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ORIGINAL ARTICLE

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# CD4$^+$ T cells support polyfunctionality of cytotoxic CD8$^+$ T cells with memory potential in immunological control of tumor

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## Abstract

Polyfunctionality/multifunctionality of effector T cells at the single cell level has been shown as an important parameter to predict the quality of T cell response and immunological control of infectious disease and malignancy. However, the fate of polyfunctional CD8$^+$ CTLs and the factors that control the polyfunctionality of T cells remain largely unknown. Here we show that the acquisition of polyfunctionality on the initial stimulation is a sensitive immune correlate of CTL survival and memory formation. CD8$^+$ T cells with high polyfunctionality, assessed with γ-interferon and tumor necrosis factor-α production and surface mobilization of the degranulation marker CD107a, showed enhanced Bcl-2 expression, low apoptosis, and increased CD127$^{high}$KLRG1$^{low}$ memory precursor phenotype. Consistent with these observations, CD8$^+$ T cells were found to acquire high frequency of cells with polyfunctionality when stimulated in conditions known to enhance memory formation, such as the presence of CD4$^+$ T cells, interleukin (IL)-2, or IL-21. Utilizing T-cell receptor (TCR) transgenic mouse-derived CD8$^+$ T cells that express a TCR specific for a tumor-derived neoantigen, we showed that polyfunctional tumor-specific CTLs generated in the presence of CD4$^+$ T cells showed long persistence in vivo and induced enhanced tumor regression when adoptively transferred into mice with progressing tumor. Acquisition of polyfunctionality thus impacts CTL survival and memory formation associated with immunological control of tumor.

**KEYWORDS**

cancer immunotherapy, CD4$^+$ T cell, CTL, IL-2, polyfunctionality

## 1 | INTRODUCTION

It is becoming increasingly evident that adoptive transfer of tumor-reacting effector cells is a promising immunotherapy for tumor.[1,2] Recent success on the treatment of B-cell malignancy by the infusion of CD19-chimeric antigen receptor (CAR)-T cells is a milestone in the clinical application of adoptive cell therapy (ACT) of malignancy.[1,2] T cells genetically engineered to express tumor-specific T cell receptor (TCR) also showed clinical efficacy when transferred into patients with solid tumors such as malignant melanoma[3,4] and synovial cell sarcoma.[5,6] However, it remains largely unclear what limits the generation of genetically engineered T cells with high efficacy when transferred into patients

and what correlates the response of these T cell products in vivo in immunological control of tumor.[1,2]

Polyfunctionality is the ability of T cells to carry out multiple functions, such as the secretion of an array of cytokines, chemokines, or cytotoxic granules simultaneously at the single cell level. It has been suggested that the appearance of polyfunctional T cells is a sensitive immune correlate for immunological disease control.[7,8] The importance of T cell polyfunctionality has been reported in preclinical infection models[9,10] as well as in humans reacting to HIV, cytomegalovirus, hepatitis B virus, or tuberculosis.[10-17] We have reported that the appearance of polyfunctional $CD8^+$ effector cytotoxic T cells (CTLs) in vivo is a critical determinant of the success of immunological control of tumor and regulatory T cells (Tregs) play a role in the mechanism inhibiting the induction of polyfunctionality in tumor antigen-specific T cells as a means of tumor escape from immunosurveillance.[18,19] These results, together with other reports,[20] indicated the importance of polyfunctionality of effector T cells in antitumor immune response. However, the fate of polyfunctional T cells and the factors that determine the polyfunctionality in CTLs remain largely unknown.

It has been shown that the type of vaccine formulation influences the functionality of induced antigen-specific T cells.[10,21] Additionally, infections with different viruses induced different magnitudes of polyfunctionality in antigen-specific $CD4^+$[22,23] and $CD8^+$[24] T cells. These results suggest that the amount and duration of antigen presentation influenced the quality of the response. However, the cellular and the molecular mechanisms underlying the control of T cell polyfunctionality have not been fully elucidated.

Central memory and effector memory $CD4^+$ and $CD8^+$ T cells were reported to produce multiple cytokines.[21,25,26] In contrast, T cells generated in animals during chronic lymphocytic choriomeningitis virus[27] or HIV[28] infection showed limited ability to produce multiple cytokines, and terminally differentiated effector T cells were suggested as γ-interferon (IFN-γ) single producers.[24] Humans that showed high frequency of IFN-γ plus tumor necrosis factor-α (TNF-α) production against smallpox vaccination,[29] and animals that showed high-quality response in infections were reported to show sustained T cell response with long-lasting protection.[10,21] These reports suggest a relationship between the quality of T cell response and their maintenance. However, a critical question remains unclear as to whether distinct populations of T cells, in terms of their polyfunctionality after initial priming, have different potential to survive.

In this study, we asked whether $CD8^+$ T cells with different functionality in the response to initial stimulation have different survival capacity and memory formation. We then tested whether the factors associated with memory formation, such as $CD4^+$ T cells or interleukin (IL)-2/-21, increase CTL polyfunctionality. Finally, we examined whether the acquisition of polyfunctionality in tumor-specific $CD8^+$ T cells influence their in vivo survival and antitumor efficacy when adoptively transferred into the hosts with progressing tumor.

## 2 | MATERIALS AND METHODS

### 2.1 | Mice and tumor lines

Female BALB/c and SCID mice were obtained from CLEA Japan. DUC18 mice, which are transgenic (Tg) for αβ-T-cell receptors (TCRs) specific for the $K^d$-restricted 136-144 peptide from a mutated form of ERK2 (mERK2), were established on BALB/c background as described previously.[30] All mice were used at 8-10 weeks of age. Mice were maintained at the Animal Center of the Mie University Graduate School of Medicine. All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of the Mie University Graduate School of Medicine. CMS5 is a methylcholanthrene-induced sarcoma cell line of BALB/c origin that expresses mERK2.[31] P1.HTR is a subline derived from P815 mastocytoma cells of DBA/2 origin.[32]

### 2.2 | Antibodies and reagents

PerCP-Cy5.5-conjugated anti-CD8 (53-6.7), V500-conjugated anti-CD8 (53-6.7), phycoerythrin (PE)-conjugated anti-TCR Vβ8.3 (1B3.3), PE-conjugated anti-CD107a (1D4B), PE-Cy7-conjugated anti-CD107a (1D4B), PerCP-Cy5.5-conjugated anti-CD127 (A7R34), PE-Cy7-conjugated anti-KLRG1 (2F1), allophycocyanin (APC)-Cy7-conjugated anti-TNF (MP6-XT22), APC-conjugated anti-IFN-γ (XMG1.2), PE-conjugated anti-Bcl-2 (3F11), and FITC-conjugated anti-active caspase-3 (C92-605) were purchased from BD Biosciences. Anti-CD16/CD32 FcR blockade (93) and PE-conjugated anti-CD122 (5H4) were purchased from eBioscience and Invitrogen, respectively. The previously described mERK2-derived synthetic $mERK2_{136\text{-}144}$ peptide (9m peptide: QYIHSANVL)[31] and human epidermal growth factor receptor 2 (HER2) oncoprotein-derived $K^d$-binding $HER2_{63\text{-}71}$ peptide (p63 peptide: TYLPTNASL)[33] was purchased from Qiagen.

