with CD19-CAR T cells, those co-expressing IL-21 showed enhanced levels of STAT3 phosphorylation, a surrogate marker of IL-21 signaling. Analysis of T cell subsets among transduced cells indicated that their phenotypes were those of central or effector memory T cells. Both types of cells exhibited cytotoxicity against Raji cells, a CD19-positive B lymphoma cell line, and produced interferon-γ upon stimulation with CD19. *In vivo* analysis using Rag2$^{-/-}$ γc$^{-/-}$ immunodeficient mice with systemic Raji tumors that were treated with transduced T cells revealed suppression of tumor progression. Our findings suggest that co-expression of IL-21 did not augment the anti-tumor effects of T cells with a second-generation CD19-CAR in the mouse xenograft model of human B cell lymphoma that we investigated.

（Key words：B cell lymphoma, chimeric antigen receptor, engineered T cell therapy, IL-21）

Introduction

Engineered T cell therapy is a new strategy for the treatment of advanced malignancies, in which autologous T cells are genetically modified to target tumor associated antigens（TAA）, either through transduced T cell receptors（TCRs）or chimeric antigen receptors（CARs）. Unlike TCRs, which recognize human leukocyte antigen（HLA）-peptide complexes, CARs recognize specific TAAs that do not require peptide processing and presentation by HLAs. Thus, CARs are more broadly applicable to patients with a diverse array of HLAs.[1,2]

CARs are recombinant proteins comprising an extracellular single chain fragment variable region（scFv）linked to the intracellular signaling domain, most commonly CD3ζ. This enables activation of transduced T cells, and these proteins are referred to as first-generation CARs.[3] When T cells express this molecule, they can recognize TAAs and kill tumor cells in an HLA-independent manner. However, first-generation CARs with CD3ζ alone failed to elicit T cell expansion and had little anti-tumor activity *in vivo*, possibly owing to the absence of co-stimulatory signals.[4] To overcome this problem, second-generation CARs, which have co-stimulatory intracellular signaling domains（CD28 or 4-1BB）in addition to CD3ζ, have

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been developed.[4,5] Second-generation CARs can transduce sufficient signals upon exposure to antigens, leading to full activation of T cells ; these are now widely used in preclinical and clinical studies.[4-7]

CD19 is an attractive target for CAR-based T cell therapy as it is a pan-B cell marker, which is expressed on normal, and most malignant B cells. It has been demonstrated that T cells expressing anti-CD19 second-generation CARs (CD19-CARs) effectively eradicated $CD19^+$ B cell malignancies in mouse xenograft models.[4,8] Early phase clinical studies investigating B cell malignancies, such as chronic lymphocytic leukemia and acute lymphocytic leukemia, refractory to chemotherapies could be treated by CAR. It has been shown that autologous T cells transduced with CD19-CARs are safe, and result in tumor regression.[6,7]

Interleukin (IL) -21 is a common gamma chain (γc) cytokine, produced by $CD4^+$ T cells and natural killer T (NKT) cells, that modulates the functions of lymphoid lineages, including $CD8^+$ T cells.[9] Because IL-21 supports proliferation and survival of cytotoxic $CD8^+$ T cells, it has been used to enhance the anti-tumor effects of tumor-specific T cells in preclinical and clinical studies, and has exhibited acceptable toxicity profiles.[10] Furthermore, IL-21 induces apoptosis of B cells under certain conditions, and has been shown to suppress the growth of B cell leukemias and lymphoma cells in preclinical studies.[11-13] Therefore, IL-21 is a potential adjuvant for the anti-tumor activity of T cells against B cell tumors. Markley *et al*. showed that co-expression of IL-21 with an anti-CD19 first-generation CAR improved the anti-tumor effects of CAR^+ T cells in a mouse xenograft model of human $CD19^+$ B cell tumors.[14]

In this study, we generated human T cells co-expressing a second-generation CD19-CAR and IL-21, and investigated whether IL-21 could augment the anti-tumor effects of CAR^+ T cells *in vitro* and in a mouse xenograft model.

Material and methods

Plasmids

A retroviral vector, SFG-1928z, encoding the CD19-CAR with the CD28 gene has been described previously.[4] Human IL-21 cDNA was cloned from peripheral blood mononuclear cells (PBMCs) activated with anti-CD3 and -CD28 antibodies, using reverse transcription polymerase chain reation (RT-PCR). A co-expression vector, SFG-1928z-P2A-IL21, was generated by linking the CAR and IL-21 with a P2A bicistronic sequence using an overlapping PCR method (Fig. 1).[15]

Cell lines

The human Burkitt lymphoma cell, Raji, was obtained from the Health Science Research Resources Bank (Osaka, Japan). The human erythroleukemia K562, and mouse fibroblast NIH3T3 cell lines were obtained from the RIKEN Bioresource Center (Tsukuba, Japan). Retroviral packaging cells (PG13) have been described before and were used in this study.[16] Raji cells expressing luciferase (Raji-Luc), and K562 and NIH3T3 cells expressing human CD19 (K562-CD19 and NIH3T3-CD19, respectively) have been reported.[8] Mouse pro-B BaF-3 cells that express the IL-21 receptor (BaF-3-IL-21R) were also used.[17] Cell lines were cultured with either Dulbecco's modified Eagle's medium or RPMI1640 supplemented with 10% fetal bovine serum (FBS).

