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Brief Communication

Monovalent Anti-CD3 Antibodies Effectively Eliminate the TCR-Positive Fraction of TCR-Deleted Allogeneic CAR-T Cells to Prevent GVHD

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Conflict of Interest
Choi K is a founder and shareholder of Ticaros Inc. Kim JH and Park HB are currently employees of Ticaros. Choi K, Kim JH, Kim H and Lee AN are co-inventors on the pending patent related to this study.

ABSTRACT

Chimeric antigen receptor-transduced T (CAR-T) cell therapy is an effective cell therapy against advanced hematological tumors. However, the use of autologous T cells limits its timely and universal generation. Allogeneic CAR-T cell therapy may be a good alternative as a ready-to-use therapeutic. Graft-versus-host disease (GVHD) is an obstacle for allogeneic CAR-T cells, but can be prevented by TCR deletion through genome editing. However, the remaining TCR-positive cells must be eliminated by costly, large-scale magnetic cell separation. Therefore, an alternative method for removing TCR-positive cells is needed. In this study, we found that monovalent anti-CD3 Abs such as Fab and single-chain variable fragment (scFv), but not whole IgG, induce apoptosis of *in vitro* expanded T cells, thereby effectively depleting residual TCR-positive T cells during TCR-deleted CAR-T cell generation and ultimately preventing xenogeneic GVHD *in vivo*. Thus, monovalent anti-CD3 treatment during allogeneic CAR-T cell manufacturing would be an efficient method to prevent GVHD.

Keywords: Anti-CD3 antibody; Receptors, chimeric antigen; Allogeneic cells; Graft-versus-host disease; T-lymphocytes; Apoptosis

INTRODUCTION

Chimeric antigen receptor-transduced T (CAR-T) cell therapy has opened an era of personalized immune cell therapy, showing remarkable responses against hematological malignancies (1,2). However, the current CAR-T cells must be individually prepared from autologous T cells, which takes 1–2 wks. In addition, the quality control process of the manufactured CAR-T cells requires another few weeks, making the total process 1–2 months before injection into the patient (3). Moreover, there is a chance of production failure (1%–10%) due to poor fitness of resource T cells from patients (4,5). Therefore, the need for CAR-T cells manufactured from healthy donor T cells (allogeneic CAR-T cells) has been continuously raised. The allogeneic CAR-T cells can be manufactured, stored frozen and ready to be administered to the patient whenever needed, thus realizing "off-the-shelf cell therapy" (6).

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Abbreviations

7-AAD, 7-amino-actinomycin D; AICD, activation-induced cell death; ATCC, American Type Culture Collection; CAR-T, chimeric antigen receptor-transduced T; Cκ, human kappa chain constant region; dJurkat, CD3ε-deleted Jurkat; GVHD, graft-versus-host disease; HvG, host versus graft reaction; MACS, magnetic cell separation; NSG, NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ; Lck, lymphocyte-specific protein tyrosine kinase; PKC, protein kinase C; scFv, single-chain variable fragment.

Author Contributions

Conceptualization: Choi K; Data curation: Kim JH; Formal analysis: Kim JH; Funding acquisition: Choi K; Investigation: Kim JH, Kim H, Lee AN, Park HB; Methodology: Kim JH, Kim H, Lee AN, Park HB; Project administration: Choi K; Supervision: Choi K; Visualization: Kim JH, Kim H, Lee AN; Writing - original draft: Kim JH, Choi K; Writing - review & editing: Choi K.

However, Allogeneic CAR-T cell therapy faces critical problems to overcome due to allogeneic immune responses induced by donor CAR-T cells and host immune cells. Allogeneic CAR-T cells can recognize host alloantigens and exhibit cytotoxicity to normal host tissues (graft-versus-host disease [GVHD]) (6). In addition, host immune cells can recognize alloantigens expressed by donor CAR-T cells and eliminate the CAR-T cells, reducing therapeutic efficacy (host versus graft reaction [HvG]). HvG could be prevented in the early phase of CAR-T cell therapy because host lymphocytes are usually depleted by non-myeloablative chemotherapy prior to CAR-T cell administration, although the reduced long-term efficacy of CAR-T cells due to HvG is still problematic. GVHD prevention of allogeneic CAR-T cells has been achieved by eliminating the TCR on their surface that recognizes host alloantigens, mostly using genome editing tools such as zinc finger nucleases (7), TALENs (8,9), and CRISPR/Cas9 nucleases (10,11). However, the TCR-deletion efficiency of these genome editing technologies is never 100%, always leaving a small percentage of residual TCR-positive CAR-T cells that can still induce GVHD when administered to the patient (9,12,13). Therefore, most TCR-deleted CAR-T cell manufacturing is accompanied by the step of removing these TCR-positive T cells by column purification using magnetic cell separation (MACS) (9). The MACS process has been effectively used for large-scale clinical production of TCR-deleted CAR-T cells, and the resulting allogeneic CAR-T cells have been safely infused into patients in early clinical trials. However, this process increases the overall cost of manufacturing, but is still imperfect in eliminating all residual TCR-positive cells, posing a potential risk of inducing GVHD side effect. Therefore, the development of a more convenient and cost-effective method to deplete TCR-positive cells is needed in this field.

Anti-CD3 Abs are known to induce T-cell apoptosis *in vitro* in some cases by inducing TCR/CD3 complex signaling (14,15). Therefore, the addition of anti-CD3 Abs during the expansion phase of TCR-deleted CAR-T cells may deplete the remaining TCR-positive T cells. To this end, we evaluated the potential use of various forms of anti-CD3 Abs to deplete the residual TCR-positive T cells after CRISPR/Cas9-mediated TCR-deletion of human T cells. In contrast to the previous report (15), the full-length anti-CD3 Abs could not induce apoptosis of the *in vitro* expanded T cells. Surprisingly, however, we found that monovalent forms of anti-CD3 Abs such as single-chain variable fragment (scFv) and Fab effectively induce apoptosis of the expanded T cells, which has never been reported. Subsequently, the simple addition of anti-CD3 scFvs to the TCR-deleted CAR-T cell culture during the expansion phase effectively eliminated the remaining TCR-positive CAR-T cells. We further demonstrate that anti-CD3 scFv treatment of the TCR-deleted CAR-T cells could completely prevent the development of xenogeneic GVHD in an *in vivo* mouse model of CAR-T cell therapy, whereas TCR-deleted CAR-T cells without this treatment developed a severe lethal GVHD side effect. Thus, the simple addition of anti-CD3 scFvs to TCR-deleted CAR-T cell manufacturing may facilitate the generation of safe allogeneic CAR-T cells without large-scale MACS purification.