### 2.3 | Stimulation of lymphocytes

The $CD8^+$ T cells and $CD4^+$ T cells were isolated from the spleens or lymph nodes of BALB/c and DUC18 Tg mice using CD8a (Ly-2) MicroBeads and CD4 (L3T4) MicroBeads according to manufacturer's protocol (Miltenyi Biotec). The purity of isolated cells was greater than 95%. $CD8^+$ T cells were stimulated by Dynabeads Mouse T-Activator CD3/CD28 (Invitrogen) at several bead-to-cell ratios in the absence or presence of syngeneic $CD4^+$ T cells (CD8 / CD4 ratio = 0.5). In some experiments, recombinant human IL-2 (provided Takeda Pharmaceutical), recombinant mouse IL-21 (R&D Systems), and/or recombinant mouse IFN-γ (R&D Systems) were added. In some experiments, Transwell system (BD Biosciences) was used to separate $CD8^+$ T cells from $CD4^+$ T cells in the absence or presence of neutralizing mAb to cytokines, such as anti-IL-2 mAb (S4B6) (BD Biosciences), anti-IL-21 polyclonal Ab (BD Biosciences), or anti-IFN-γ mAb (H22) (kindly provided Dr RD Schreiber, Washington University School of Medicine).

### 2.4 | Multiparameter flow cytometry

Stimulated lymphocytes were incubated in the presence of 0.1 μg/mL anti-CD107a for 1 hour. We then incubated these samples for an additional 4 hours after the addition of 1 μL/mL GolgiPlug (BD Biosciences). Cells were incubated for 15 minutes at 4°C with anti-CD16/CD32 FcR blockade, and then stained with Abs against surface markers. After permeabilization and fixation, cells were stained intracellularly with anti-IFN-γ and anti-TNF mAbs in combination with anti-Bcl-2 or anti-active caspase-3. Stained cells were acquired using a FACSCanto II flow cytometer (BD Biosciences), and data were analyzed using FACSDiva and FlowJo (BD Biosciences) software. For the assessment of polyfunctionality, $CD8^+$ T cells were grouped into all 3 functions as IFN-$\gamma^+$TNF-$\alpha^+$CD107a$^+$ (3), any 2 functions including IFN-$\gamma^+$TNF-$\alpha^+$CD107a$^-$, IFN-$\gamma^+$TNF-$\alpha^-$CD107a$^+$, and IFN-$\gamma^-$TNF-$\alpha^+$CD107a$^+$ (2), any single function including IFN-$\gamma^+$TNF-$\alpha^-$CD107a$^-$, IFN-$\gamma^-$TNF-$\alpha^+$CD107a$^-$, and IFN-$\gamma^-$TNF-$\alpha^-$CD107a$^+$ (1), or no function (0), then calculated the relative frequency of each group and represented in the pie chart.[18]

### 2.5 | Adoptive transfer

The unstimulated or stimulated $CD8^+$ T cells from DUC18 TCR Tg mice were used for adoptive transfer. For the unstimulated $CD8^+$ T cells transfer, freshly isolated DUC18 spleen $CD8^+$ cells were transferred with or without freshly isolated WT BALB/c $CD4^+$ T cells. When indicated, 5-(and-6)-carboxyfluorescein succinimidyl ester (Molecular Probes) labeled (2.5 μmol/L) cells were used to track the transferred cells in vivo. For adoptive transfer of stimulated $CD8^+$ T cell transfer, the $CD8^+$ T cells from the spleens of DUC18 TCR Tg mice were stimulated with anti-CD3/CD28 microbeads at a bead-to-cell ratio of 0.5, in the absence or presence of $CD4^+$ T cells utilizing the Transwell system for 2 days. Then $CD8^+$ cells ($1 \times 10^6$ in 0.2 mL PBS) were suspended in 0.2 mL PBS and injected i.v. into a lateral tail vein.

### 2.6 | Tumor challenge

CMS5 tumor cells ($1 \times 10^6$ in 0.2 mL PBS) were s.c. inoculated into the right flanks of mice. Tumor size was determined by averaging 2 orthogonal diameters measured with calipers every other day. Mice were killed when the mean diameter of the tumor reached 20 mm according to institutional guidelines.

### 2.7 | Enzyme-linked immunosorbent assay

Culture supernatants were collected and IL-2 concentration was assessed by ELISA according to the kit instructions (eBioscience).

### 2.8 | γ-Interferon enzyme-linked immunospot assay

The enzyme-linked immunospot assay was undertaken as described previously[34] with modification to detect $mERK2_{136\text{-}144}$-specific $CD8^+$ T cells. Briefly, LN cells were cultured with irradiated (45 Gy) P1.HTR cells pulsed with 1 μmol/L 9m peptide or control p63 peptide. Spots were counted with the aid of a dissecting microscope and analyzed by ImmunoSpot S5 Versa Analyzer (Cellular Technology).