Transduction of PBMCs

Our human study was approved by the Institutional Review Board of Jichi Medical University, with written informed consent obtained from each donor. Human PBMCs were cultured with X-Vivo 15 (Takara Bio, Shiga, Japan) supplemented with 10% human AB serum (Nova Biologics, Oceanside, CA) and 1 nM recombinant human IL-2 (hIL-2 ; Life technologies, Carlsbad, CA, USA). PBMCs were pre-stimulated in 24-well plates coated with an anti-CD3 ε antibody (R&D Systems, Minneapolis, MN, USA) and RetroNectin (Takara Bio) on day 0. Retroviral supernatants were obtained from PG13 producer cells established by transduction with either SFG-1928z or SFG-1928z-P2A-IL21. On days 3 and 4, retroviral supernatants were applied to RetroNectin-coated culture plates and centrifuged (2000×*g*, 32℃, 2 h). Supernatants were removed, and stimulated PBMCs were applied to preloaded plates and centrifuged (1000×*g*, 32℃, 10 min), then incubated overnight. For selective expansion ex vivo, cells were co-cultured with NIH3T3-CD19 on days 5 and 12 at a T cell to NIH3T3-CD19 ratio of 10 : 1.

Flow cytometry

CD19-CAR surface expression was assessed using a biotin-conjugated goat anti-mouse IgG Fab-specific antibody (Jackson Immunoresearch, West Grove, PA, USA) and avidin-FITC or -PE (Dako, Glostrup, Denmark). Anti-CD3 FITC, anti-CD8 APC, anti-CD62L PE, anti-CD45RO APC and anti-CD27 APC were obtained from Biolegend (San Diego, CA, USA). Anti-CD4 FITC, anti-CD8 PE and anti-CD45RA APC were obtained from BD Biosciences (San Jose, CA, USA). Anti-CCR7 PE was obtained from R&D systems. Anti-CD28 PE was obtained from eBioscience (San Diego, CA, USA). For intracellular phopho-STAT3 (pSTAT3) detection, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with Perm buffer III, then stained with a PE-labeled pSTAT3 antibody (BD Biosciences). Samples were analyzed using a BD LSR™ with Cell Quest™ Pro software, or a BD LSR Fortessa™ with FACSDiva™ software (BD Biosciences).

Enzyme-linked immunosorbent assays (ELISAs)

For IL-21 detection, PBMCs were washed and resuspended in cytokine-free X-Vivo 15 medium at a concentration of 10^7 cells /ml. After 12 h, IL-21 levels in culture supernatants were measured by a human IL-21 ready-set-go ELISA kit (eBioscience). For interferon (IFN) - γ detection, 10^5 PBMCs were washed and cultured with 200 µl of cytokine-free X-Vivo 15, either in the presence or absence of NIH3T3-CD19 cells, at a 1 : 1 ratio, for 72 h. IFN- γ levels in culture supernatants were measured using a human IFN- γ ready-set-go ELISA kit (eBioscience).

Western blotting

Whole cell lysates were prepared from transduced cells. Protein extracts (20 μg) were separated on NuPage Bis-Tris 4%–12% gels and transferred to polyvinylidene difluoride (PVDF) membranes (Life technologies). Membranes were incubated with anti-human CD3 ζ for CAR detection, and anti-hIL-21 for IL-21 detection (Biolegend), and then stained with horseradish peroxidase-conjugated secondary antibodies. Visualization of positive signals was facilitated with enhanced chemiluminescence systems (GE Healthcare, Buckinghamshire, UK).

Cytotoxicity assays

Cytotoxicity assays employing calcein-acetomethyl ester (calcein AM) were conducted as described previously.[18] Target cells were incubated with 15 µM calcein AM (Dojin, Kumamoto, Japan) at 37℃ for 30 min, and mixed with effector cells at the indicated effector/target ratios. Effector cell numbers were calculated based on the total number of CAR^+ cells. After incubation at 37℃ for 4 h, 75 µl of supernatant was harvested and transferred into the well of a 96-well plate. Samples were measured using a Fluoroskan Ascent FL (excitation at 485 nm, emission at 530 nm) (Thermo scientific, Waltham, MA, USA). The proportion of lysis was calculated according to the following formula : [(test release - spontaneous release) / (maximum release - spontaneous release)] × 100.

Mouse xenograft model

Balb/c $Rag2^{-/-}\gamma c^{-/-}$ mice (8–12-weeks-old) were kindly provided by Dr. M. Ito (Central Institute for Experimental Animals, Kanagawa, Japan). On day 0, 5×10^4 Raji-Luc tumor cells were infused into mice by tail vein injection. Four days later, for mice in the treatment group, 7×10^6 CAR^+ PBMCs were intravenously injected. *In vivo* bioluminescent imaging was conducted every 7 days. Mice were intraperitoneally injected with luciferin (75 mg/kg body weight) (Ieda, Tokyo, Japan). Photons emitted from Raji-Luc were acquired and quantified using an IVIS imaging system (PerkinElmer, Waltham, MA) and living image software (PerkinElmer). All mice experiments were carried out in a humane manner following approval from the Institutional Animal Experiment Committee of Jichi Medical University.