MATERIALS AND METHODS

Mice and cells

NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were from The Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed in a specific pathogen-free animal facility at the Seoul National University College of Medicine. The experimental use of animals was approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-201129-2-5). Raji and Jurkat (clone E6-1) cells were purchased from the American Type

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Culture Collection (ATCC, Manassas, VA, USA). Raji-Luc cells were generated by transducing lentivirus carrying luciferase-P2A-EGFP. CD3ε-deleted Jurkat (dJurkat) was generated by CRISPR/Cas9-mediated CD3ε deletion. Lenti-X 293T cells were purchased from Takara Bio (Shiga, Japan). For human T cells, PBMCs were obtained by leukapheresis from healthy volunteers at Seoul National University Hospital (IRB No. H-2008-114-1150).

Generation of various forms of anti-CD3 Abs

Full-length OKT3-IgG and UCHT1-IgG were purchased from Bio X Cell (Lebanon, NH, USA) and BioLegend (San Diego, CA, USA), respectively. For OKT3- and UCHT1-Fabs, the cDNAs encoding the VH-CH1 and VL-CL domains of each Ab were cloned into separate pCEP4 plasmids and co-transfected into FreeStyle™ 293-F cells (Thermo Fisher Scientific, Waltham, MA, USA). For scFvs (OKT3, UCHT1, 1-4-2, 1-4-7), the cDNAs encoding VL-$(G4S)_3$ linker-VH were cloned into the human kappa chain constant region (Cκ)-containing pCEP4 expression vector (Invitrogen, Waltham, MA, USA) and transfected into 293-F cells. Abs were purified using KappaSelect resin (Cytiva, Marlborough, MA, USA) and tested for cell binding by flow cytometry.

To screen novel anti-CD3 scFvs, white leghorn chickens were immunized and boosted twice with primary human PBMCs and once with a mixture of human CD3ε/CD3γ and CD3δ/CD3ε heterodimeric proteins (Sino Biological, Beijing, China). One week after receiving the third booster dose, spleens, bone marrow, and bursa of Fabricius were harvested. ScFv-displayed phage libraries were constructed and were subjected to biopanning against recombinant human CD3 proteins. The selected scFv clones were cloned into Cκ-containing pCEP4 for further purification and validation of the antigen specificity by ELISA and flow cytometry as described previously (16).

CRISPR/Cas9-mediated TCR deletion in T cells

Human PBMCs were activated with plate-bound anti-CD3 (OKT3, 10 μg/ml), anti-CD28 (CD28.2, 2 μg/ml), and human IL-2 (200 U/ml, Proleukin) for 2 days. Activated T cells were electroporated with Cas9 ribonucleoprotein containing anti-TCRA sgRNA using the Neon Transfection System (Thermo Fisher Scientific). The synthesized sgRNA (IDT, Coralville, IA, USA) including the guide sequence (GAGAATCAAAATCGGTGAAT) against TCRA constant region was reported previously (17). TCR deletion was analyzed by flow cytometry using anti-TCRαβ-PE/Cy5 (IP26, BioLegend) and anti-CD3ε-PE (UCHT1, BioLegend) staining. For MACS-mediated TCR-positive cell depletion, T cells expanded in IL-2 (200 U/ml) for 6 days after TCR deletion were incubated with CD3 microbeads and applied to an LD column (Miltenyi Biotec, Gaithersburg, MD, USA). Cells that passed through the column were analyzed for TCR positivity by flow cytometry.

T cell apoptosis induction by anti-CD3 Abs

Various forms of anti-CD3 Abs (12.5 pmol/1×10^5 cells/ml) were added to the 7-day expanded T cells (2-day activation and further expansion in IL-2 for 5 days). Subsequently, at the indicated time points, cells were stained with a dead cell exclusion dye, 7-amino-actinomycin D (7-AAD, BioLegend) and AnnexinV-FITC (BioLegend), or with anti-active caspase3-PE (BD Biosciences, Franklin Lakes, NJ, USA) after fixation and permeabilization (BD Cytofix/Cytoperm, BD Biosciences), and analyzed by flow cytometry. In some experiments, Abs were co-treated with the following chemicals: cyclosporine A (10 μM in 0.01% DMSO, Sigma-Aldrich, St. Louis, MO, USA), dasatinib (100 nM in 0.01% DMSO, Selleckchem, Houston, TX, USA), or control vehicle (0.01% DMSO).

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Immunoblot analysis

Total lysates of cultured T cells were subjected to SDS-PAGE, transferred to a nitrocellulose membrane (Millipore, Burlington, MA, USA), and probed with primary Abs (anti-NFATc2, Santa Cruz, Dallas, TX, USA; anti-IKB, anti-p-ERK, and anti-ERK, Cell Signaling Technology, Danvers, MA, USA). Blots were stained with HRP-conjugated secondary Abs (anti-mouse IgG-HRP, anti-rabbit IgG-HRP; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and visualized by a chemiluminescence reaction using Super Signal West Pico (Thermo Fisher Scientific).

Anti-CD19 CAR-T cell generation

Anti-CD19 CAR cDNA consists of anti-CD19 scFv, CD8 hinge and transmembrane domain, 41BB cytoplasmic domain, and CD3ζ cytoplasmic domain (US Patent: 2013/0287748 A1). The synthesized CAR cDNA (IDT) was cloned into a lentiviral vector and then co-transfected into 293T cells (ATCC) with packaging plasmids (pMD.2G, pMDLg/pRRE, pRSV-rev) using Lipofectamine 3000 (Invitrogen). The viral supernatant was collected and concentrated by ultracentrifugation (Beckman, Brea, CA, USA). For lentiviral transduction of T cells, 2-day activated T cells were incubated with the concentrated lentivirus for 2 days and further expanded in IL-2 for 6 days. For TCR-deleted CAR-T cells, activated T cells were electroporated with anti-TCRA RNP and then transduced with CAR lentivirus for 2 days before cell expansion.