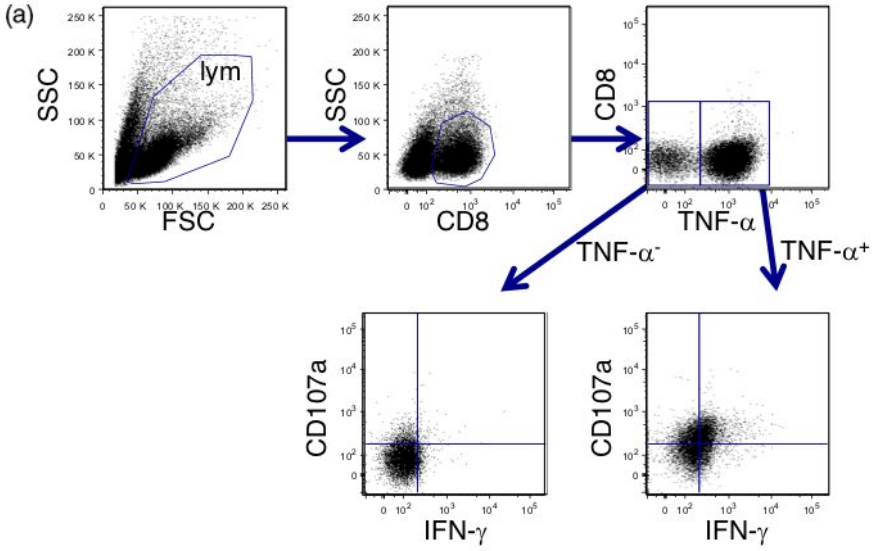
### 2.9 | Statistical analysis

Data were expressed as the means + SD. Differences between groups were examined for statistical significance using Student's *t* test. A *P* value less than .05 denoted a statistically significant difference (*$P < .05$ and **$P < .01$).

## 3 | RESULTS

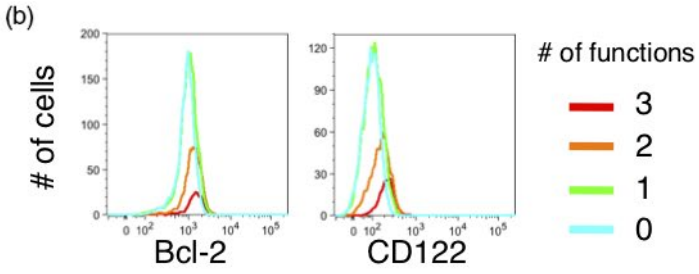
### 3.1 | Polyfunctional $CD8^+$ T cells show antiapoptotic and memory precursor phenotype

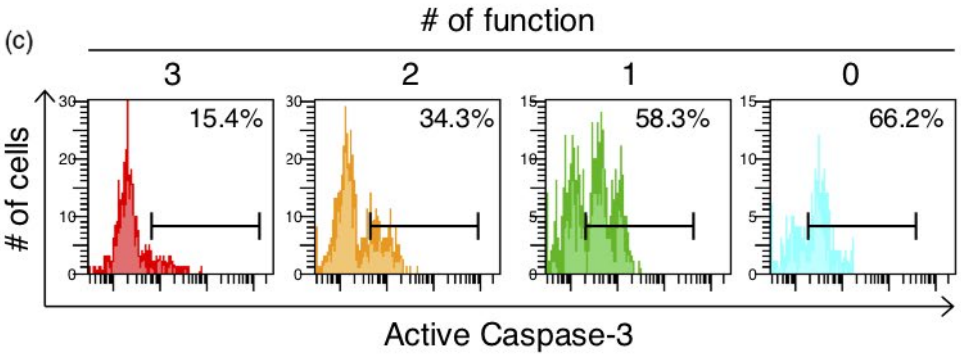
To evaluate the character of distinct functional $CD8^+$ T cell populations, we addressed the expression of a member of apoptosis regulator proteins, Bcl-2, memory marker CD122, and a key mediator of apoptosis, active caspase-3, in each functional population. $CD8^+$ T cells purified from spleen of BALB/c mice were stimulated by microbeads conjugated with anti-CD3 and anti-CD28 mAbs for 2 days and evaluated with CD107a mobilization assay and intracellular cytokine staining. We determined the number of functions of $CD8^+$ T cells according to the IFN-γ and TNF-α production and CD107a mobilization in $CD8^+$ T cells at the single-cell level, and responses were grouped and color-coded by calculating the relative frequency of several unique functional subsets: cells with all 3 functional characteristics (red), populations with varying combinations of 2 functions (orange), subsets displaying only 1 function (green), and those without any of the 3 functions (blue) (Figure 1A). These functions were selected because the polyfunctionality assessed by these factors in $CD8^+$ T cells has been shown to define a sensitive correlate of the immunological control of tumor.[18,19] $CD8^+$ T cells with 1 function showed similar expression of Bcl-2 and CD122 to the cells with no function. In contrast, $CD8^+$ T cells with 2 or 3 functions showed higher expression of Bcl-2 and CD122 compared to those expressing 1 or no function (Figure 1B). As shown in Figure 1C, highly polyfunctional T cells showed decreased expression of active caspase-3, a key mediator of the signal cascade of apoptosis. These results indicated that $CD8^+$ T cells that acquired high polyfunctionality after the initial stimulation through CD3 possess favorable capacities of survival. Because highly polyfunctional T cells preferentially expressed Bcl-2 and CD122, some of the hallmarks of memory development, we next addressed the acquisition of memory precursor phenotype in each functional

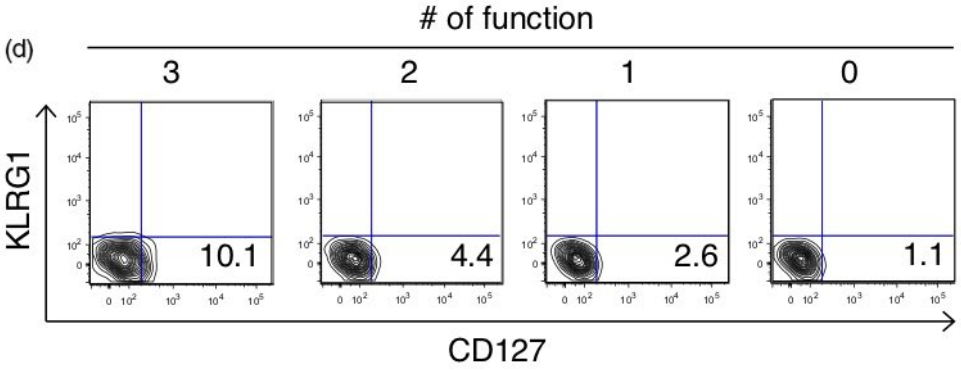



# of functions

| | TNF-α | IFN-γ | CD 107a |
|---|---|---|---|
| 3 | + | + | + |
| 2 | + | + | − |
| | + | − | + |
| | − | + | + |
| 1 | + | − | − |
| | − | + | − |
| | − | − | + |
| 0 | − | − | − |