Results

We generated the SFG-1928z-P2A-IL21 co-expression vector using overlapping PCR (Fig. 1). We then generated CD19-CAR (CAR^+) and CD19-CAR and IL-21 ($CARIL21^+$) T cells by transducing human PBMCs obtained from healthy donors with SFG-1928z and SFG-1928z-P2A-IL21. CAR^+ and $CARIL21^+$ T cells showed approximately 100-fold expansion within 2 weeks, with no difference between both cell types (data not shown). We analyzed cell surface CAR expression of transduced PBMCs by flow cytometry. At day 17 of culture, we observed 80% CAR^+ and 87% $CARIL21^+$ T cells (Fig. 2a). We also confirmed CAR expression in both T cell types by western blotting (WB) (Fig. 2b). Mean IL-21 levels in culture supernatants obtained from CAR^+ and $CARIL21^+$ T cells were 5 and 185 pg/ml respectively (Fig. 2c), while IL-21 was not detected in non-transduced T cells (data not shown). Expression of IL-21 was confirmed by WB in cell lysates from $CARIL21^+$ T cells, but not from CAR^+ T cells (Fig. 2d).

To assess the function of IL-21 from $CARIL21^+$ T cells, we investigated the phosphorylation status of STAT3, a surrogate marker of IL-21 signaling.[9,17,19] Intracellular flow cytometry showed enhanced phosphorylation of STAT3 in $CARIL21^+$ T cells compared with CAR^+ T cells. STAT3 phosphorylation levels of $CARIL21^+$ T cells was comparable to that in CAR^+ T cells stimulated with recombinant hIL-21 (Fig. 3a). To address signals specific to IL-21, we used an indicator cell line, BaF-3-IL-21R, which was genetically transduced with the IL-21 receptor. BaF-3-IL-21R cells stimulated with culture supernatant obtained from $CARIL21^+$ T cells showed STAT3 phosphorylation levels comparable to that in cells stimulated with hIL-21. Supernatants obtained from CAR^+ T cells did not exhibit enhanced STAT3 phosphorylation levels (Fig. 3b).

We examined the immunophenotype of these T cells by flow cytometry. At day 17 of culture, more than 99% of $CAR^+$and $CARIL21^+$ T cells were positive for CD3, with a large number of $CD8^+$ T cells present (Fig. 4a). T cell subset analysis for both T cell types showed that they differentiated and possessed either $CD45RA^-CD62L^+$ central memory (T_{CM}) or $CD45RA^-CD62L^-$ effector memory (T_{EM}) phenotypes. T_{EM} subpopulation analysis using CD27 and CD28 markers showed large numbers of $CD27^+CD28^+$ EM1 and CD27-$CD28^+$ EM4 memory-like subpopulations,[20] with no differences observed between both T cell types (Fig. 4b).

We assessed antigen-specific IFN- γ production of transduced PBMCs by ELISA. $CARIL21^+$ T cells stimulated with NIH3T3-CD19 produced slightly lower levels of IFN- γ compared with CAR^+ T cells (Fig. 5a). CAR^+ and $CARIL21^+$

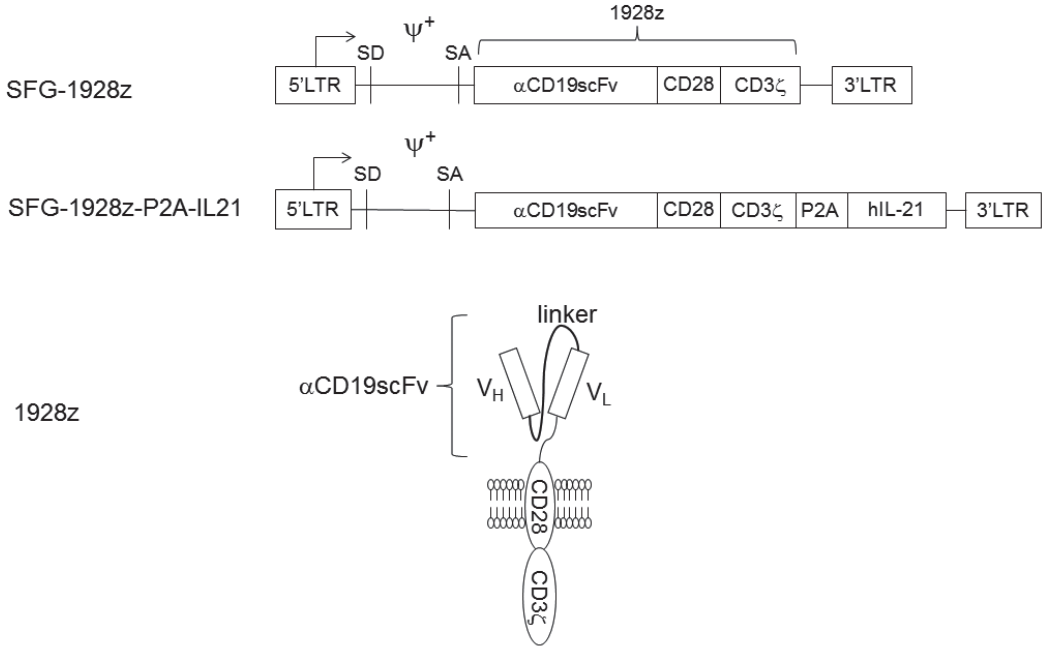

Fig. 1. Schematic of the SFG-1928z retroviral vector, SFG-1928z-P2A-IL21, and 1928z protein. SD：splice donor；SA：splice acceptor；LTR：long terminal repeat；Ψ：packaging signal, P2A：porcine teschovirus-1-derived 2A peptide；ScFv：single chain variable fragment region；linker：$(GGGGS)_3$ linker