In vitro assessment of CAR-T cell functionality

For tumor cytotoxicity assay, 3×10^4 Raji-Luc cells were incubated with various numbers of CD19 CAR-T cells for 18 h in a 96 well plate. The 50 μl of luciferin (6 mg/ml, Promega, Madison, WI, USA) was added to each well and the luminescence signal was measured using a luminometer (PerkinElmer, Waltham, MA, USA). Percent cytotoxicity was calculated using the following formula:

$$100 - \left(\frac{\text{Test Well Signal} - \text{Background Well Signal}}{\text{Tumor Only Well Signal} - \text{Background Well Luciferase Signal}} \times 100\right)$$

For CAR-T cell activation, 3×10^4 CD19 CAR-T cells were incubated with 1.5×10^5 Raji cells. After 24 h, secreted IFN-γ in the culture supernatant was measured by ELISA (R&D Systems, Minneapolis, MN, USA).

In vivo model of xenogeneic GVHD by CAR-T cells

NSG mice were irradiated (1.5 Gy) and inoculated with 5×10^5 Raji-Luc cells intravenously on day 0. After 3 days, CAR-T cells (5×10^6) were administered intravenously. Tumor burden was measured by weekly peritoneal injection of D-luciferin (2 mg/head, Promega) and bioluminescence imaging using IVIS 100 (PerkinElmer). GVHD score was monitored twice weekly using 5 clinical parameters (skin, fur texture, posture, activity, ocular inflammation) on a scale of 0 to 3 (18).

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RESULTS

Incomplete TCR deletion by CRISPR/Cas9-mediated TCR genome editing

To evaluate the efficiency of CRISPR/Cas9-mediated TCR-deletion and MACS-mediated removal of residual TCR-positive cells in human T cells, we performed TCR-deletion experiments using a previously reported anti-TCRα guide RNA sequence (**Fig. 1A**) (17). The TCR-deletion efficiency was very impressive, leaving only 5%–10% TCR-positive cells (**Fig. 1B**). However, these residual TCR-positive cells persisted throughout the 2-wk culture period. Furthermore, anti-CD3 MACS-mediated elimination of TCR-positive cells was not complete, leaving approximately 1% of TCR-positive cells (**Fig. 1C and D**). Thus, the residual TCR-positive T cells after TCR deletion are not easily eliminated by conventional MACS purification.

T cell apoptosis induced by monovalent anti-CD3 Abs

To investigate the possibility that anti-CD3 Ab-induced apoptosis could be used for TCR-positive cell depletion, we first tested the cell death inducing ability of different forms (full-length IgG, Fab, and scFv-Cκ [scFv fused with Cκ; hereafter simply scFv]) of the anti-CD3 Ab, OKT3, treated on the pre-activated and subsequently expanded human T cells (hereafter expanded T cells) (**Supplementary Fig. 1A**). The full-length OKT3 (OKT3-IgG) failed to induce cell death. However, both monovalent forms of OKT3 (OKT3-Fab and OKT3-scFv) induced massive cell death as measured by 7-AAD/Annexin V staining (**Fig. 2A**). Since the Annexin V staining kinetically preceded the 7-AAD staining, it was very likely to be apoptotic cell death (**Fig. 2B**). Apoptotic cell death was further confirmed by active caspase-3 staining, in which only OKT3-Fab- and OKT3-scFv-treated cells showed a dramatic increase in active caspase-3-positive apoptotic cells, whereas OKT3-IgG did not (**Fig. 2C and D**). Thus, monovalent OKT3 treatment of expanded human T cells induced rapid apoptosis within 24 h.