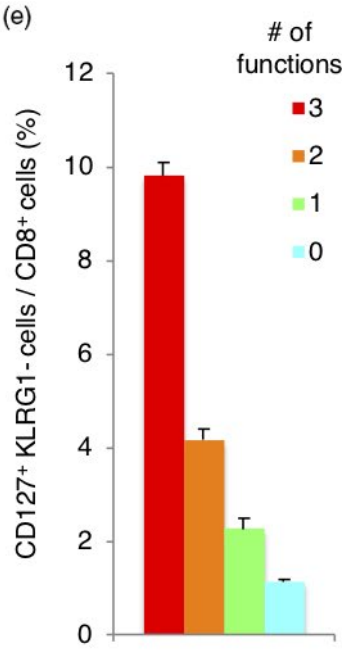



**FIGURE 1** $CD8^+$ T cells with polyfunctionality showed the antiapoptotic and memory precursor phenotype. $CD8^+$ T cells purified from BALB/c mice-derived spleen cells were stimulated by microbeads conjugated with anti-CD3 and anti-CD28 mAbs for 2 days, and CD107a mobilization assay and intracellular cytokine staining was carried out. A, The polyfunctionality of cells was defined by the number of functions as indicated. Representative staining of γ-interferon (IFN-γ), tumor necrosis factor-α (TNF-α), and CD107a is shown. FSC, Forward Scatter; SSC, Side Scatter. B, Expression of Bcl-2 and CD122 in cells with each functionality is indicated. C, Expression of active caspase-3 in cells with each functionality is indicated. D, E, FACS staining (D) and summary (E) of $CD127^{high}KLRG1^{low}$ cells in $CD8^+$ cells with each functionality is indicated. Results are representative of 3 independent experiments

population. As shown in Figure 1D,E, highly polyfunctional T cells were found to preferentially acquire $CD127^{high}KLRG1^{low}$ memory precursor phenotype compared to single or no functional T cells.

## 3.2 | $CD4^+$ T cell help increased polyfunctionality in $CD8^+$ T cells through soluble factors

Based on the above data suggesting the close relationship between CTL polyfunctionality and their memory formation, we hypothesized that the factors known to support the memory formation of T cells could determine the T cell polyfunctionality. It has been reported that $CD4^+$ T cell help was required in the generation of functional $CD8^+$ T cell memory.[35] Therefore, we examined the impact of the presence of $CD4^+$ T cells on the acquisition of polyfunctionality in $CD8^+$ T cells when stimulated by anti-CD3/CD28. Among $CD8^+$ T cells stimulated without $CD4^+$ T cells, 3.9% of all cells showed cells with 3 functions and 21.0% of the cells gaining 2 functions at a bead-to-cell ratio of 1. When stimulated with $CD4^+$ T cells, the $CD8^+$ T cell response appeared more robust, showing that 12.6% of all cells gained 3 functions and 32.0% of the cells were bifunctional at day 1 (Figure 2A). Similar results with limited frequency of polyfunctional T cells were observed at bead-to-cell ratio of 0.1. These results suggested that $CD4^+$ T cell help promoted $CD8^+$ T cells to gain polyfunctionality with enhanced frequency. We next addressed whether soluble factors play a critical role in the induction of polyfunctionality of $CD8^+$ T cells utilizing the Transwell culture system. As shown in Figure 2A, $CD8^+$ T cells separated from $CD4^+$ T cells by a permeable membrane acquired higher polyfunctionality compared to those stimulated as $CD8^+$ T cells only. The polyfunctionality in this group was not completely similar but close to those in the group stimulated with $CD4^+$ T cells in a contact-sufficient manner. The number of $CD8^+$ T cells cultured without $CD4^+$ cells and with $CD4^+$ cells in the Transwell system on day 1 was 60% and 51% of the initial number of cultured $CD8^+$ cells, respectively, which became 108% and 204% on day 7, respectively. These results indicated that soluble factors played an important role in the $CD4^+$ T cell help on the $CD8^+$ T cell polyfunctionality.

