

**ORIGINAL ARTICLE**

# Improved survival of chimeric antigen receptor-engineered T (CAR-T) and tumor-specific T cells caused by anti-programmed cell death protein 1 single-chain variable fragment-producing CAR-T cells

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**Abstract**

Chimeric antigen receptor-engineered T (CAR-T)-cell therapy holds significant promise for the treatment of hematological malignancies, especially for B-cell leukemia and lymphoma. However, its efficacy against non-hematological malignancies has been limited as a result of several biological problems characteristic of the tumor microenvironment of solid tumors. One of the main hurdles is the heterogeneous nature of tumor-associated antigens (TAA) expressed in solid tumors. Another hurdle is the inefficient activation and limited persistence of CAR-T cells, mainly as a result of T-cell exhaustion caused by immunosuppressive factors in the tumor microenvironment. In the present study, to address these problems, we engineered CAR-T cells to produce antagonistic anti-programmed cell death protein 1 (PD-1) single-chain variable fragment (scFv), by which PD-1-dependent inhibitory signals in CAR-T cells and adjacent tumor-specific non-CAR-T cells are attenuated. In mouse solid tumor models, PD-1 scFv-producing CAR-T cells induced potent therapeutic effects superior to those of conventional CAR-T cells, along with a significant reduction of apoptotic cell death not only in CAR-T cells themselves but also in TAA-specific T cells in the tumor tissue. In addition, the treatment with anti-PD-1 scFv-producing CAR-T cells resulted in an increased concentration of PD-1 scFv in tumor tissue but not in sera, suggesting an induction of less severe systemic immune-related adverse events. Hence, the present study developed anti-PD-1 scFv-producing CAR-T cell technology and explored its cellular mechanisms underlying potent antitumor efficacy.

**KEYWORDS**

activation-induced cell death, CAR-T cell, PD-1, tumor microenvironment, tumor-specific T cell

## 1 | INTRODUCTION

Chimeric antigen receptor-engineered T (CAR-T)-cell therapy is one of the most promising approaches to revolutionize the field of cancer immunotherapy.<sup>1</sup> CAR is a synthetic receptor that contains an antigen-binding domain of single-chain variable fragment (scFv) derived from a mAb against a tumor-associated antigen (TAA) linked to various costimulatory domains such as CD28 and 4-1BB, and a CD3 $\zeta$  signaling motif to induce optimal T-cell activation.<sup>2</sup> Adoptive transfer of CAR-T cells has emerged as an effective treatment modality capable of generating outstanding clinical responses in otherwise treatment-refractory patients with hematological malignancies. There are currently two FDA-approved CAR-T-cell therapies targeting the CD19 molecule, one is tisagenlecleucel for adult B-cell acute lymphoblastic leukemia (ALL) and large B-cell non-Hodgkin lymphoma, and the other is axicabtagene ciloleucel for large B-cell non-Hodgkin lymphoma.

In contrast to the potent therapeutic effects of CAR-T cells in hematological malignancies, the effects of CAR-T cell therapy against solid tumors have shown only modest clinical outcomes so far.<sup>3</sup> Several factors which could limit the antitumor responses of CAR-T-cell therapy in solid tumors have been considered. First, cytotoxic function and persistency of CAR-T cells is suppressed in the tumor microenvironment, a so-called exhaustion state, through multiple inhibitory cosignals.<sup>4</sup> Among them, interaction between programmed cell death protein 1 (PD-1), expressed on activated or exhausted CAR-T cells, and its ligand, programmed death-ligand 1 (PD-L1), expressed on tumor cells and various non-tumor cells in the tumor microenvironment, plays a major role in the suppression of CAR-T cells.<sup>5</sup> In order to address this problem, various approaches to attenuate PD-1 inhibitory signal in CAR-T cells have been reported, including a combination with systemic administration of anti-PD-1/PD-L1 Ab, CAR-T cells that coexpress a dominant-negative form of PD-1 or PD-1/CD28 chimeric switch-receptor, and genetic disruption of PD-1 on CAR-T cells.<sup>5-8</sup>

The second hurdle of CAR-T-cell therapy in solid tumors is the heterogeneity of TAA. As CAR-T cells are usually designed to recognize only one target molecule expressed on tumor cells, CAR-T-cell therapy could induce tumor immune escape due to antigen loss, which results in limited therapeutic potential.<sup>9,10</sup> We recently reported that enhanced accumulation and persistency of endogenous tumor-specific T cells in addition to the transferred CAR-T cells is necessary for the potent therapeutic effects of next-generation CAR-T cells expressing interleukin (IL)-7 and chemokine (C-C motif) ligand 19 (CCL19).<sup>11</sup> Thus, additional strategies to mobilize tumor-specific endogenous T cells with polyclonal specificity are essential to overcome the heterogeneity of TAA in solid tumors and to improve CAR-T-cell therapy.

In the present study, we developed CAR-T cells which produce anti-PD-1 scFv to attenuate PD-1 inhibitory signal in the tumor tissues where CAR-T and tumor-specific T cells reside, and found potent and durable therapeutic efficacy in mouse solid tumor models. Molecular and cellular mechanisms underlying the effects of anti-PD-1 scFv-producing CAR-T cells were investigated.

## 2 | MATERIALS AND METHODS

### 2.1 | Mice and cell lines

Male or female 6- to 10-week-old DBA/2 and C57BL/6 mice were purchased from Japan SLC. P1A-specific T-cell receptor (TCR)-transgenic mice<sup>12</sup> were kindly provided by Dr Yang Liu, and backcrossed with DBA/2 mice in our animal facility. P815 mastocytoma, 3LL Lewis lung carcinoma, and B16F10 melanoma were transfected to express human CD20 in our laboratory, referred to as P815-hCD20, 3LL-hCD20, and B16F10-hCD20, respectively. Culture medium used for T cells, 3LL, 3LL-hCD20, and B16F10-hCD20 was RPMI-1640 supplemented with 10% FBS, 1% penicillin-streptomycin, 25 mmol/L HEPES, and 50 mmol/L 2-mercaptoethanol. Culture medium used for P815 and P815-hCD20 cells was DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Antimouse PD-1 mAb was isolated from the supernatants of hybridoma by using Protein A column.<sup>13</sup> All animal procedures were approved by the Institutional Animal Care and Use Committee of Yamaguchi University.