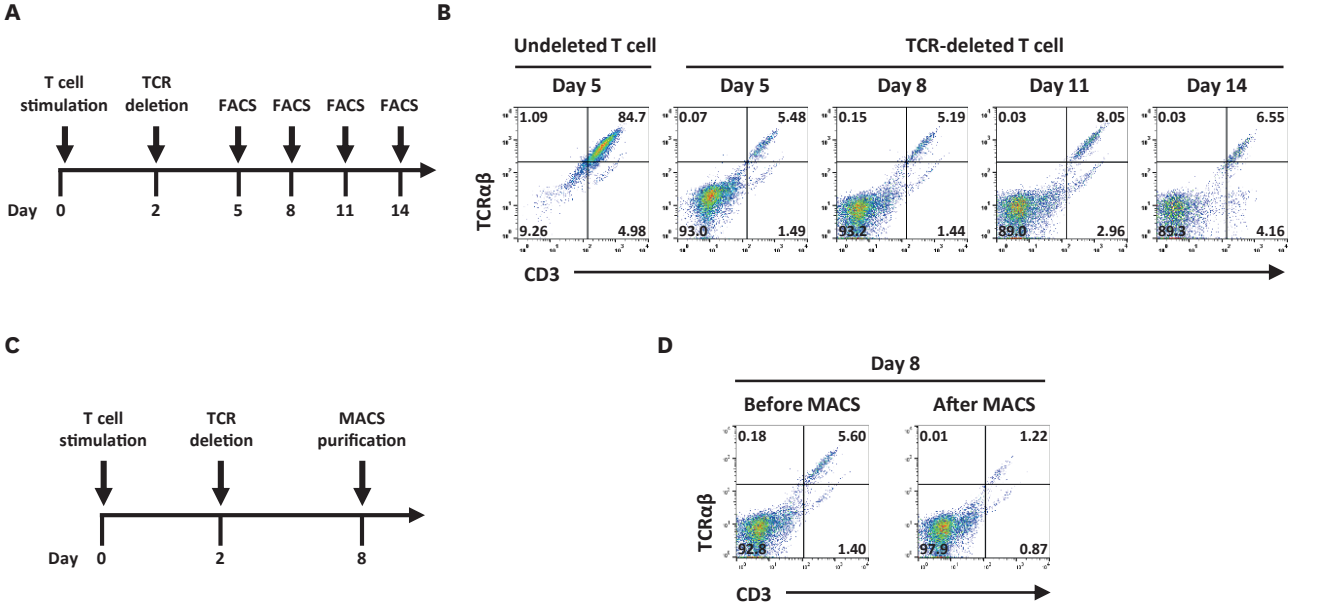

Figure 1. TCR-positive T cells cannot be readily eliminated after CRISPR/Cas9-mediated TCR deletion. (A) Experimental scheme for TCR deletion in human T cells using CRISPR/Cas9 gene editing. T cells were stimulated on day 0, transfected with anti-TCRA sgRNA/Cas9 RNP for TCR deletion on day 2, and analyzed by FACS for TCR/CD3 deletion efficiency on days 5, 8, 11, and 14. (B) TCRαβ and CD3ε expression on TCR-deleted T cells on the indicated days. (C) Experimental scheme for MACS purification to deplete residual TCR-positive T cells from TCR-deleted T cell populations. (D) Representative flow cytometry plot of TCR-deleted T cells after MACS purification. Data are representative of at least 2 independent experiments.

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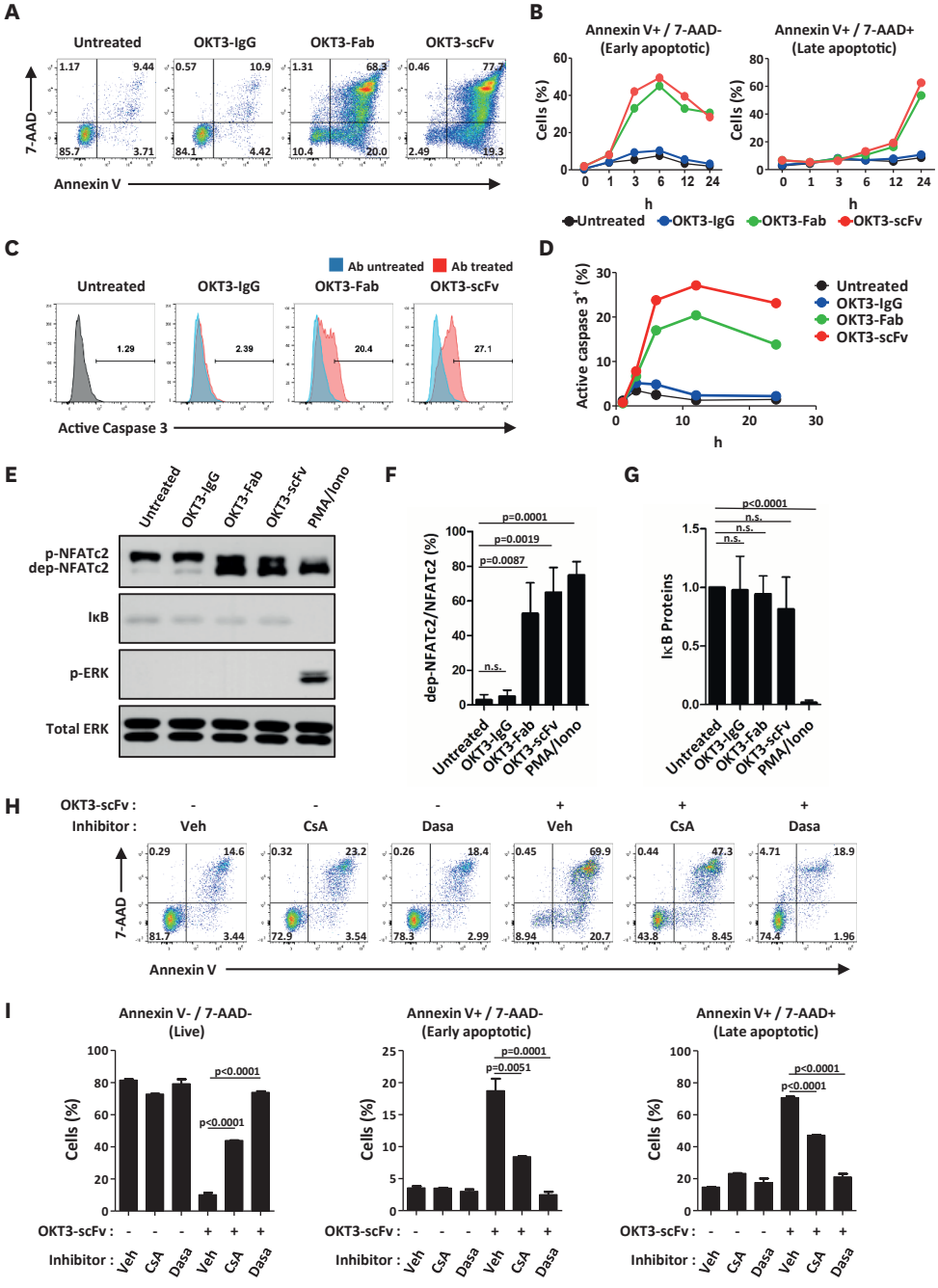

Figure 2. Monovalent OKT3 Abs eliminate expanded T cells by inducing apoptosis. (A-D) 7-day expanded human T cells were incubated with various forms of OKT3 (12.5 pmol/1×10^5 cells) for different durations. Apoptotic cells were analyzed by staining with 7-AAD and Annexin V and shown as a flow cytometry plot at 24 h (A) and a kinetics graph (B). Apoptotic cells were intracellularly stained with anti-active caspase 3 and shown as a flow cytometry plot at 12 h (C) and a kinetics graph (D). (E) Immunoblot analysis of NFATc2, IκB and ERK. Human T cells expanded for 7 days were incubated with different forms of OKT3 (12.5 pmol/1×10^5 cells) or PMA (50 ng/ml) plus ionomycin (1 μg/ml) as a positive control for 24 h, and total cell lysates were subjected to immunoblotting. For NFATc2 blot, NFATc2 is activated by dephosphorylation. The upper band represents inactive phosphorylated NFATc2 (p-NFATc2) and the lower band represents active dephosphorylated form (dep-NFATc2). For IκB blot, decreased IκB band intensity indicates NFκB activation. For ERK, p-ERK represents ERK activation. Total ERK is the sum of active and inactive ERK. (F) Degree of NFATc2 activation measured by dep-NFATc2/p-NFATc2 ratio: p-NFATc2 Intensity/(p-NFATc2 + dep-NFATc2 Intensity)×100. (G) IκB band intensities relative to that of the untreated sample. Equal protein loading was normalized to total ERK intensity. Graphs in (F) and (G) represent the average of 3 independent experiments. (H, I) Seven-day expanded human T cells were treated with OKT3-scFv in the presence of cyclosporine or dasatinib for 24 h. Apoptotic cells were stained with 7-AAD and Annexin V and shown as flow cytometry plot (H) and quantitative graph of triplicate samples (I). Data are representative of at least 2 independent experiments. All data are presented as mean ± SD. Statistical significance was determined by Student's *t*-test (F, G, H, J).
Veh, vehicle; CsA, cyclosporine A; Dasa, dasatinib; n.s., not significant; p-NFATc2, phosphorylated NFATc2; dep-NFATc2, dephosphorylated NFATc2; p-ERK, phosphorylation of ERK.