## 3.3 | Interleukin-2 and IL-21 are critical soluble factors in the induction of polyfunctionality in CTLs

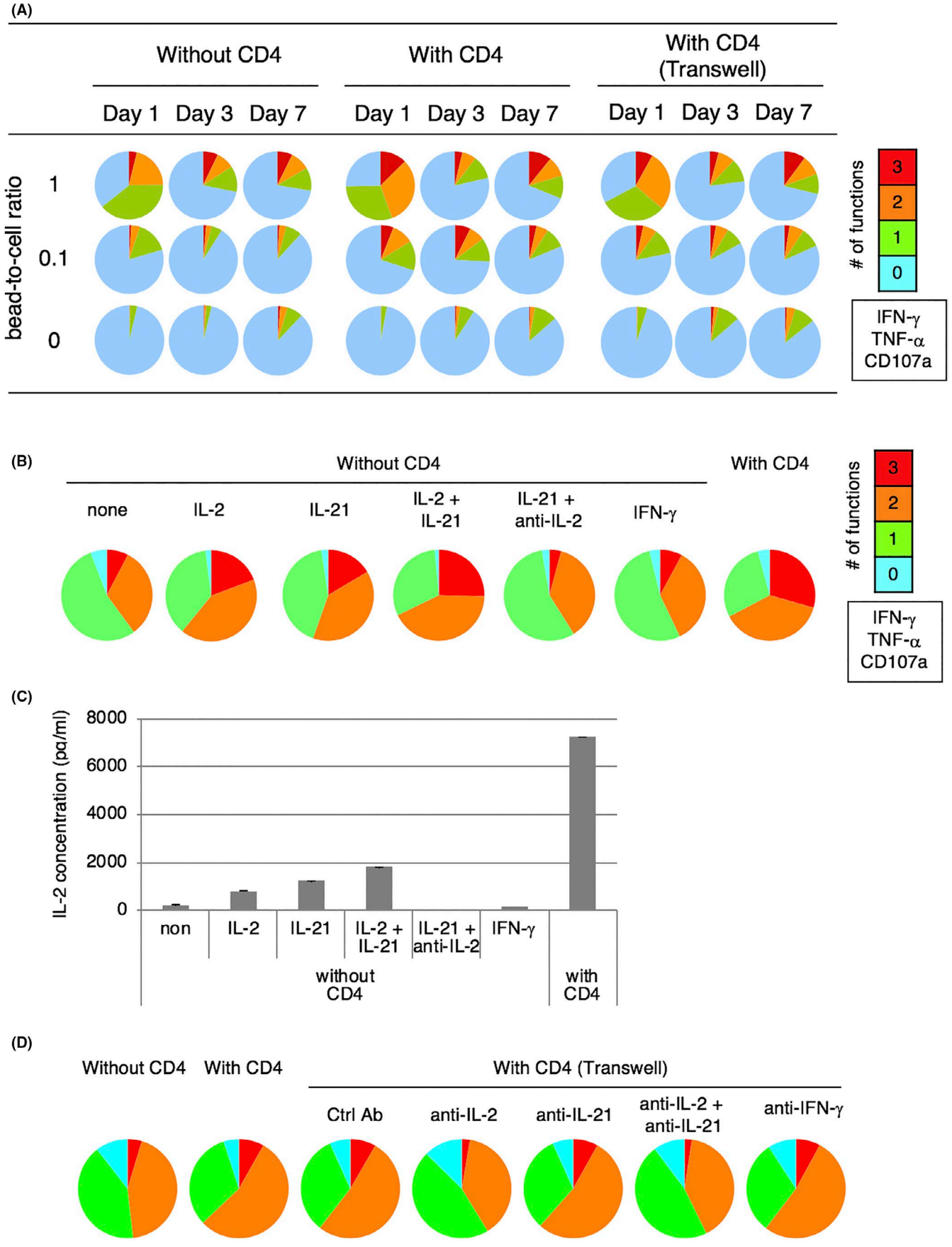
To clarify the factors that increase the polyfunctionality, we examined the induction of polyfunctionality in $CD8^+$ T cells when stimulated in the presence and absence of cytokines with memory promoting capacity. Polyfunctionality of CTLs was found to increase when IL-2 or IL-21 was added to the culture media. The combination of IL-2 and IL-21 was slightly more effective than either IL-2 or IL-21 alone (Figure 2B). Addition of anti-IL-2 mAb abolished the effect of IL-21, suggesting that the action of IL-21 was indirect and mediated through IL-2 (Figure 2B). Consistent with this finding, the IL-2 concentration was increased when the cells were cultured with IL-21 (Figure 2C). The mAb against IL-2 but not against IL-21 inhibited the induction of CTL polyfunctionality when cultured with $CD4^+$ T cell-derived factors through the Transwell membrane, further supporting the predominant role of IL-2 on the induction of CTL polyfunctionality (Figure 2D). γ-Interferon did not show any influence on CTL polyfunctionality. These results suggest that IL-2 and IL-21 are the factors that could determine polyfunctionality in CTLs, although IL-21 might act through IL-2.

## 3.4 | Consistent relationship between polyfunctionality and memory precursor phenotype

We next addressed whether the existence of $CD4^+$ T cells and the exposure to IL-2/-21 confer the capacity to form memory cell populations in $CD8^+$ T cells regardless of their functionality, or only highly polyfunctional $CD8^+$ T cells possess the superior potential of survival and memory formation, even in these conditions. When $CD8^+$ T cells were stimulated by anti-CD3 and CD28 in the presence of $CD4^+$ T cells for 2 days, these T cells showed higher Bcl-2 and lower active caspase-3 expression compared to the cells stimulated without $CD4^+$ T cells (Figure 3A,B). However, even in the presence of $CD4^+$ T cells, only the $CD8^+$ T cells with high functionality showed this antiapoptotic phenotype, leaving low functional cells as apoptotic populations (Figure 3C,D). The existence of $CD4^+$ T cells or IL-2/-21 also increased the frequency of $CD127^{high}KLRG1^{low}$ memory precursor cells (Figure 3E). However, highly polyfunctional $CD8^+$ T cells preferentially acquired the memory precursor phenotype in both conditions (Figure 3F). These data suggest the consistent relationship between the acquisition of polyfunctionality and capacity of memory formation. Therefore, the increased cellular population with high polyfunctionality in the cells exposed to $CD4^+$ T cells or IL-2/-21 resulted in the overall increase of cells with increased memory formation potential.

## 3.5 | Polyfunctional tumor-specific CTLs preferentially survive in vivo and induce tumor regression

As highly polyfunctional T cell populations were suggested to possess favorable capacities of survival and memory formation, we


(A)
Without CD4
With CD4
With CD4 (Transwell)
Day 1 Day 3 Day 7
bead-to-cell ratio
1
0.1
0
# of functions
3
2
1
0
IFN-γ
TNF-α
CD107a
(B)
Without CD4
With CD4
none
IL-2
IL-21
IL-2 + IL-21
IL-21 + anti-IL-2
IFN-γ
(C)
IL-2 concentration (pg/ml)
8000
6000
4000
2000
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non
IL-2
IL-21
IL-2 + IL-21
IL-21 + anti-IL-2
IFN-γ
without CD4
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Without CD4
With CD4
With CD4 (Transwell)
Ctrl Ab
anti-IL-2
anti-IL-21
anti-IL-2 + anti-IL-21
anti-IFN-γ

**FIGURE 2** Existence of $CD4^+$ T cells and interleukin (IL)-2/-21 help the acquisition of polyfunctionality in $CD8^+$ T cells. Responses are grouped and color-coded according to the number of acquired functions (γ-interferon [IFN-γ], tumor necrosis factor-α [TNF-α], and CD107a). A, BALB/c mice-derived $CD8^+$ T cells were stimulated by anti-CD3/CD28 in the presence or absence of syngeneic $CD4^+$ T cells. BALB/c mice-derived $CD8^+$ T cells were stimulated by anti-CD3/CD28 in the presence or absence of IL-2, IL-21, and IFN-γ, then the polyfunctionality of $CD8^+$ T cells (B) and IL-2 concentration in the culture supernatant (C) were assessed. D, Acquisition of polyfunctionality of $CD8^+$ T cells stimulated with $CD4^+$ T cells in the presence and absence of Abs against IL-2, IL-21, or IFN-γ is indicated. Results are representative of 3 independent experiments