### 2.2 | Plasmid construction and CAR-T-cell generation

To design human CD20-specific second-generation CAR (conv. CAR), antihuman CD20 scFv was connected to the transmembrane domain of the mouse CD8a chain and the cytoplasmic regions composed of mouse CD28 and CD3 $\zeta$ , and then cloned into the retroviral vector MSGV1.<sup>11,14,15</sup> Antimouse PD-1 scFv was generated from the sequence of antimouse PD-1 mAb.<sup>13</sup> The DNA sequence encoding the anti-PD-1 scFv was designed by connecting heavy chain variable domain, a (GGGG) $\times$ 3 linker, light chain variable domain, and FLAG tag. To construct a vector encoding anti-PD-1 scFv expressing CAR (scFv CAR), 2A self-cleavable linker sequence was inserted between conv. CAR and anti-PD-1 scFv. Vectors coexpressing eGFP through a self-cleavable 2A peptide sequence after conv. CAR or scFv CAR sequence were also constructed. Retroviral transduction with mouse T cells was conducted as previously described.<sup>11</sup>

### 2.3 | Flow cytometry

The following mAbs were used in this study; anti-CD8 $\alpha$ , anti-TIGIT, anti-CD107a, and anti-Bcl-2 (BioLegend), anti-CD90.2, anti-PD-1, anti-LAG-3, anti-TIM-3, and anti-V $\alpha$ 8.3 mAb (Thermo Fisher Scientific), antihuman CD20 (BD Biosciences), anti-Bcl-xL (Abcam), and anti-Bim (CST). Annexin V (BD Biosciences) and Zombie Yellow viability dye (BioLegend) were also used. Biotinylated recombinant protein L (GenScript) was used to detect CAR-T cells, as previously reported.<sup>16</sup> Intracellular protein staining buffer set (Thermo Fisher Scientific) was also used in some experiments. APC-conjugated antihuman IgG mAb (BioLegend) was used to detect the binding of mouse PD-L1-human Fc fusion protein (R&D Systems). Antimouse CD16/CD32 mAb was used for blockade of non-specific binding of mAb to Fc $\gamma$  receptors.

Flow cytometric data were acquired by EC800 (SONY) or BD LSRFortessa X-20 cell analyzer (BD Biosciences), and analyzed using FlowJo software (FlowJo, LLC).

## 2.4 | In vitro functional analyses of CAR-T cells

For cytotoxicity assay, conv. CAR-T or scFv CAR-T cells ( $1 \times 10^5$  cells/well) were cocultured with 3LL-hCD20 tumor cells at various effector/target (E/T) ratios in 24-well tissue culture plates. As a negative control, activated T cells without gene transduction were used at the same total cell number. After 2 days, the culture cells were harvested and stained with Zombie Yellow viability dye, anti-hCD20 mAb, and anti-CD90.2 mAb, followed by analysis with a flow cytometer in order to detect residual tumor and CAR-T cells.

For in vitro stress tests,  $1 \times 10^6$  CAR-T cells were stimulated with  $1 \times 10^6$  mitomycin C-treated 3LL-hCD20 in 24-well tissue culture plates. After 48 hours, CAR-T cells were harvested and restimulated with mitomycin C-treated 3LL-hCD20 tumor cells in a 1:1 ratio. One day after the second stimulation, CAR-T cells were harvested to assess cell number, level of apoptosis, expression of apoptosis-related proteins, exhaustion markers and CD107a.

For the proliferation assay, CAR-T cells labeled with CytoTell Blue (AAT Bioquest) were stimulated with mitomycin C-treated 3LL-hCD20 cells in a 1:1 ratio in a 96-well tissue culture plate. After 3 and 5 days, the intensity of CytoTell Blue was analyzed by flow cytometry.

## 2.5 | In vivo models to assess antitumor effects of CAR-T cells

C57BL/6 mice were injected s.c. with  $2.5 \times 10^6$  3LL-hCD20 on day 0. The mice were exposed to sublethal irradiation (3 Gy) on day 6, and then treated on day 7 with i.v. injection of  $1 \times 10^6$  conv. CAR-T cells, scFv CAR-T cells, or activated T cells without gene transduction, in which the total cell number was adjusted to be equivalent to CAR-T-cell groups. In some experiments,  $2.5 \times 10^6$  B16F10-hCD20 were inoculated s.c. instead of 3LL-hCD20. In tumor rechallenge experiments, mice that had achieved complete eradication of initial tumor by treatment with scFv CAR-T cells were reinoculated s.c. with  $2.5 \times 10^6$  3LL-hCD20. In all experiments, tumor size and mouse survival were assessed twice a week.

## 2.6 | In vivo models to assess kinetics of CAR-T cells and tumor-specific T cells

DBA/2 mice were injected s.c. with  $5 \times 10^5$  P815-hCD20 tumor cells on day 0 and exposed to sublethal irradiation (3 Gy) on day 13. On day 14, the mice were injected i.v. with  $1 \times 10^6$  P1A-specific T cells together with  $1 \times 10^6$  scFv CAR-T cells or conv. CAR-T cells. P1A-specific T cells were isolated from spleen and lymph nodes of naïve P1A-specific TCR-transgenic mice. On day 21, tumor-infiltrating lymphocytes (TIL) were harvested from tumor tissues, and analyzed for apoptosis of CAR-T cells and P1A-specific T cells.

## 2.7 | Assessment of biodistribution of anti-PD-1 scFv and anti-PD-1 mAb

C57BL/6 mice were injected s.c. with  $2.5 \times 10^6$  3LL-hCD20 tumor cells on day 0. The mice were exposed to sublethal irradiation (3 Gy) on day 6, and then treated on day 7 with i.v. injection of  $1 \times 10^6$  scFv CAR-T cells alone or conv. CAR-T cells together with i.p. injections of anti-PD-1 mAb (200  $\mu$ g) on days 7, 12, and 17. On day 18, tumor tissues and sera were harvested from the mice and measured for concentrations of anti-PD-1 scFv and anti-PD-1 mAb.

## 2.8 | Enzyme-linked immunosorbent assay

Interferon (IFN)- $\gamma$  ELISA kit (BD Biosciences) was used to measure IFN- $\gamma$  concentration. Concentrations of anti-PD-1 scFv and anti-PD-1 mAb in the homogenized tumor tissue and mouse serum were determined by ELISA established in our laboratory as follows: recombinant mouse PD-1-human Fc fusion protein (R&D Systems) as a capture reagent, anti-PD-1 scFv with FLAG tagged or hamster anti-PD-1 mAb as standard reagents, HRP-conjugated anti-FLAG Ab (Sigma-Aldrich) or HRP-conjugated antihamster IgG Ab (Jackson ImmunoResearch) as detection reagents.