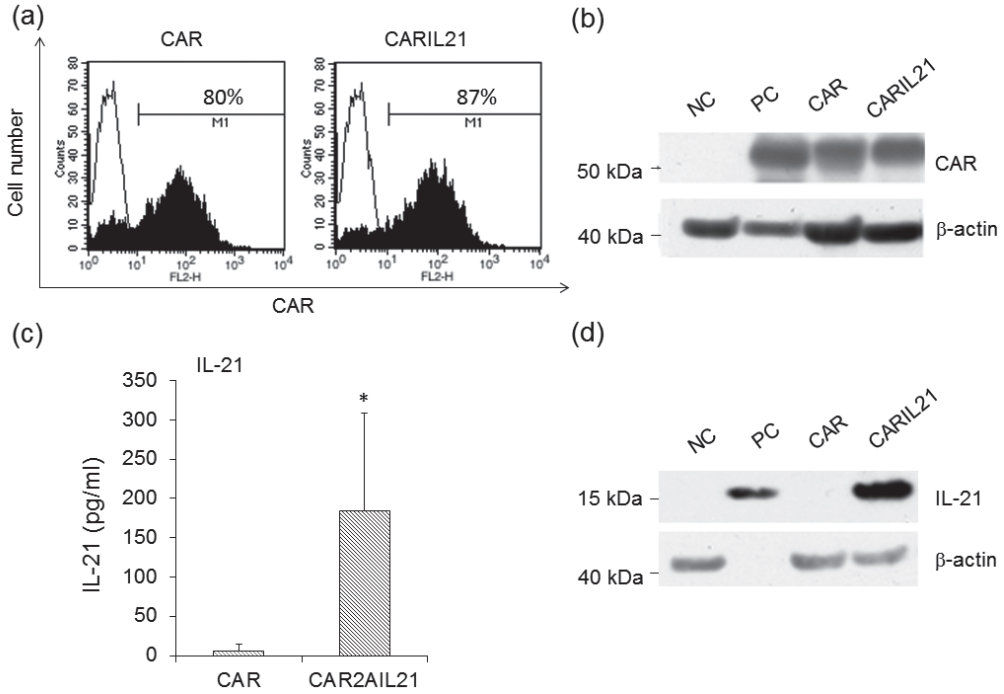

Fig. 2. Generation of CD19-CAR（CAR）- or CD19-CAR and hIL-21（CARIL21）-expressing T cells.（a）Surface expression of CAR was assessed by flow cytometry. Values represent the percentage of CAR^+ cells. Solid black lines represent isotype controls.（b）Expression of CAR was assessed by western blotting. PG13 cells transfected with SFG-1928z and non-transduced T cells served as positive（PC）and negative（NC）controls, respectively.（c）ELISAs were used to measure IL-21 concentrations in culture supernatants of CAR^+ and $CARIL21^+$ T cells. Values are mean ± SD from four different donors. *$p < 0.05$.（d）Cellular expression of IL-21 was assessed by western blotting. Recombinant human IL-21（rhIL-21；1 ng/lane）and non-transduced T cells served as positive（PC）and negative（NC）controls, respectively, with β-actin used as a loading control.