further assessed the in vivo persistence of these populations. DUC18 TCR Tg mouse-derived $CD8^+$ T cells that recognize a neoantigen generated from mERK2 were stimulated in vitro with or without WT mouse-derived $CD4^+$ T cells in Transwell plates. These $CD8^+$ Tg T cells were adoptively transferred into WT BALB/c mice. Forty days later (in the memory phase), lymph node cells were harvested and evaluated for the maintenance of transferred CTLs by assessing the number of cells that showed IFN-γ secretion against $mERK2_{136\text{-}144}$ antigen peptide recognized with the Tg TCR. As shown in Figure 4A, the number of spots for IFN-γ secretion was significantly higher in the group receiving highly polyfunctional $CD8^+$ T cells that were stimulated with $CD4^+$ T cells compared to the groups transferred with cells stimulated as $CD8^+$ T cells alone, or unstimulated. These cells did not show the reactivity to target cells pulsed with control p63 peptide, suggesting that the effect of $CD4^+$ T cells on $CD8^+$ effector T cells functioned in an antigen-dependent manner. We further addressed the antitumor effect of these cells. We adoptively transferred these cells into mice that bear CMS5 tumor expressing cognate neoantigen and the tumor growth was monitored over time. As shown in Figure 4B, the tumor growth was significantly inhibited when mice were inoculated with tumor-specific $CD8^+$ T cells that showed high polyfunctionality endowed by the presence of $CD4^+$ T cells compared to mice that were inoculated with CTLs of limited polyfunctionality imprinted by stimulation without $CD4^+$ T cells. To further address whether tumor-specific CTLs show better tumor control in the presence of $CD4^+$ T cells in vivo, we transferred TCR Tg mouse-derived CTLs with or without polyclonal $CD4^+$ T cells into CMS5 tumor-bearing SCID mice. The tumor growth was significantly inhibited in the mice receiving tumor-specific CTLs with $CD4^+$ cells (Figure 5A). The CTLs transferred with $CD4^+$ T cells were confirmed to show high polyfunctionality compared to CTLs transferred without $CD4^+$ T cells in the draining lymph node of the mice that received adoptive transfer of CTLs (Figure 5B).

## 4 | DISCUSSION

$CD4^+$ T cells have been shown to be essential in CTL response to noninflammatory antigens, infectious agents, and cancer.[36-39] They are required for secondary expansion and memory generation in $CD8^+$ T cells after acute infection.[35,40,41] However, the role of $CD4^+$ T cells in the control of CTL polyfunctionality has not been addressed. Previously, we observed that depletion of Tregs in vivo facilitated the induction of polyfunctional effector $CD8^+$ CTLs.[18] Additionally, we and others have reported that in vivo injection of mAb that blocked the coinhibitory molecule CTLA-4 or agonistic mAb for costimulatory molecule GITR induced increased the polyfunctionality of CTLs.[19,20] Because all of these interventions could target $CD4^+$ helper T cells, we hypothesized that activation of $CD4^+$ T cells played a role in the control of CTL polyfunctionality in these observations. Our study here identified a cellular population that directly regulates T cell polyfunctionality. Moreover, IL-2 and IL-21 were found to represent molecules that acted on T cells to enhance their polyfunctionality. It has been shown that $CD4^+$ T cells exert their help on $CD8^+$ T cells by activating APCs through CD40-CD154 interaction,[36-38] or by direct $CD4^+$-$CD8^+$ T cell communication through cytokines or cell-to-cell interaction.[42,43] It is noteworthy that our results with adoptive transfer of both $CD4^+$ and $CD8^+$ T cells into SCID mice indicated that in vivo $CD4^+$ T cell help, which could involve the activation of APCs,[44,45] increased CTL polyfunctionality as well as their antitumor effect (Figure 5).

As a soluble factor in the direct $CD4^+$-$CD8^+$ T cell communication, IL-2 and IL-21 were found to be important in our model (Figure 2). Interleukin-2 has been shown to play a modest role in vivo in the primary $CD8^+$ T cell response to acute infection despite the initial reports of IL-2 as a critical factor for $CD8^+$ T cell expansion.[46-49] It should be noted that we stimulated T cells in vitro by anti-CD3 and anti-CD28 mAb-coated microbeads without the involvement of APC. In this assay, the cognate interaction of $CD8^+$ and $CD4^+$ T cells in the presence of APCs could be excluded. In addition to IL-2 and IL-21, the importance of the factors that depend on APC needs future investigation. Therefore, evaluation of the effect of IL-2 in vivo on the induction of CTL polyfunctionality is an issue to be addressed in future studies. Interleukin-21 was reported to promote the generation of IL-2-producing $CD8^+$ T cells in an acute infection model,[50] and cytolytic $CD8^+$ T cell formation in chronic infection and cancer,[39] consistent with our finding on the importance of IL-2/-21 in CTL polyfunctionality. Our neutralizing anti-mouse IL-2 rat mAb clone S4B6 is known to disrupt the interaction of IL-2 and the IL-2Rα subunit.[51] The absence of detection of IL-2 in Figure 2C indicates the absence of functional IL-2 because the detection of Ab clone JES6-5H4 in our ELISA is known to compete with S4B6 in IL-2 binding.[52] Moreover, it is likely that polyfunctionality of T cells was induced by both quantitative and qualitative differences of stimulation because both the increased bead-to-cell ratio and the presence of $CD4^+$ cells enhanced polyfunctionality of $CD8^+$ T cells (Figure 2A). Whether the heterogeneity in the responses of individual T cells derives from the differences in their imprinted program or their stochastic variation in the strength and quality of the received stimulation is currently unknown. Further analysis utilizing single cell technology to track and analyze single T cells could address the question.