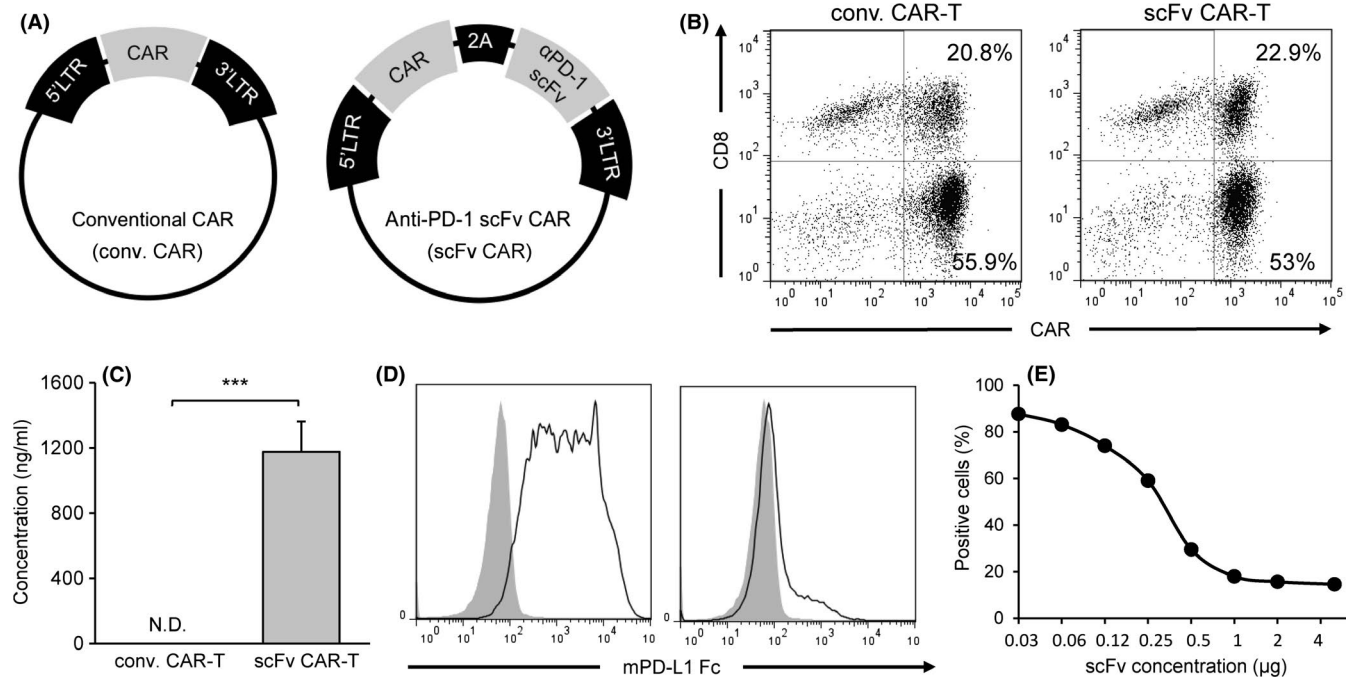
## 2.9 | Statistical analysis

Two-tailed Student's *t* test was used for statistical analyses in all assays except survival experiments. For mouse survival, Kaplan-Meier curves were depicted, and the log-rank test was used for statistical analysis. Differences at *P* values < .05 were considered significant.

# 3 | RESULTS

## 3.1 | Generation of anti-PD-1 scFv-producing CAR-T cells

We first constructed a second-generation CAR targeting hCD20, composed of anti-hCD20 scFv, CD8 transmembrane domain, and intracellular signaling motifs of CD28 and CD3 $\zeta$  (referred to as conv. CAR). To design an anti-hCD20 CAR which produces anti-PD-1 scFv, the conv. CAR construct was further engineered to connect with anti-PD-1 scFv by self-cleavable 2A peptide linker (referred to as scFv CAR) (Figure 1A). Retroviral transduction of mouse T cells with scFv CAR vector displayed efficient induction of CAR expression approximately 70%-80%, which was equivalent to conv. CAR vector (Figure 1B). To confirm the production of anti-PD-1 scFv, culture supernatants of scFv CAR-T cells were measured for the level of anti-PD-1 scFv by ELISA. Significant production of anti-PD-1 scFv at approximately 1  $\mu$ g/mL was detected in the supernatants of scFv CAR-T cells but not conv. CAR-T cells (Figure 1C). We further evaluated the functional activity of anti-PD-1 scFv to interfere with the interaction of PD-1 and its ligand, PD-L1. Binding of PD-L1 fusion protein with PD-1 receptor transiently expressed on 293 T cells was significantly attenuated in the presence of anti-PD-1 scFv



**FIGURE 1** Generation and functional characterization of single-chain variable fragment (scFv) chimeric antigen receptor-engineered T (CAR-T) cells. A, Schematic representation of anti-hCD20 conv. CAR and scFv CAR retroviral vectors. B, Two days after retroviral transduction, CAR expressions were analyzed. C, Four days after retroviral transduction, production of anti-programmed cell death protein 1 (PD)-1 scFv in the culture supernatants were analyzed by ELISA (mean  $\pm$  SD,  $n = 3$ , \*\*\* $P < .001$ ). D, 293T cells expressing PD-1 were first incubated with 2  $\mu$ g control hamster immunoglobulin (left panel) or anti-PD-1 scFv (right panel), and then stained with control immunoglobulin (filled lines) or PD-L1-Fc protein (open lines), followed by APC-conjugated antihuman IgG mAb. E, In the assay similar to (D), binding of programmed death-ligand 1 (PD-L1)-Fc protein to PD-1-expressing 293T cells in the presence of titrated doses of anti-PD-1 scFv was assessed by flow cytometry. Representative data from at least three independent experiments are shown