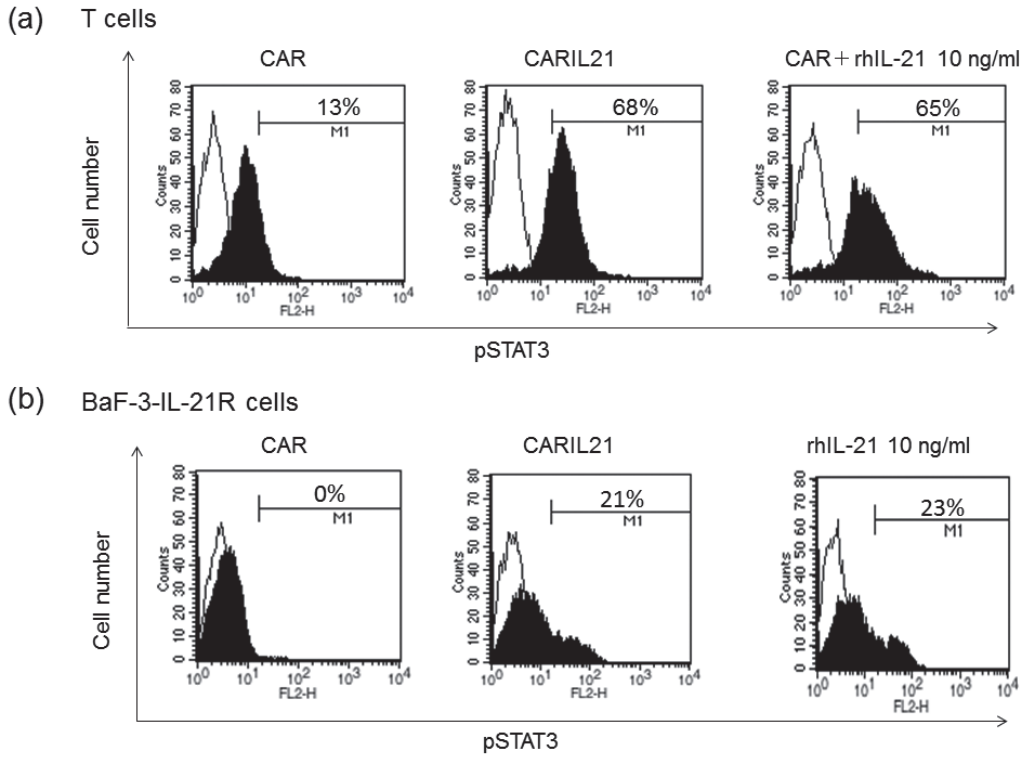

Fig. 3. Functional assessment of IL-21 produced by CARIL21$^+$ T cells was conducted using flow cytometry to detect phosphorylated STAT3 (pSTAT3). (a) Levels of pSTAT3 in CAR$^+$, CARIL21$^+$, and rhIL-21-stimulated CAR$^+$ T cells. (b) Levels of pSTAT3 in BaF-3-IL21R cells stimulated with each culture supernatant and rhIL-21. Values represent percentages of pSTAT3-positive cells. Solid black lines represent isotype controls.

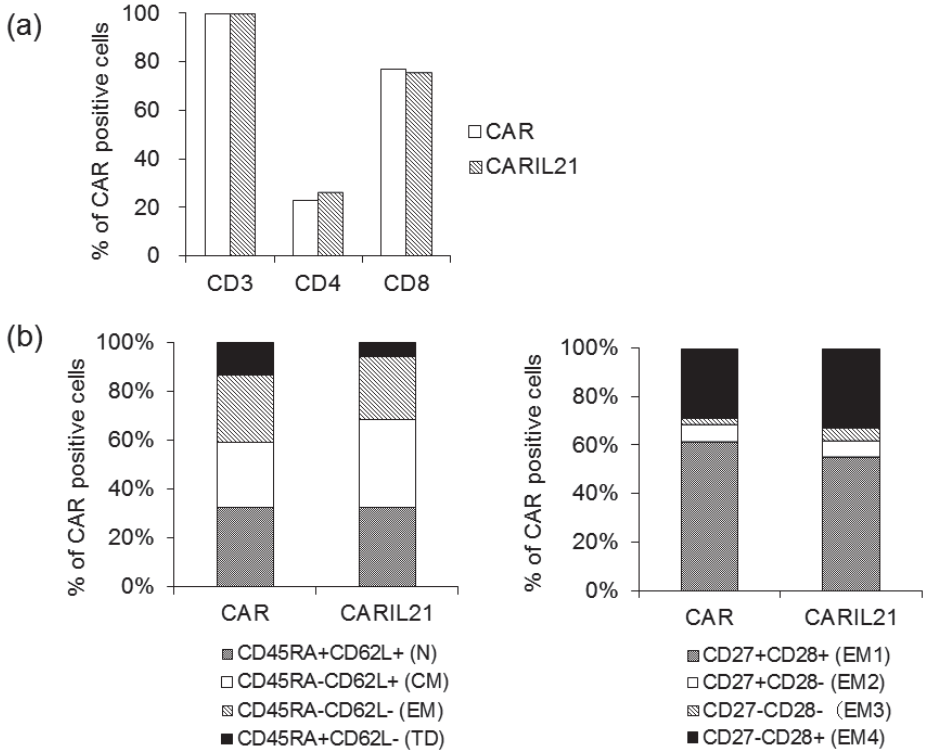

Fig. 4. T cell subset analysis of transduced T cells. After gating for CAR$^+$ cells, cells were analyzed by flow cytometry on day 17. Values shown represent the mean from two different donors. (a) CD3, CD4 and CD8 expression of transduced cells. (b) Left, proportions of CD45RA- and CD62L-expressing cells. Right, proportions of CD27$^+$ and CD28$^+$ cells. N, naïve ; CM, central memory ; EM, effector memory ; TD, terminally differentiated effector.