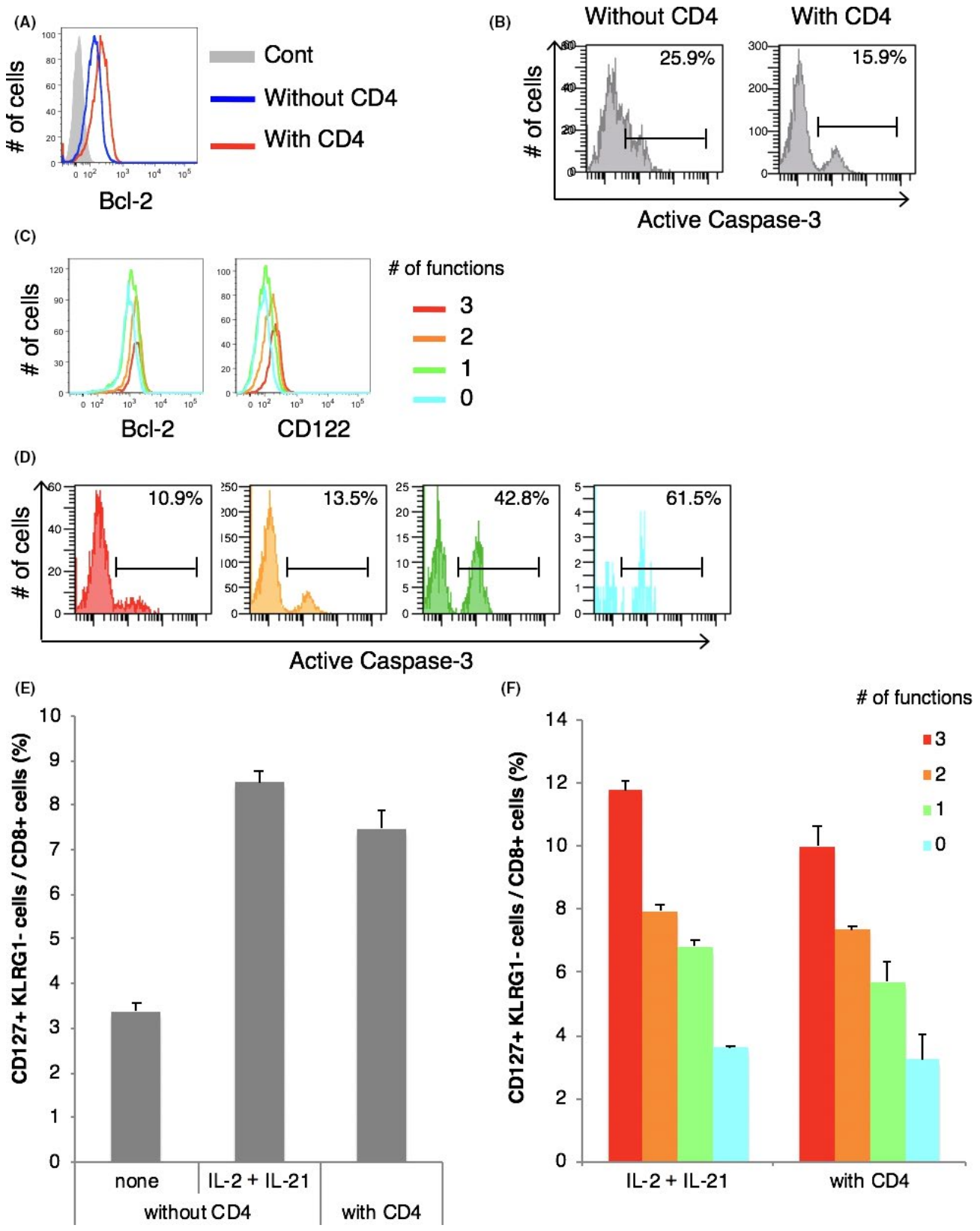


**FIGURE 3** Skewed memory formation and survival capacity in polyfunctional $CD8^+$ T cells when stimulated with $CD4^+$ T cells or interleukin (IL)-2/-21. $CD8^+$ T cells purified from BALB/c mice-derived spleen cells were stimulated by microbeads conjugated with anti-CD3 and anti-CD28 mAbs for 2 days in the presence or absence of $CD4^+$ T cells or IL-2/-21. A-D, Bcl-2 expression (A) and active caspase-3 expression in $CD8^+$ T cells (B) are shown. Expression of bcl-2/CD122 (C), or active caspase-3 (D) in $CD8^+$ T cells with each functionality was assessed. E, F, Percentages of $CD127^{high}$ $KLRG1^{low}$ in all $CD8^+$ T cells (E) or $CD8^+$ T cells with each functionality (F) are indicated. Results are representative of 3 independent experiments. Cont, control