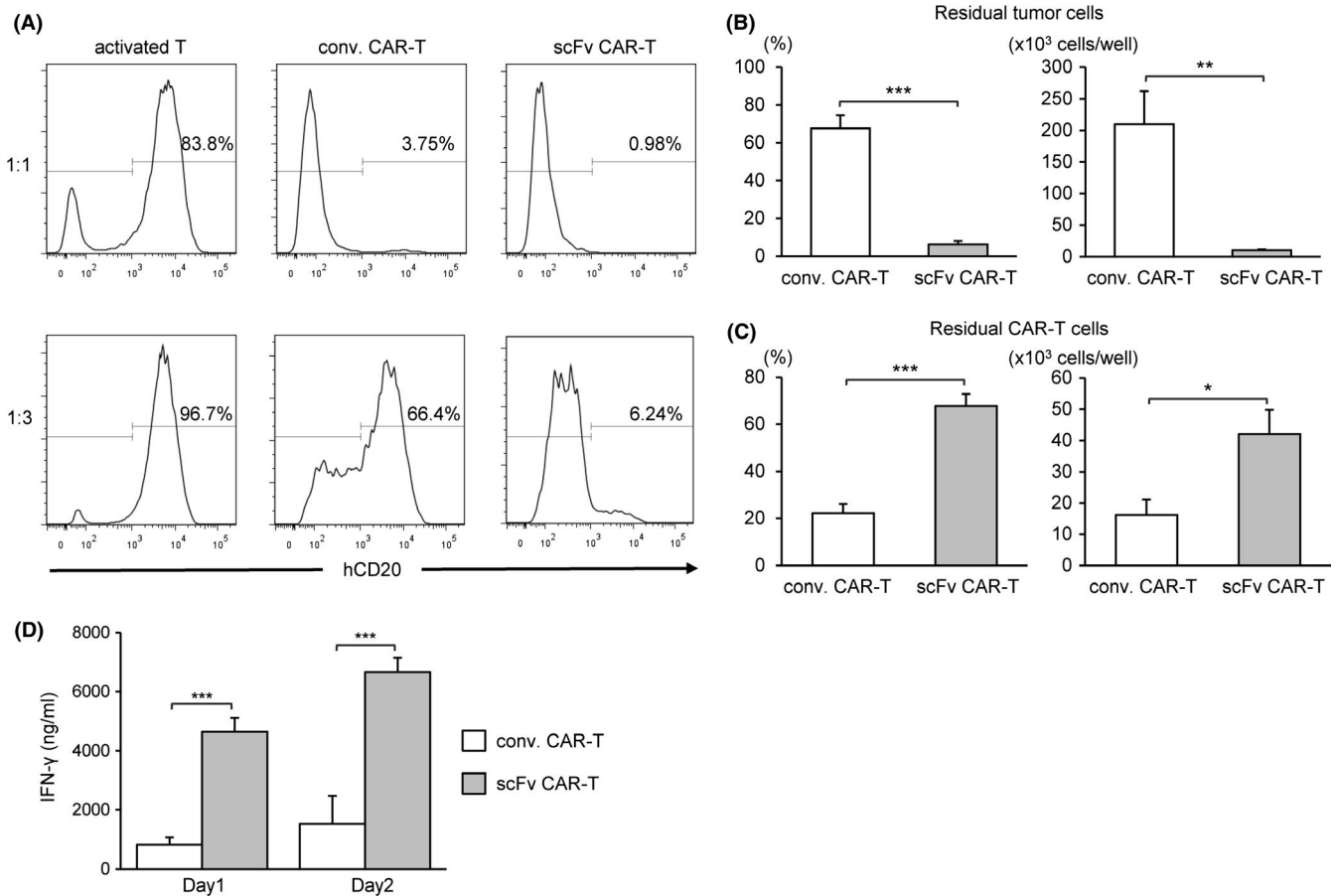
(Figure 1D). The blockade was shown in a dose-dependent way, with almost complete inhibition at 1  $\mu$ g/mL anti-PD-1 scFv (Figure 1E). These results indicated that scFv-CAR T cells have a capacity to produce anti-PD-1 scFv which attenuates the PD-1 signal.

### 3.2 | Enhanced tumor-killing ability of scFv CAR-T cells in association with decreased apoptosis

To investigate the potential of scFv CAR-T cells to kill tumor cells, anti-hCD20 conv. CAR-T, anti-hCD20 scFv CAR-T, or activated T cells without gene transfection were cocultured with 3LL-hCD20 for 2 days at various effector to target (E:T) ratios. For this assay, it was confirmed that PD-L1 was inducibly expressed on 3LL-hCD20 by IFN- $\gamma$  stimulation *in vitro* (data not shown). It was found that conv. CAR-T cells and scFv CAR-T cells showed almost equivalent cytotoxic activity at an E:T ratio of 1:1 (Figure 2A). In contrast, in the presence of higher tumor cell numbers at an E:T ratio of 1:3, conv. CAR-T cells significantly impaired the tumor-killing activity, whereas scFv CAR-T cells maintained the activity. Consistently, in this condition, the number of residual tumor cells was significantly decreased by coculture with scFv CAR-T cells compared to conv. CAR-T cells (Figure 2B). It was also found that the number of scFv CAR-T cells after coculture was significantly higher than that of conv. CAR-T cells (Figure 2C), and that scFv CAR-T cells produced much greater

amounts of IFN- $\gamma$  compared to conv. CAR-T cells (Figure 2D). Taken together, these results indicated that the blockade of PD-1/PD-L1 interaction by anti-PD-1 scFv derived from scFv CAR-T cells improved the antitumor activity of CAR T cells, especially when CAR-T cells were confronted with a high number of tumor cells, a situation resembling the microenvironment of solid tumors.

To investigate mechanisms of the enhanced antitumor activity of scFv CAR-T cells, we carried out so-called *in vitro* "stress tests",<sup>17</sup> in which CAR-T cells were repeatedly stimulated with target-positive tumor cells, closely mimicking a condition of tumor-immune cell interaction *in vivo*. In this model, conv. CAR-T cells and scFv CAR-T cells were stimulated with mitomycin C-treated 3LL-hCD20 cells on days 0 and 3 in an E:T ratio of 1:1, then further incubated for an additional 2 days. We first examined the exhausted status of CAR-T cells by staining the expression markers of LAG-3, TIM-3, and TIGIT. It was found that expression levels of exhaustion markers were almost comparable between conv. CAR-T and scFv CAR-T cells (Figure S1A), whereas the expression of TIGIT was slightly, but significantly, decreased in scFv CAR-T cells compared to conv. CAR-T cells (Figure S1B). We also examined expression of CD107a, a marker reflecting cytotoxic activity of individual CAR-T cells, and cellular division of CAR-T cells by using a fluorescent probe-dilution assay. It was found that conv. CAR-T cells and scFv CAR-T cells showed a comparable level of CD107a expression and proliferation responses (Figure



**FIGURE 2** In vitro antitumor activity of single-chain variable fragment (scFv) chimeric antigen receptor-engineered T (CAR-T) cells. Anti-hCD20 CAR-T cells or activated T cells were cocultured with 3LL-hCD20 in an E:T ratio of 1:1 and 1:3 for 2 d. Representative data of residual viable tumor cells (A), percentages and cell numbers of tumor cells (B) and CAR-T cells (C) are shown (mean  $\pm$  SD,  $n = 3$ ). D, On days 1 and 2, culture supernatants were harvested and assessed for concentration of interferon (IFN)- $\gamma$  by ELISA (mean  $\pm$  SD,  $n = 3$ ). Representative data from at least three independent experiments are shown. \*\*\* $P < .001$ , \*\* $P < .005$ , \* $P < .01$