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To determine whether this monovalent anti-CD3-induced cell death is a unique property of OKT3, similar experiments were performed with another anti-CD3 Ab, UCHT1. Similar to OKT3, full-length UCHT1-IgG did not induce apoptosis of the expanded T cells, whereas UCHT1-scFv induced dramatic apoptosis (**Supplementary Fig. 1B**). To extend this observation to other anti-CD3 Abs, we generated new anti-CD3 Abs by screening scFv libraries from the immunized chickens. Clone 1-4-2 and 1-4-7 scFvs were obtained and their specificity to the recombinant CD3 proteins and cell surface-expressed CD3 was confirmed by ELISA and flow cytometry, respectively (**Supplementary Fig. 2A-C**). When these scFvs were then added to the expanded T cell culture, they induced massive cell death just like OKT3 and UCHT1 (**Supplementary Fig. 2D**). Thus, monovalent anti-CD3 Abs, particularly the scFv form of the Abs, induce expanded T cell death. Interestingly, when OKT3 Fab and scFv were added to unactivated fresh human T cells, they did not induce cell death (**Supplementary Fig. 1C**). Therefore, the apoptosis-inducing capacity of monovalent anti-CD3 Abs is restricted to pre-activated T cells.

Monovalent anti-CD3-mediated T cell apoptosis is partially dependent on NFAT signaling

Since anti-CD3 Ab-induced T cell apoptosis is usually driven by TCR cross-linking by solid-phase bound Abs or bivalent soluble Abs (full-length IgG or F(ab')2), it was of interest whether monovalent anti-CD3 Abs, such as Fab and scFv, could actually provide TCR signaling to induce apoptosis without TCR cross-linking. TCR signaling is mediated by 3 signaling pathways, including the calcium-NFAT pathway, the protein kinase C (PKC)θ-NFκB pathway, and the Ras-MAPK(ERK)-AP1 pathway (19). Therefore, we examined the activation of these 3 pathways in T cells treated with different forms of OKT3 by immunoblot analysis of NFATc2 dephosphorylation (NFAT activation), IκB degradation (NFκB activation), and ERK phosphorylation (ERK activation) (**Fig. 2E**). PMA and ionomycin treatment (PMA for activation of the PKCθ-NFκB and Ras-MAPK(ERK)-AP1 pathways, ionomycin for activation of the calcium-NFAT pathway) was used as a positive control. As expected, OKT3-IgG did not induce activation of any of the 3 signaling molecules. In contrast, OKT3-Fab and -scFv significantly activated NFATc2 as evidenced by the decrease in phosphorylation of NFATc2 with a concomitant increase in dephosphorylation of the molecule (**Fig. 2E and F**). IκB band intensity was only slightly decreased in monovalent OKT3-treated cells compared to the PMA/Ionomycin-treated positive control (**Fig. 2E and G**). However, phosphorylated ERK could not be detected in OKT3-Fab and -scFv-treated cells, indicating that the ERK pathway was not affected (**Fig. 2E**).

To confirm that TCR signaling by monovalent OKT3 does indeed provide an apoptotic signal, we co-treated a TCR signaling inhibitor (dasatinib, a lymphocyte-specific protein tyrosine kinase [Lck] inhibitor) with OKT3-scFv. Dasatinib completely inhibited OKT3-scFv-induced apoptosis, suggesting that TCR-induced Lck activation and downstream TCR signaling are critical for OKT3-scFv-induced cell death (**Fig. 2H and I**). In addition, co-treatment with cyclosporin A (an inhibitor of calcium-activated calcineurin) partially inhibited OKT3-scFv-induced cell death, implying that calcium-NFAT signaling contributes to this cell death (**Fig. 2H and I**). Thus, anti-CD3 scFv delivers apoptotic TCR signaling partially through the calcium-NFAT pathway.

Efficient removal of TCR-bearing CAR-T cells by anti-CD3 scFv

Next, we evaluated whether the apoptosis-inducing ability of anti-CD3 scFv could be applied to the elimination of residual TCR-positive cells in the TCR-deleted T cells. When OKT3-scFv was

added to TCR-deleted T cells cultured for 7 days, almost all TCR-positive T cells were eliminated within 3 days (**Fig. 3A and B**). We then investigated in CAR-T cells whether the remaining TCR-positive T cells could be depleted in the same way after TCR deletion. CD19-targeting CAR-T cells (hereafter CD19 CAR-T cells) that had been TCR-deleted were generated and expanded as a regular procedure for 7 days, and OKT3-scFv was treated for 3 days (**Fig. 3C**). Strikingly, the residual TCR-positive cells could not be detected at all, indicating the complete elimination of TCR-positive cells (**Fig. 3D**). We also confirmed that these OKT3-scFv-treated TCR-deleted (TCR-null) CD19 CAR-T cells were fully functional. When TCR-null CAR-T cells were co-cultured with CD19-positive tumor cells (Raji cells) *in vitro*, they produced IFN-γ and showed anti-tumor cytotoxicity equivalent to that of conventional CD19 CAR-T cells (**Fig. 3E and F**).