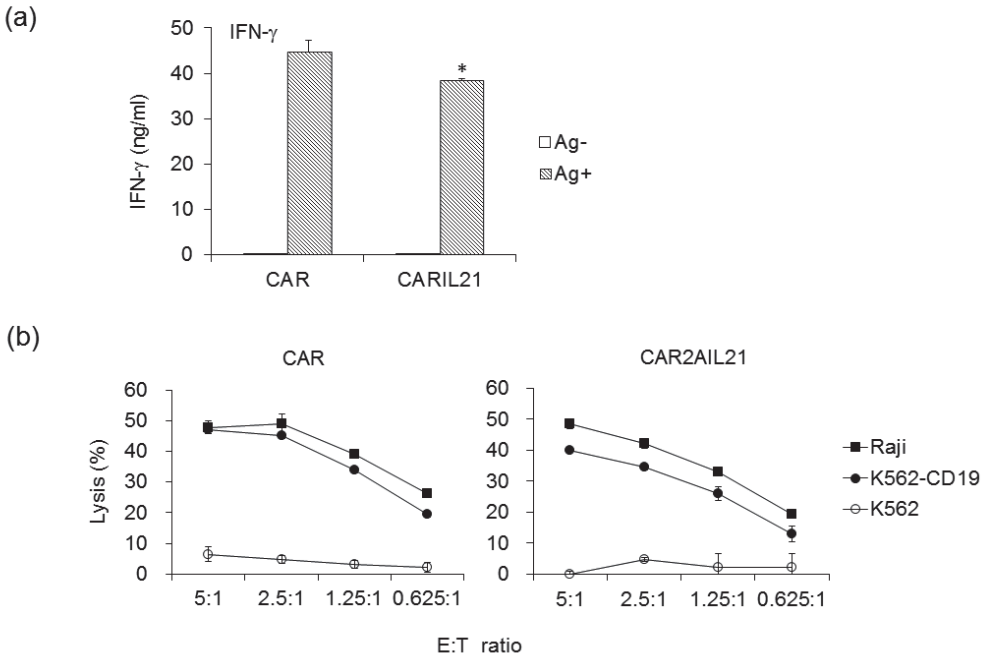

Fig. 5. Cytotoxicity of transduced T cells. (a) IFN-γ production from transduced T cells was assessed using ELISAs. T cells were cultured for 72 h in the presence (Ag^+) or absence (Ag^-) of NIH3T3-CD19. Data are the mean ± SD of triplicate wells from one of two donors, with similar results seen for both. *$p < 0.05$. (b) Cytotoxicity assays showing proportions of Raji ($CD19^+$ human B lymphoma cell line ; filled squares) and K562-CD19 (human leukemia cell line transduced with CD19 ; filled circles) cells lysed by CAR^+ or $CARIL21^+$ T cells. Values represent the mean ± SD from triplicate wells. K562 cells serve as $CD19^-$ targets (circles). T cell numbers were adjusted according to the number of CAR^+ cells.

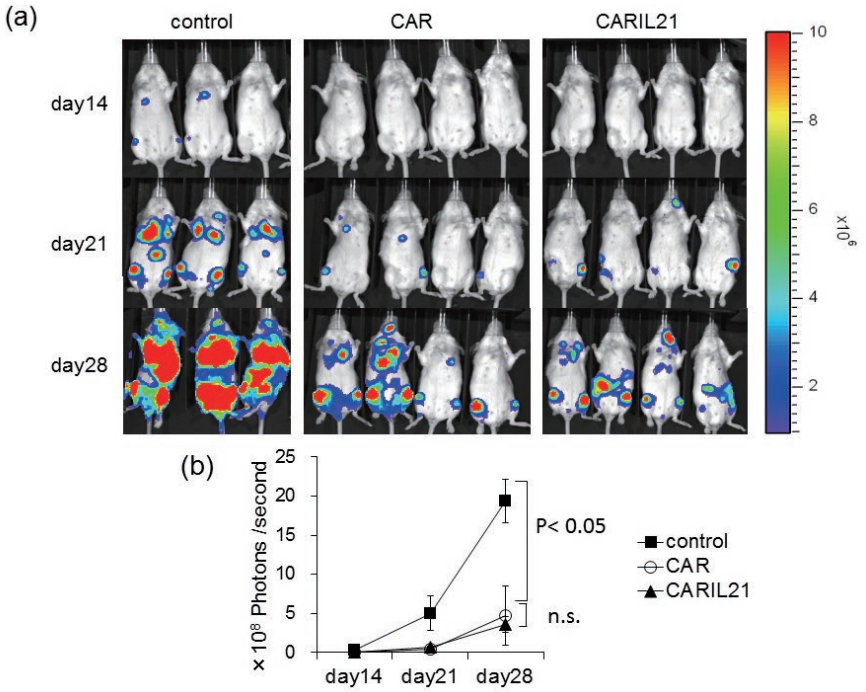

Fig. 6. Anti-lymphoma effects of transduced T cells in a mouse xenograft model. $Rag2^{-/-}$ $\gamma c^{-/-}$ immunodeficient mice were intravenously inoculated with 5×10^4 Raji-Luc cells on day 0. On day 3, 7×10^6 CAR^+ or $CARIL21^+$ T cells were infused (treatment groups, n=4 each), whereas mice in the control group (n=3) did not receive T cells. Bioluminescent imaging was conducted on days 14, 21, and 28. Data are representative from one of three independent experiments using different donors, and yielded similar results. (a) Progression of inoculated Raji-Luc cells. (b) Values presented are the mean ± SD of quantified biophotons at each time point.

T cells showed similar levels of cytotoxicity against Raji and K562-CD19 cells, but not against control CD19- K562 cells (Fig. 5b).

We evaluated the anti-tumor activity of transduced PBMCs in a mouse xenograft model. We intravenously injected human B lymphoma Raji cells expressing luciferase into Rag2$^{-/-}$ γc$^{-/-}$ immunodeficient mice and assessed tumor burden using bioluminescent imaging. Tumor-bearing mice infused with CAR$^+$ and CARIL21$^+$ T cells showed significantly suppressed tumor growth compared with control mice (Fig. 6a and b). Non-transduced T cells did not suppress tumor growth (data not shown). However, tumor growth was not significantly different between mice injected with CAR$^+$ or CARIL21$^+$ T cells.

Discussion

The use of engineered T cells with second-generation CD19-CARs is a potential therapeutic strategy against B cell lymphomas. Autologous T cells are activated through signals 1 and 2 provided by the CD3ζ chain and CD28 signaling domains, respectively. IL-21 provides signal 3, which is known to boost effector functions of antigen-specific CD8$^+$ T cells, and cooperates with signals 1 and 2 of T cell activation.[21] In this study, we generated T cells co-expressing CD19-CAR and IL-21 to augment the anti-lymphoma effects of engineered T cells.