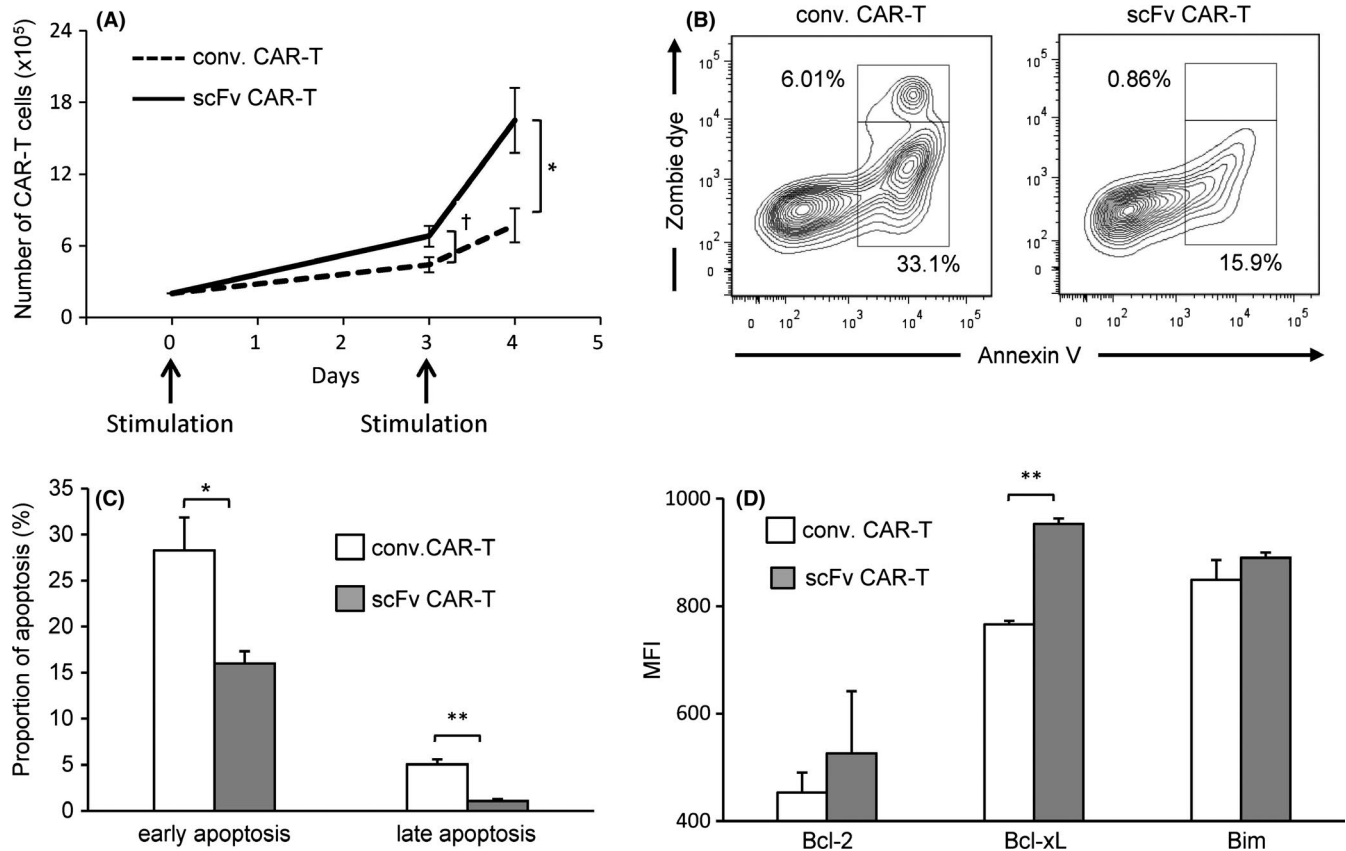
S1C,D). These results suggested that the enhanced antitumor activity of scFv CAR-T cells had no apparent association with the prevention or restoration of T-cell exhaustion, augmentation of cytotoxic potential per individual CAR-T cells, or accelerated proliferation of CAR-T cells.

It was found that the number of scFv CAR-T cells was significantly higher than that of conv. CAR-T cells after coculture (Figure 2C). A similar result was observed in the experiments with repeated stimulations with tumor cells (Figure 3A). In order to interpret these findings, we hypothesized that blockade of PD-1/PD-L1 interaction by anti-PD-1 scFv would inhibit activation-induced apoptotic cell death in CAR-T cells. Therefore, we next examined the percentages of early and late apoptosis in CAR-T cells, which were identified as Zombie Yellow dye-negative/Annexin V-positive population and Zombie Yellow dye/Annexin V-double positive population, respectively. It was found that percentages of early and late apoptosis in scFv CAR-T cells were significantly lower than those of conv. CAR-T cells (Figure 3B,C), indicating that blockade of PD-1 signal in scFv CAR-T cells enhanced the survival of CAR-T cells through the inhibition of activation-induced cell death (AICD). To further investigate the mechanism of this observation, we analyzed protein levels of

anti- and pro-apoptotic molecules after the repeated antigen stimulations. It was found that expression of Bcl-xL, an anti-apoptotic molecule, in scFv CAR-T cells was significantly higher than that of conv. CAR-T cells, whereas Bcl-2, another anti-apoptotic molecule, and Bim, a pro-apoptotic molecule, were expressed at comparable levels between these CAR-T cells (Figure 3D). Collectively, these results suggested that scFv CAR-T cells induced the enhanced antitumor effects through decreased AICD of CAR-T cells in association with upregulation of Bcl-xL.

### 3.3 | Improved therapeutic effects of scFv CAR-T cells against solid tumors in vivo

We next examined in vivo efficacy of scFv CAR-T cells to treat pre-established solid tumors in mouse models. C57BL/6 mice were inoculated s.c. with 3LL-hCD20, exposed to pretreatment with sublethal irradiation, and then treated by i.v. injection with anti-hCD20 conv. CAR-T cells, scFv CAR-T cells, or activated T cells without gene transfection. Treatment with scFv CAR-T cells inhibited tumor growth (Figure 4A) and significantly prolonged mouse survival, which could not be achieved by conv. CAR-T cell