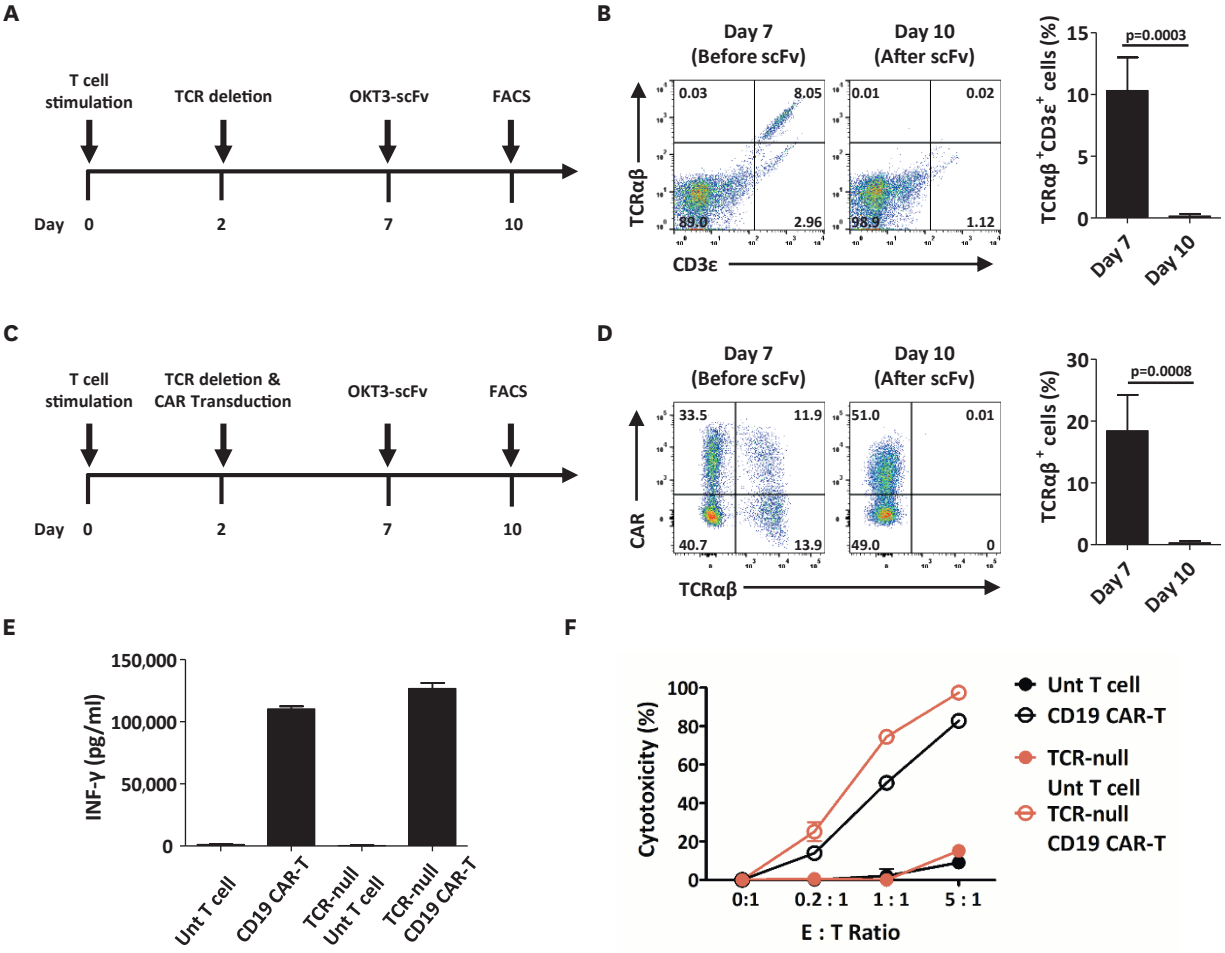

Figure 3. OKT3-scFv efficiently depletes residual TCR-positive T cells in TCR-deleted CAR-T cells. (A) Experimental scheme for depletion of residual TCR-positive T cells after TCR deletion. T cells were stimulated on day 0, transfected with anti-TCRA sgRNA/Cas9 RNP for TCR deletion on day 2, treated with OKT3-scFv on day 7, and analyzed by FACS for TCR/CD3 deletion efficiency on day 10. (B) TCRαβ and CD3ε expression on TCR-deleted T cells on the indicated days. A flow cytometry plot (left) and a quantitative graph representing the average of 4 independent experiments (right). (C) Experimental scheme for depletion of residual TCR-positive T cells after TCR deletion and CD19 CAR lentiviral transduction in CD19 CAR-T cell generation. (D) TCRαβ and CD3ε expression on TCR-deleted CAR-T cells on the indicated days. A flow cytometry plot (left) and a quantitative graph representing the average of 4 independent experiments (right). (E) Ten-day expanded conventional CD19 CAR-T cells (CD19 CAR-T) or OKT3-scFv-treated TCR-deleted CD19 CAR-T cells (TCR-null CD19 CAR-T) were co-cultured with Raji tumor cells for 24 h. Ten-day expanded T cells (Unt T cell) or OKT3-scFv-treated TCR-deleted expanded T cells (TCR-null Unt T cell) were used as negative controls. The amount of IFN-γ in the culture supernatants was measured by ELISA. (F) CD19 CAR-T cells or TCR-null CD19 CAR-T cells (effector, E) were co-cultured with luciferase-expressing Raji cells (Raji-Luc) (target, T) at the indicated E:T ratios for 18 h. The luciferase activity of the cell lysates was measured by a luminometer and the percent cytotoxicity was quantified. The data are representative of at least 3 independent experiments. All data are presented as mean ± SD. Statistical significance was determined by Student's *t*-test (B, D).
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Anti-CD3 scFv prevents GVHD induced by TCR-deleted CAR T cells

We extended this observation *in vivo* to test whether this anti-CD3 scFv-mediated elimination of TCR-positive cells could prevent GVHD induced by TCR-deleted CAR-T cells. When TCR-deleted CD19 CAR-T cells were administered to the tumor-bearing immunodeficient NSG mice, they eliminated tumor cells as efficiently as conventional (TCR-undeleted; TCR-positive) CD19 CAR-T cells, as measured by tumor cell bioluminescence imaging (**Fig. 4A and B**). However, both conventional and TCR-deleted CAR-T cells gradually developed severe GVHD and ultimately killed the mice (**Fig. 4C and D**). Therefore, TCR-deletion in CAR-T cells without removal of residual TCR-positive cells was not sufficient to prevent xenogeneic GVHD by TCR-positive T cells in this experimental setting. In contrast, when TCR-null CAR-T cells were administered to the tumor-bearing mice, they did not induce GVHD, indicating that GVHD-inducing TCR-positive cells were completely eliminated (**Fig. 4C**). Furthermore, these CAR-T cells could also efficiently eliminate tumor cells, ensuring 100% survival of the mice (**Fig. 4B and D**). Thus, monovalent anti-CD3 treatment during the generation of TCR-deleted CAR-T cells is an efficient method to deplete GVHD-inducing TCR-positive T cells.