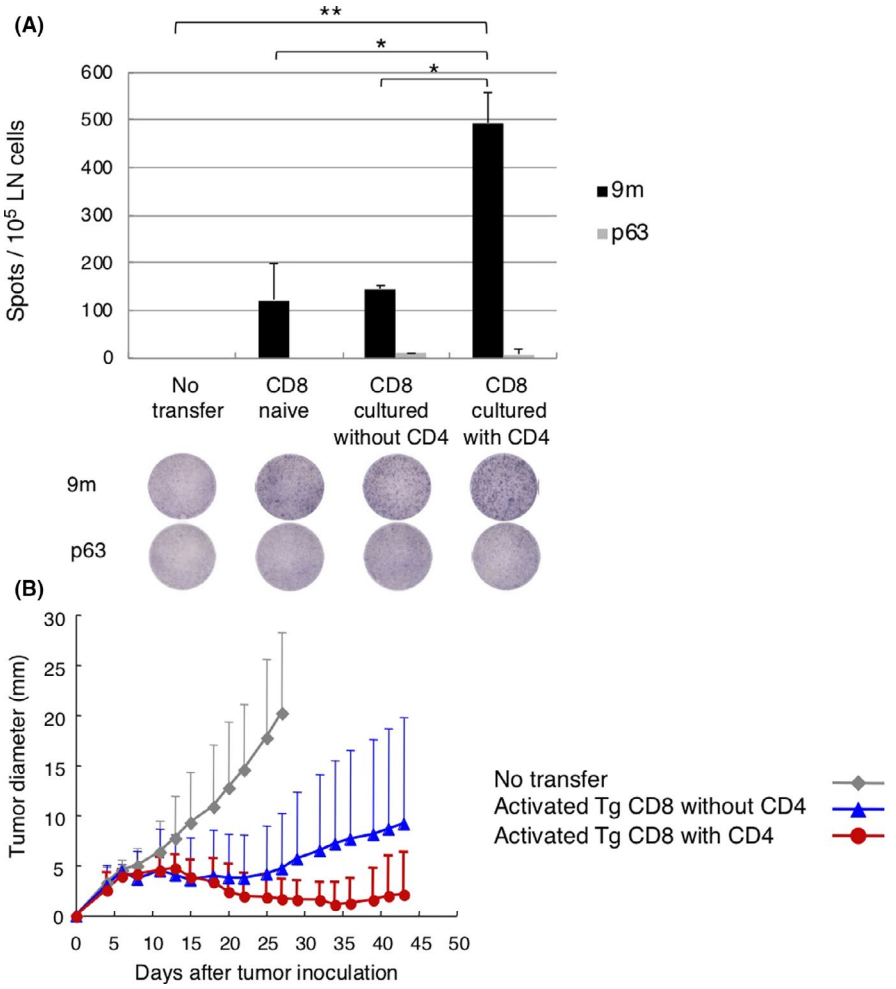


**FIGURE 4** Increased in vivo survival and antitumor effect of adoptively transferred $CD8^+$ T cells when cultured with $CD4^+$ T cells. DUC18 TCR Tg mouse-derived mutated $ERK2_{136\text{-}144}$-specific $CD8^+$ T cells were stimulated in vitro with or without WT $CD4^+$ T cells in Transwell plates. A, Stimulated $CD8^+$ CTLs were adoptively transferred into WT mice, and lymph node (LN) cells were harvested on day 40 to evaluate γ-interferon (IFN-γ) secretion against $mERK2_{136\text{-}144}$ antigen peptide or control p63 peptide by enzyme-linked immunospot assay. B, BALB/c mice (n = 5) were inoculated s.c. with $1 \times 10^6$ CMS5 cells on day 0. A total of $1 \times 10^6$ activated DUC18 $CD8^+$ CTLs were adoptively transferred into mice on day 7 and tumor growth was monitored over time. Results are representative of 3 independent experiments. Tg, transgenic

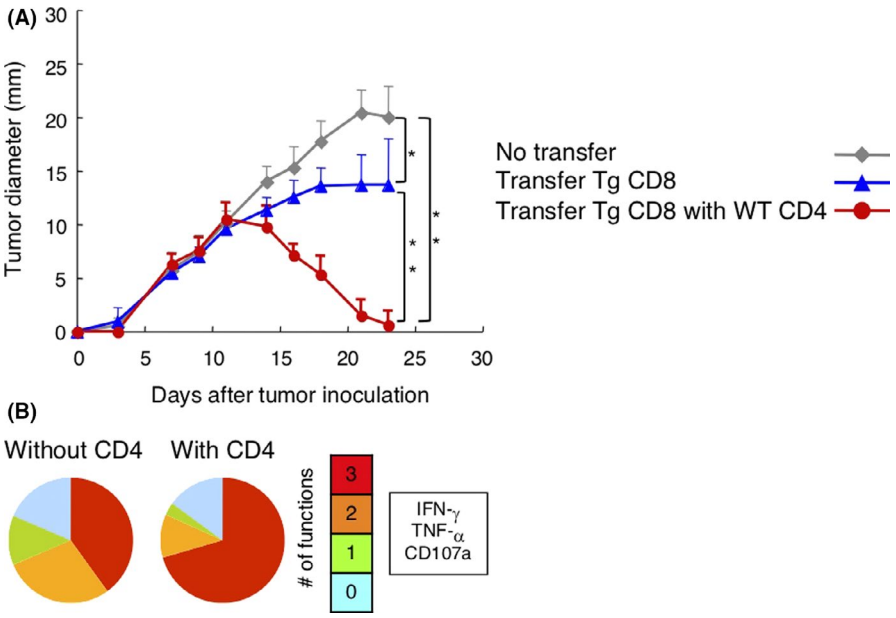


**FIGURE 5** Existence of $CD4^+$ T cells is required for antitumor effect and acquisition of polyfunctionality of tumor-specific $CD8^+$ T cells. A, SCID mice (n = 5) were inoculated s.c. with $1 \times 10^6$ CMS5 cells on day 0. A total of $1 \times 10^6$ unstimulated DUC18 $CD8^+$ cells with or without $1 \times 10^6$ WT BALB/c mice-derived unstimulated $CD4^+$ T cells were adoptively transferred into the mice on day 7 and tumor growth was monitored over time. B, CSFE-labeled DUC18 $CD8^+$ cells in the draining lymph node 4 d after the transfer were assessed for their polyfunctionality. Results are representative of 3 independent experiments. IFN-γ, γ-interferon; Tg, transgenic; TNF-α, tumor necrosis factor-α

Adoptive cell therapy with tumor infiltrating lymphocytes as well as genetically engineered lymphocytes that express tumor-reacting TCR have shown to be effective in controlling tumors including malignant melanoma, synovial cell sarcoma, and other tumor types.[1,2] Genetically engineered lymphocytes that express CAR against CD19 have shown remarkable efficacy to control $CD19^+$ hematological malignancies.[2] However, the functional characteristics and phenotypic markers of effector T cells that are appropriate for effective immunotherapy remain controversial.[1,2,53,54] T cells with naïve or stem cell memory phenotypes were suggested as superior to cells for adoptive

immunotherapy despite their limited production of cytokines or cytotoxic granules in vitro.[53,55] The favorable in vivo antitumor effects of cells with these phenotypes have been attributed to their capacities for survival and persistence. In contrast, in vitro activation of effector cells prior to transfer, or engineering of T cells to produce IL-12 that endow them to secrete enhanced IFN-γ and increased T-bet expression have been reported to show enhanced tumor eradiation.[56,57] CD19-CAR-T cells with disrupted *Tet2* gene were reported to have the advantage to expand in vivo with central memory phenotype in a patient who experienced complete response after ACT.[58] CAR-T cells with disruption of all 3 subtypes of *NRA4* genes were reported to have increased antitumor effect with gene expression patterns similar to effector T cells in a murine model.[59] Our study here provides a potential link among these previous reports. The results here support a differentiation model for CTLs in which an appropriate primary activation that is well assessed with polyfunctionality endow T cells with the capacities for survival and presumably differentiation into memory T cells. We have previously shown that highly polyfunctional CTLs were responsible for tumor eradication in a CMS5 tumor model by comparing the transfer of the same number of $CD8^+$ T cells with high or low polyfunctionality into tumor-bearing mice,[18] consistent with the result in Figures 4 and 5. Our results here, together with other reports, indicate that polyfunctionality represents a sensitive immune correlate of the efficacy for the in vitro propagated T cells in immunotherapy, and will be useful in assessing the quality of T cells generated by different methods. The presence of $CD4^+$ T cells will be beneficial, and sometimes might be critical, in the preparation and infusion of adoptive transfer of tumor-reacting CTLs into patients.

The data here shed new light on the understanding of a program in T cells that links the expression of T cell effector functions with their fate. Moreover, this study should be important when one needs a sensitive assessment for the quality of in vitro propagated effector CTLs for immunotherapy as well as the monitoring of the T cell response in vivo in immunological therapy of cancer.

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## CONFLICT OF INTEREST

HI is provided with a research grant from Takara Bio Inc. The other authors have no conflict of interest.

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