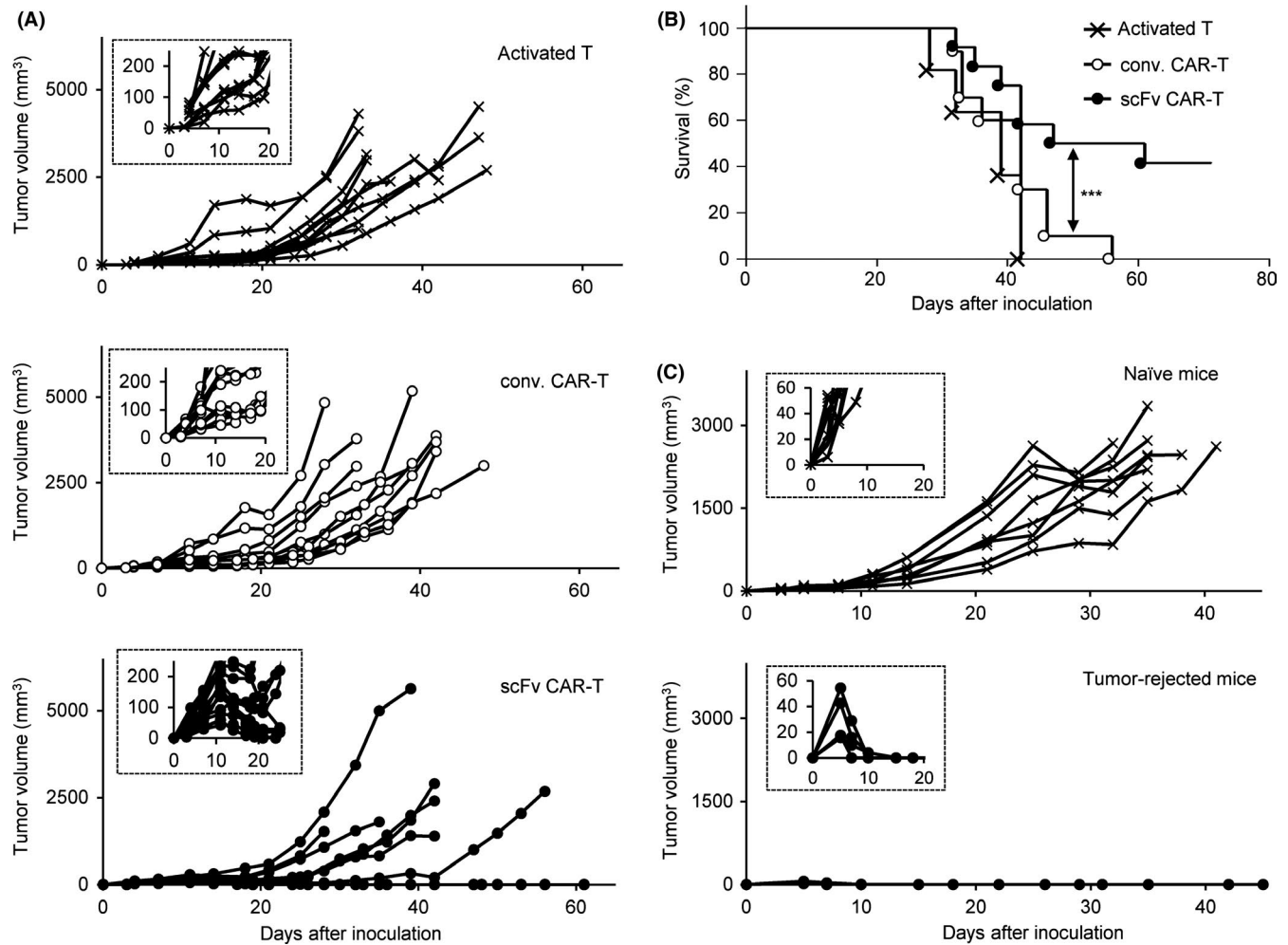
**FIGURE 3** In vitro survival and activation-induced cell death (AICD) of single-chain variable fragment (scFv) chimeric antigen receptor-engineered T (CAR-T) cells. Anti-hCD20 CAR-T cells were stimulated with mitomycin C-treated 3LL-hCD20 on days 0 and 3 in a 1:1 E:T ratio. A, Number of CAR-T cells was counted on days 3 and 4 (mean  $\pm$  SD,  $n = 3$ ). B–D, On day 5, level of apoptosis of CAR-T cells was analyzed by flow cytometry. Zombie Yellow dye/Annexin V-staining of CAR-T cells (B), its percentages (mean  $\pm$  SD,  $n = 3$ ) (C), and the expression of apoptosis-related proteins (mean  $\pm$  SD,  $n = 3$ ) (D) are shown. Representative data from three independent experiments are shown.  $^{**}P < .005$ ,  $^{*}P < .01$ ,  $^{\dagger}P < .05$

treatment (Figure 4B). The enhanced therapeutic effects of scFv CAR-T cells were further confirmed by another solid tumor model of B16F10-hCD20, in which a transfer of anti-hCD20 scFv CAR-T cells also inhibited tumor growth (Figure S2A) and significantly prolonged mouse survival compared to anti-hCD20 conv. CAR-T cells or activated T cells (Figure S2B).

To determine whether long-term antitumor memory responses can be generated by scFv CAR-T cell therapy, the mice which have survived 80 days by this treatment were rechallenged s.c. with 3LL-hCD20. These mice were completely resistant to the rechallenge with 3LL-hCD20 cells, showing an apparent contrast to naïve mice, in which inoculation of 3LL-hCD20 cells resulted in progressive tumor growth (Figure 4C). We also examined the presence of scFv CAR-T cells in tumor-draining lymph nodes and their level of apoptosis, and found that a low but stable number of scFv CAR-T cells without apoptosis were detected (data not shown), implicating an important role of the persistent existence of scFv CAR-T cells in the memory responses. These results showed that scFv CAR-T-cell therapy can induce therapeutic effects against solid tumors which were otherwise resistant to regular CAR-T-cell therapy, and lead to durable memory responses to prevent tumor relapse.