It has been shown that naïve and memory T cells are suitable sources of cells for engineered T cell therapy as they persist for long periods after infusion, and provide protection against tumors over an extended time period.[22-24] Hinrichs *et al*. showed that antigen-specific CD8$^+$ T cells primed with IL-21 sustained expression of CD62L, a marker expressed on T_{CM} cells. This resulted in enhanced anti-tumor effects in mouse melanoma models.[25] Markley *et al*. reported that T cells with CARs and IL-21 had an EM1 phenotype, a less differentiated subset of T_{EM}, and showed improved anti-tumor effects *in vivo*.[14] However, IL-21 did not affect T cell phenotypes in the present study, in which the majority of transduced T cells, regardless of type, differentiated into CD8$^+$ naïve or memory T cells.

We also assessed the cytotoxicity of CAR$^+$ and CARIL21$^+$T cells, as the effect of IL-21 on cytotoxic T cells is subject to some controversy. Singh *et al*. reported that addition of IL-21 up-regulates IFN-γ expression in CAR$^+$ T cells,[21] while Hinrichs *et al*. reported that IL-21 down-regulates IFN-γ expression in T cells, leading to lower cytotoxicity *in vitro*.[25] In our study, IL-21 did not improve IFN-γ production upon antigen stimulation, nor was cell lytic activity increased against CD19$^+$ lymphoma cells.

Finally, we assessed the *in vivo* efficacy of CARIL21$^+$ T cells. Improved cytotoxicity *in vitro* does not translate to improved anti-tumor effects *in vivo* because differentiation towards the effector T cell phenotype leads to exhaustion of T cells. Markley *et al*. compared four γc cytokines in combination with first-generation CD19-CAR T cells. They found that IL-21 had little effect upon T cell phenotype and cytotoxicity *in vitro*, and enhanced tumor activity *in vivo*.[14] However, in our study, IL-21 did not improve the *in vivo* efficacy of T cells.

In our study, we showed that CARIL21$^+$ T cells were not superior over CAR$^+$ T cells, although IL-21 signaling was confirmed by enhanced STAT3 phosphorylation. Kaka *et al*. demonstrated that T cells expressing high levels of IL-21 under the control of the cytomegalovirus promoter exhibited a central memory phenotype and enhanced cytotoxicity.[26] IL-21 expression levels could be important in enhancing effector functions of T cells. However, Markley *et al*. reported enhanced anti-tumor effect of T cells co-expressing first-generation CARs and IL-21, using similar IL-21 concentrations that we used. A possible explanation is that second-generation CARs containing CD28 effectively activate T cells, therefore is no need for additional stimulation by IL-21. In contrast, Singh *et al*. showed improved anti-tumor effects of T cells with second-generation CARs using a membrane-bound form of IL-21.[21] The method of delivery for IL-21 possibly contributes to the discrepancy in these results. We should also point out that the mice used in our current study lacked NK and NKT cells, primary responders to IL-21,[9] which could have led to insufficient immune reactions against tumor cells. IL-21 could be effective under conditions where CAR$^+$ T cells alone are unable to suppress tumors,[14] or in immunocompetent animals where host immune cells can respond to IL-21.

In conclusion, we showed that IL-21 did not improve the function of CAR$^+$ T cells *in vitro*, or in a human B lymphoma xenograft mouse model. Our collective findings suggest that unlike first-generation CARs, second-generation CARs with CD28 activate T cells sufficiently for adoptive transfer without the need for a 'third signal' such as IL-21.

Declaration of interest

The authors declare that they have no potential conflicts of interest.

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References

1）Pule M, Finney H, Lawson A. Artificial T-cell receptors. *Cytotherapy*. 2003；5：211-226.

2）Jena B, Dotti G, Cooper LJ. Redirecting T-cell specificity by introducing a tumor-specific chimeric antigen receptor. *Blood* .2010：116：1035-1044.

3）Brentjens RJ, Latouche JB, Santos E, et al. Eradication of systemic B-cell tumors by genetically targeted human T lymphocytes co-stimulated by CD80 and interleukin-15. *Nat Med*. 2003；9：279-286.

4）Brentjens RJ, Santos E, Nikhamin Y, et al. Genetically targeted T cells eradicate systemic acute lymphoblastic leukemia xenografts. *Clin Cancer Res*. 2007；13：5426-5435.

5）Kochenderfer JN, Yu Z, Frasheri D, Restifo NP, et al. Adoptive transfer of syngeneic T cells transduced with a chimeric antigen receptor that recognizes murine CD19 can eradicate lymphoma and normal B cells. *Blood*. 2010；116：3875-3886.

6）Brentjens RJ, Riviere I, Park JH, et al. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. *Blood*. 2011；118：4817-4828.

7）Brentjens RJ, Davila, M, Riviere, I, et al. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. *Sci Transl Med*. 2013；5：177ra38

8）Tsukahara T, Ohmine K, Yamamoto C, et al. CD19 target-engineered T-cells accumulate at tumor lesions in human B-cell lymphoma xenograft mouse models. *Biochem Biophys Res Commun*. 2013；438：84-89.

9）Sondergaard H, Skak K. IL-21：roles in immunopathology and cancer therapy. *Tissue Antigens*. 2009；74：467-479.