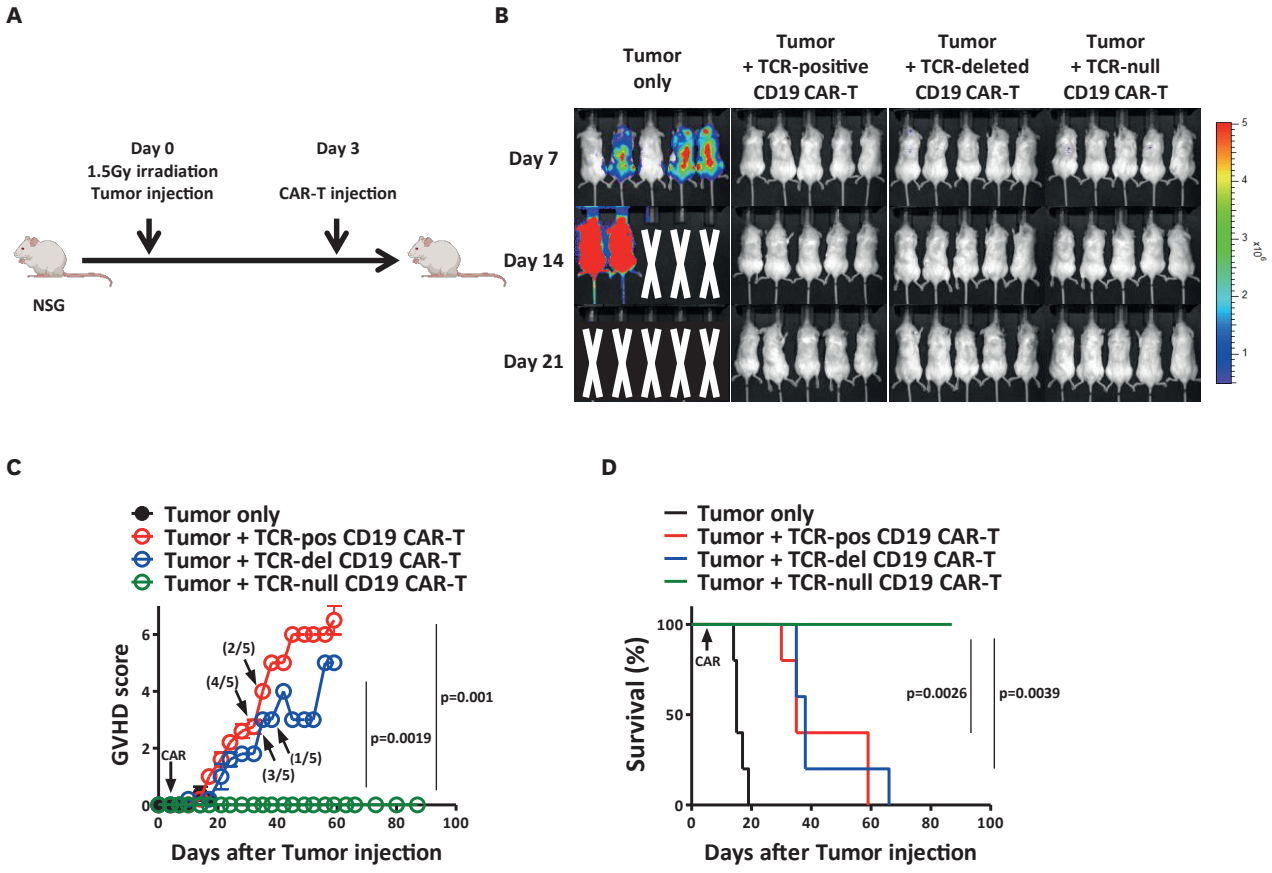

Figure 4. OKT3-scFv-mediated depletion of TCR-positive cells prevents xenogeneic GVHD induction by TCR-deleted CAR-T cells. (A) Experimental scheme for CD19 CAR-T cell therapy model in human lymphoma-bearing immunodeficient mice. NSG mice were irradiated (1.5 Gy), intravenously injected with Raji-Luc cells (5×10^5) on day 0, and intravenously injected with CD19 CAR-T cells (5×10^6) on day 3. (B) Bioluminescence imaging of tumor burden at the indicated time points after Raji-Luc cell injection. Ten-day expanded conventional TCR-positive CD19 CAR-T cells (TCR-pos CD19 CAR-T), TCR-deleted CD19 CAR-T cells (TCR-del CD19 CAR-T), or OKT3-scFv-treated TCR-deleted CD19 CAR-T cells (TCR-null CD19 CAR-T) were used as therapeutic CAR-T cells (n=5 for each group). (C, D) Clinical GVHD score (C) and mouse survival (D) were measured twice weekly throughout the experiments. Numbers in parentheses are surviving mice at the indicated time points (C). Data are representative of at least 3 independent experiments. Data in (C) are presented as mean ± SEM. Statistical significance was determined by 2-way ANOVA followed by Tukey's multiple comparison test (TCR-del CD19 CAR-T versus TCR-null CD19 CAR-T: p=0.0019 at day 28, TCR-pos CD19 CAR-T versus TCR-null CD19 CAR-T: p=0. 001 at day 28, when all 5 mice were alive) (C) or log-rank (Mantelcox) test (TCR-del CD19 CAR-T versus TCR-null CD19 CAR-T: p=0.0039, TCR-pos CD19 CAR-T versus TCR-null CD19 CAR-T: p=0.0026) (D).