### 3.4 | Inhibition of apoptosis in tumor-specific TIL by treatment with scFv CAR-T cells

Our recent study indicated that synergistic responses of endogenous tumor-specific T cells are important for the optimal antitumor effects of CAR-T-cell therapy against solid tumors.<sup>11</sup> As therapeutic effects of scFv CAR-T cells are associated with decreased apoptosis of CAR-T cells (Figure 3), we next examined whether anti-PD-1 scFv produced by scFv CAR-T cells can also prevent apoptosis of antigen-specific non-CAR-T cells in tumor tissue by paracrine effects. Mice inoculated s.c. with P815-hCD20 were exposed to sublethal irradiation and then injected i.v. with either conv. CAR-T cells or scFv CAR-T cells, together with P1A-specific TCR-transgenic T cells (P1A-CTL). In this model, P1A-CTL which recognize P1A tumor antigen derived from P815 were used as surrogate tumor-specific endogenous T cells. TIL were harvested from the tumor tissues and analyzed for the number and levels of early and late apoptosis in CAR-T cells and P1A-CTL. Consistent with the in vitro results, the number of scFv CAR-T cells in TIL was significantly higher than in conv. CAR-T cells (Figure 5A), along with a significant decrease in apoptosis (Figure 5B,C). Importantly, we also found that the number



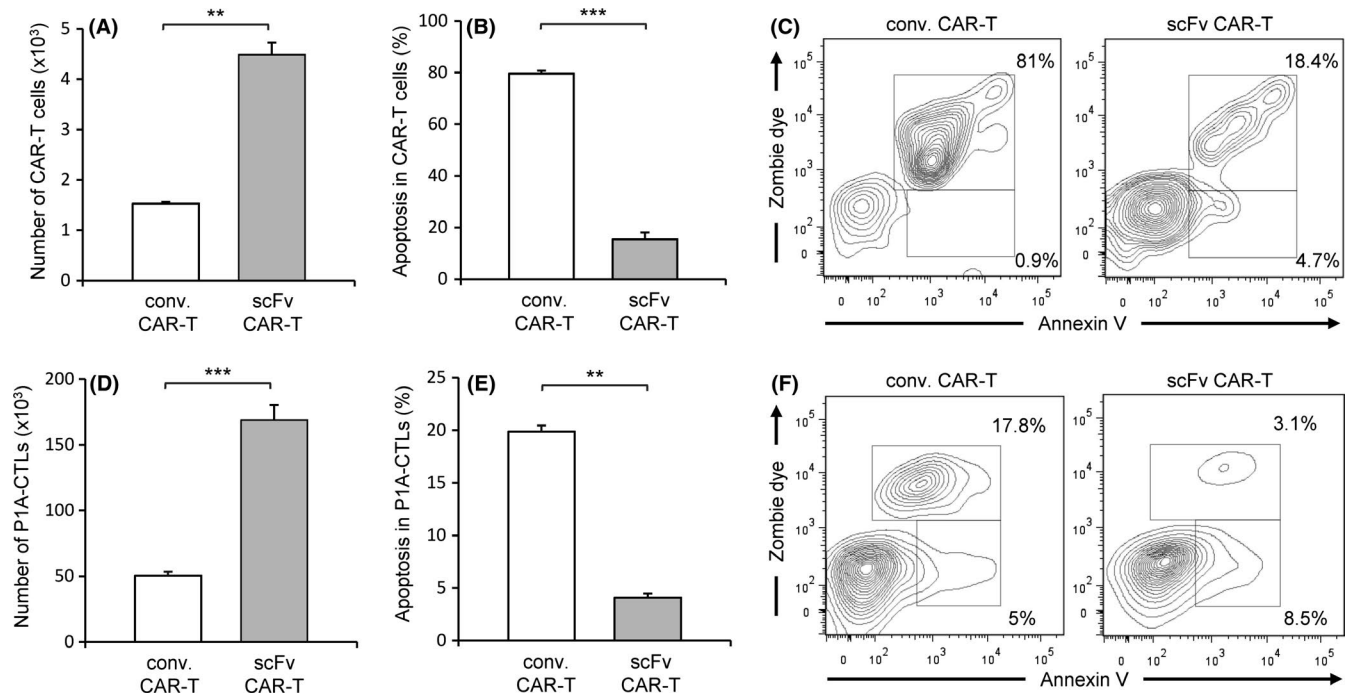
**FIGURE 4** In vivo therapeutic effects of single-chain variable fragment (scFv) chimeric antigen receptor-engineered T (CAR-T) cells against a pre-established solid tumor model. 3LL-hCD20-bearing C57BL/6 mice were exposed to sublethal irradiation and treated with anti-hCD20 conv. CAR-T cells ( $n = 12$ ), scFv CAR-T cells ( $n = 12$ ), or activated T cells ( $n = 10$ ). A, Tumor growth was measured periodically. Each line indicates an individual mouse. B, Survival of mice was assessed. \*\*\* $P < .001$ . C, Mice which had rejected initial 3LL-hCD20 by treatment with scFv CAR-T cells were rechallenged s.c. with 3LL-hCD20 ( $n = 4$ ). As a control, naïve C57BL/6 mice were inoculated s.c. with 3LL-hCD20 ( $n = 8$ ). Tumor growth was measured periodically. Representative data from at least two independent experiments are shown

of tumor-infiltrating P1A-CTL was significantly increased in the mice treated with scFv CAR-T cells compared to those with conv. CAR-T cells (Figure 5D). Percentage of apoptotic cells in P1A-CTL was also significantly lower in the treatment with scFv CAR-T cells than that with conv. CAR-T cells (Figure 5E,F). These results suggested that anti-PD-1 scFv produced by scFv CAR-T cells in the tumor micro-environment could mediate its functions in a paracrine method as well as in an autocrine method, which inhibited apoptosis in both endogenous tumor-specific T cells and CAR-T cells, leading to superior therapeutic effects against solid tumors by synergistic actions of these cells.

### 3.5 | Selective presence of anti-PD-1 scFv in tumor tissues by treatment with scFv CAR-T cells

Although anti-PD-1 Ab can induce potent therapeutic effects in advanced and refractory cancer patients, immune-related adverse

events (irAE) represent major clinical disadvantages associated with the treatment. Simple combination of CAR-T cell therapy with systemic administration of anti-PD-1 Ab cannot avoid this problem, and may deteriorate symptoms of cytokine-release syndrome (CRS), a major adverse event of CAR-T-cell therapy. Therefore, we assessed biodistribution of anti-PD-1 scFv by measuring its concentrations in serum and tumor tissue of the mice that were treated with i.v. injection of scFv CAR-T cells. As a control, the concentrations of anti-PD-1 Ab in the serum and tumor tissue of the mice treated with i.v. injection of conv. CAR-T cells together with i.p. injections of anti-PD-1 Ab were measured. Anti-PD-1 scFv produced by scFv CAR-T cells was detectable in the tumor tissue extract but not in serum, whereas anti-PD-1 Ab was detectable both in the serum and tumor tissue extract after systemic administration (Figure S3). These data strongly suggested that scFv CAR-T-cell therapy could have the advantage of safety over combined therapy of CAR-T cells and systemic injection of anti-PD-1



**FIGURE 5** Anti-apoptotic effects of single-chain variable fragment (scFv) chimeric antigen receptor-engineered T (CAR-T) cells in tumor-specific T cells. P815-hCD20-bearing DBA/2 mice were exposed to sublethal irradiation and treated with P1A-CTL together with either anti-hCD20 conv. CAR-T or scFv CAR-T cells. On day 21, tumor tissues were harvested, prepared for single-cell suspension, and analyzed by flow cytometry. Cell numbers of CAR-T cells (A) and P1A-CTL (D) were assessed (mean  $\pm$  SD,  $n = 3$ ). Percentages of late apoptotic cells among CAR-T cells (B) and P1A-CTL (E) were also examined (mean  $\pm$  SD,  $n = 3$ ). Representative flow cytometric data of (B) and (E) are shown in (C) and (F), respectively. Representative data from three independent experiments are shown. \*\*\* $P < .001$ , \*\* $P < .005$

Ab while maintaining superior therapeutic efficacy compared to regular CAR-T cells.