10）Hashmi MH, Van Veldhuizen PJ. Interleukin-21：updated review of Phase I and II clinical trials in metastatic renal cell carcinoma, metastatic melanoma and relapsed/refractory indolent non-Hodgkin's lymphoma. *Expert Opin Biol Ther*. 2010；10：807-817.

11）Sarosiek KA, Malumbres R, Nechushtan H, et al. Novel IL-21 signaling pathway up-regulates c-Myc and induces apoptosis of diffuse large B-cell lymphomas. *Blood*. 2010；115：570-580.

12）Gelebart P, Zak Z, Anand M, et al. Interleukin-21 effectively induces apoptosis in mantle cell lymphoma through a STAT1-dependent mechanism. *Leukemia*. 2009；23：1836-1846.

13）Akamatsu N, Yamada Y, Hasegawa H, et al. High IL-21 receptor expression and apoptosis induction by IL-21 in follicular lymphoma. *Cancer Lett*. 2007；256：196-206.

14）Markley JC, Sadelain M. IL-7 and IL-21 are superior to IL-2 and IL-15 in promoting human T cell-mediated rejection of systemic lymphoma in immunodeficient mice. *Blood*；2010：115：3508-3519.

15）Holst J, Szymczak-Workman AL, Vignali KM, et al. Generation of T-cell receptor retrogenic mice. *Nat Protoc*. 2006；1：406-417.

16）Miller AD, Garcia JV, von Suhr N, et al. Construction and properties of retrovirus packaging cells based on gibbon ape leukemia virus. *J Virol*. 1991；65：2220-2224.

17）Rahman M, Nara H, Onoda T, et al. Cloning and characterization of an isoform of interleukin-21. *FEBS Lett*. 2007；581：4001-4009.

18）Neri S, Mariani E, Meneghetti A, et al. Calcein-acetyoxymethyl cytotoxicity assay：standardization of a method allowing additional analyses on recovered effector cells and supernatants. *Clin Diagn Lab Immunol*. 2001；8：1131-1135.

19）Davis ID, Skak K, Smyth MJ, et al. Interleukin-21 signaling：functions in cancer and autoimmunity. *Clin Cancer Res*. 2007；13：6926-6932.

20）Romero P, Zippelius A, Kurth I, et al. Four functionally distinct populations of human effector-memory CD8+ T lymphocytes. *J Immunol*. 2007；178：4112-4119.

21）Singh H, Figliola MJ, Dawson MJ, et al. Reprogramming CD19-specific T cells with IL-21 signaling can improve adoptive immunotherapy of B-lineage malignancies. *Cancer Res*. 2011；71：3516-3527.

22）Hinrichs CS, Borman ZA, Cassard L, et al. Adoptively transferred effector cells derived from naive rather than central memory CD8+ T cells mediate superior antitumor immunity. *Proc Natl Acad Sci U S A*. 2009；106：17469-17474.

23）Hinrichs CS, Borman ZA, Gattinoni L, et al. Human effector CD8+ T cells derived from naive rather than memory subsets possess superior traits for adoptive immunotherapy. *Blood*. 2011；117：808-814.

24）Klebanoff CA, Gattinoni L, Torabi-Parizi P, et al. Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. *Proc Natl Acad Sci U S A*. 2005；102：9571-9576.

25）Hinrichs CS, Spolski R, Paulos CM, et al. IL-2 and IL-21 confer opposing differentiation programs to CD8+ T cells for adoptive immunotherapy. *Blood*. 2008；111：5326-5333.

26）Kaka AS, Shaffer DR, Hartmaier R, et al. Genetic modification of T cells with IL-21 enhances antigen presentation and generation of central memory tumor-specific cytotoxic T-lymphocytes. *J Immunother*. 2009；32：726-736.

IL-21の共発現は第二世代CD19特異的キメラ抗原受容体導入T細胞の抗リンパ腫効果に影響しない
－マウス異種移植モデルにおける検討－

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要　　約

CD28を含むCD19特異的第二世代キメラ抗原受容体（CD19-CAR）による養子免疫遺伝子療法はＢ細胞腫瘍に対する有望な治療法である。本研究において我々は，CD19-CARに加えIL-21を遺伝子導入したＴ細胞を作製し，CD19-CAR単独導入Ｔ細胞に比し抗腫瘍効果の増強が得られるかを検討した。まず健常者由来の末梢血リンパ球を活性化し，CD19-CAR+IL-21共発現またはCD19-CAR発現レトロウイルスベクターを用いて遺伝子導入した。導入遺伝子の発現はいずれも良好であった。両細胞は同様の増殖を示し，CD19-CAR+IL-21導入細胞でのみIL-21シグナリングの代替マーカーであるSTAT3のリン酸化を認めた。両細胞は表面マーカー上主にCentral memoryあるいはeffector memoryの形質を示し，CD19陽性Ｂ細胞株であるRajiに対して細胞傷害性を示し，CD19刺激によりIFN-γを産生した。Rag2$^{-/-}$γc$^{-/-}$免疫不全マウスを用いた動物実験では，両細胞はいずれもRajiの増殖を抑制した。IL-21は我々のＢリンパ腫モデルでは抗腫瘍効果を修飾せず，CD19-CAR導入Ｔ細胞は単独で十分な抗腫瘍効果を発揮する可能性が示された。

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