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DISCUSSION

In this study, we propose a simple and efficient method to eliminate residual TCR-positive cells in allogeneic CAR-T cell products using T-cell depleting anti-CD3 Abs. The conventional method for removing TCR-positive cells is MACS-based column purification at the end of CAR-T cell expansion, which is associated with substantial cell loss and still shows a small percentage of residual TCR-positive cells (approximately 1%) (9,12,13). This small number of TCR-positive cells induces low-grade GVHD in clinical trials, although it could be managed with corticosteroids. Recently, several other strategies have been proposed. One was to transfect anti-CD3 CAR mRNA into TCR-deleted CAR T cells to generate transient anti-CD3 CAR-T cells to kill TCR-positive T cells during the expansion phase (20). The other was to generate an anti-CD3 CAR-expressing NK cell line and co-culture these CAR-NK cells with TCR-deleted CAR T cells to deplete TCR-positive cells (21). However, these methods are rather complicated and require the introduction of another gene or cell into the culture. Therefore, more convenient and efficient methods should be introduced into the manufacturing process, such as apoptosis-inducing Ab treatment.

TCR/CD3 signaling in T cells is critical for T cell activation. Therefore, anti-CD3 Abs are the most popular reagents for artificial activation of T cells. However, it is known that anti-CD3 Abs can also provide an apoptotic signal, especially in the absence of CD28 costimulation. In particular, plate-bound anti-CD3 Abs are known to induce T cell apoptosis (14,22). Therefore, coating the culture bag wall with anti-CD3 Abs would be a way to induce apoptosis of residual TCR-positive cells during the expansion phase of TCR-deleted CAR T cells. However, coating the culture bag with Abs would be a cumbersome process. On the other hand, soluble bivalent Abs such as full-length anti-CD3 IgG or F(ab')2, especially low-affinity Abs, are also known to induce apoptosis of activated murine and human T cells (15,23). In this case, simply adding the Abs to the culture would be a more convenient procedure than coating them onto the culture bag. However, in our hands, soluble full-length anti-CD3 IgG Abs failed to induce apoptosis of the expanded human T cells. The reasons for this difference are not clear at this time. Typically, plate-bound anti-CD3 Abs provide a strong TCR signal, thereby inducing activation-induced cell death (AICD) of T cells (14,22). On the other hand, soluble bivalent Abs cannot provide sufficient TCR signal to resting T cells. However, they can facilitate cell death of recently activated T cells (72 h after initial activation) by providing an additional weak TCR signal necessary for AICD (14). In contrast, the 7-day expanded T cells in our study may have evaded the weak TCR signal-induced AICD phase and lost sensitivity to the soluble bivalent Abs.

Surprisingly, however, soluble treatment with monovalent anti-CD3 scFv or Fab Abs could efficiently induce apoptosis of expanded T cells, which to our knowledge has not been reported in the literature. Mechanistically, monovalent anti-CD3 Ab activated NFAT, but not NFκB or ERK activation, and inhibition of NFAT pathway by CsA partially inhibited T cell apoptosis. Activation of the NFAT pathway without ERK-AP1 activation is known to induce T cell anergy (24). Calcium signaling is also known to induce T cell apoptosis in some cases (25). Because the calcium-NFAT pathway is more sensitive to low-affinity TCR signals than the NFκB and ERK pathways (26), subtle low-affinity stimulation by monovalent Abs may have activated the NFAT pathway preferentially over other pathways, leading to apoptotic cell death. This mechanism of monovalent anti-CD3-dependent cell death appears to differ from that of solid-phase-bound/soluble bivalent anti-CD3-dependent cell death, as the latter is mediated by ERK activation and subsequent FasL upregulation (15,27-29).

Although the mechanism of monovalent anti-CD3-induced apoptosis needs to be further elucidated, its practical value as a depleting reagent to remove residual TCR-positive allogeneic CAR T cells seems obvious, since this treatment almost completely eliminated residual TCR-positive cells (less than 0.1%) and completely prevented xenogeneic CAR-T cell-mediated GVHD, while maintaining the therapeutic efficacy of CAR-T cells.

Thus, treatment of the TCR-deleted allogeneic CAR T-cell culture with monovalent anti-CD3 Ab would provide a novel method to significantly improve the manufacturing efficiency of allogeneic CAR-T cells.

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SUPPLEMENTARY MATERIALS

Supplementary Figure 1

Cytotoxicity of various forms of anti-CD3 Abs against expanded and Unact T cell. (A) Illustration of the different forms of Abs used in this study. (B) Seven-day expanded human T cells were incubated with UCHT1-IgG or UCHT1-scFv (12.5 pmol/1×10^5 cells) for 24 h. Apoptotic cells were analyzed by staining with 7-AAD and Annexin V and shown as a flow cytometry plot. (C) Human T cells purified from PBMCs by MACS using mixed CD4 and CD8 microbeads (Unact T cell, Miltenyi Biotec) were incubated with different forms of OKT3 (12.5 pmol/1×10^5 cells) for 24 h. Apoptotic cells were analyzed by staining with 7-AAD and Annexin V and presented as a flow cytometry plot.

Supplementary Figure 2

Characterization of newly screened anti-CD3 scFvs. (A, B) Anti-CD3 scFv-Cκ proteins (clones 1-4-2, 1-4-7) were subjected to enzyme immunoassay against human CD3δ/CD3ε heterodimeric proteins (A) and human CD3ε/CD3γ heterodimeric proteins (B). Enzyme immunoassay was performed in triplicate and data are presented as mean ± SD. (C) Binding specificity of the novel scFvs to CD3 expressed on T cells. The 1-4-2 scFv-Cκ and 1-4-7 scFv-Cκ (25 pmol/1×10^5 cells) bound to CD3-expressing Jurkat T cells, but not to dJurkat. (D) Ten-day expanded human T cells were treated with 1-4-2 scFv-Cκ or 1-4-7 scFv-Cκ (12.5 pmol/1×10^5 cells) for 24 h. T cell death was assessed by 7-AAD staining and flow cytometry analysis. The data are representative of at least 2 independent experiments.

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