## 4 | DISCUSSION

In the present study, we developed CAR-T cells producing anti-PD-1 scFv to enhance the efficacy of CAR-T-cell therapy against solid tumors. scFv CAR-T cells showed enhanced tumor-killing activity in vitro and improved therapeutic effects against solid tumors along with long-term memory responses in vivo. Functional analysis showed that scFv CAR-T cells prevented apoptosis of not only CAR-T cells but also of tumor-specific T cells in the tumor tissues. Biodistribution analysis showed that scFv CAR-T-cell injection induced detectable levels of anti-PD-1 scFv locally in tumor tissues but not in sera. Thus, our study showed the validity and mechanisms of anti-PD-1 scFv-producing CAR-T-cell therapy.

Our study showed that scFv CAR-T cells underwent less apoptotic cell death when exposed to repeated stimulations with target-positive tumor cells. AICD is an important mechanism for eliminating autoreactive T cells and terminating the immune response of peripheral T cells.<sup>18</sup> Previous reports indicated that the PD-1/PD-L1 pathway could induce AICD of antigen-specific T cells by downregulation of anti-apoptotic protein Bcl-xL and/or upregulation of pro-apoptotic protein Bim by PI3K/Akt dephosphorylation.<sup>19,20</sup> In the field of cancer immunotherapy, it was reported

that apoptosis of TIL is a major component to limit their therapeutic efficacy, and thus rescuing TIL from apoptosis could be an important strategy.<sup>21</sup> As for CAR-T-cell therapy, recent studies indicated that AICD is one of the important mechanisms to impede the therapeutic efficacy of CAR-T cells against solid tumors.<sup>22,23</sup> However, to the best of our knowledge, a role of the PD-1/PD-L1 pathway in AICD of CAR-T cells was reported by only one study so far,<sup>24</sup> in which underlying molecular mechanisms remained unknown. In this regard, the present study showed that scFv CAR-T cells prevented both early and late apoptosis in association with an increased expression of Bcl-xL. This result is concordant with a previous report that CAR-T cells engineered to produce PD-1-CH3 fusion protein showed Bcl-xL upregulation.<sup>25</sup> Based on these studies, overexpression of Bcl-xL in CAR-T cells would be an interesting approach to enhance the antitumor efficacy, although oncogenic potential of Bcl-xL to induce leukemic transformation should be carefully investigated.<sup>26,27</sup>

In order to attenuate the PD-1 inhibitory signal in CAR-T cells, several researchers have developed CAR-T-cell systems which contain a dominant-negative form of PD-1, PD-1/CD28 chimeric switch receptor, or genetic disruption of PD-1.<sup>6-8</sup> These approaches could modify the PD-1 signal in CAR-T cells themselves, but have no effects on non-CAR-T cells including endogenous tumor-specific T cells. As we recently reported,<sup>11</sup> synergistic effects with endogenous tumor-specific T cells are essential to optimize the therapeutic potential of CAR-T cells against solid tumors. In this regard,



three recent studies reported CAR-T cells engineered to produce soluble PD-1 blockers.<sup>25,28,29</sup> Among these studies, two of them used NSG immune-deficient mice as recipients, so that effects on endogenous tumor-specific T cells could not be evaluated.<sup>25,28</sup> A report by Rafiq et al<sup>29</sup> used immune-competent mouse models and showed that anti-PD-1 scFv produced from CAR-T cells improved the antitumor activity of bystander T cells as well as CAR-T cells. In this study, however, the responses of endogenous T cells specific to TAA were not analyzed. In addition, mechanisms underlying the enhanced activity of bystander T cells were not fully revealed. In contrast, our current study used P1A-specific TCR-transgenic T cells to assess the responses of endogenous TAA-specific T cells, and found decreased apoptotic cell death in P1A-CTL as well as in CAR-T cells. Thus, our current study is the first to demonstrate the evidence and mechanisms of TAA-specific T-cell responses by treatment with CAR-T cells producing PD-1 blocker. Regarding the efficacy of scFv CAR-T cells, there was intra-cohort difference showing complete rejection in some mice and very weak effects in other mice (Figure 4A and Figure S2A). As we found a relatively large variation in intratumor concentration of anti-PD-1 scFv (data not shown), technical improvement to increase and stabilize anti-PD-1 scFv production would be necessary to achieve consistent efficacy of scFv CAR-T cells.

Immune checkpoint inhibitors have been applied against various types of advanced cancers and shown its effectiveness in the clinic. At the same time, however, irAE associated with the treatment have been recognized as serious problems.<sup>30</sup> It is also known that CAR-T-cell therapy often induces adverse events including CRS and on-target off-tumor toxicities.<sup>14,31</sup> As the adverse events associated with these treatments can sometimes be severe and even fatal, exploration of novel strategies to reduce the toxicities is highly important, especially when combined immunotherapies are explored. In this regard, our approach to engineer CAR-T cells to produce anti-PD-1 scFv resulted in detection of anti-PD-1 scFv in local tumor tissues but not in sera of the systemic circulation. This finding suggests that anti-PD-1 scFv-producing CAR-T-cell therapy could result in less severe irAE compared to systemic anti-PD-1 Ab treatment.

In conclusion, CAR-T cells which produce anti-PD-1 scFv showed enhanced antitumor efficacy against mouse solid tumor models through the reduction of apoptosis in tumor-specific non-CAR-T cells as well as in CAR-T cell themselves. Effect of anti-PD-1 scFv was induced locally in the tumor tissues but not systemically, suggesting a possibility to decrease adverse events caused by PD-1 blockade. The technology to engineer CAR-T cells to produce immune checkpoint blockers as a soluble protein is a promising approach which could be applicable to other immune checkpoint molecules.

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## CONFLICT OF INTEREST

MN, KA and HN have no conflict of interest.

## DISCLOSURE

KT and YS hold stocks of Noile-Immune Biotech Inc., and receive remuneration from Noile-Immune Biotech Inc.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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