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ORIGINAL RESEARCH

Enhancement of antitumor activity by using a fully human gene encoding a single-chain fragmented antibody specific for carcinoembryonic antigen

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Abstract: Human leukocyte antigen and/or costimulatory molecules are frequently lacking in metastatic tumor cells, and thus tumor cells are able to escape from the immune system. Although lymphocytes with a chimeric antigen receptor (CAR) is a promising approach for overcoming this challenge in cancer immunotherapy, administration of modified T cells alone often demonstrates little efficacy in patients. Therefore, in order to enhance the antitumor activity of immune cells in the cancer microenvironment, we used lymphocytes expressing CAR in combination with a fusion protein of IL-2 that contained the single-chain fragmented antibody (scFv) specific for the carcinoembryonic antigen. Among a series of CAR constructs, with or without a spacer and the intracellular domain of CD28, the CAR construct containing CD8α, CD28, and CD3ζ most effectively activated and expressed INF-γ in CAR-bearing T cells. Furthermore, in comparison with free IL-2, the combination of peripheral blood mononuclear cells expressing CAR and the fusion protein containing IL-2 significantly enhanced the antitumor activity against MKN-45 cells, a human gastric cancer cell line. In conclusion, this novel combination therapy of CAR and a fusion protein consisting of a functional cytokine and a fully human scFv may be a promising approach for adoptive cancer immunotherapy.

Keywords: chimeric antigen receptor, fusion protein, human scFv, CEA, combination therapy

Introduction

In addition to the 3 primary treatment options for cancer, viz., surgery, radiotherapy, and chemotherapy, cell-based cancer immunotherapy has become a promising approach in recent years.[1–4] In clinical studies, it has been reported that this treatment was efficacious in some cases, as indicated by the complete response, whereas, in other cases, the lower response rate, at least in part, due to loss/dysfunction of human leukocyte antigen (HLA) on cancer cells and/or immune suppression in the microenvironment of the tumor mass, remains an issue to be overcome.[5–7] As a strategy for addressing non-HLA-restriction, cytotoxic T lymphocytes (CTL) with a chimeric antigen receptor (CAR) that has an antibody-based cellular arm for antigen recognition and the intracellular domain of a T cell receptor or Fc receptor, for cell activation, is a promising approach to cancer gene therapy. To date, many tumor-associated antigens (TAAs) have been used as a target of CAR in preclinical and clinical investigations, and the results have indicated the usefulness of CAR in cancer therapy.[1,8,9]

Although aspects of the gene construction of CAR, for example, the importance and length of the spacer between the cellular arm and the intracellular domain and the type of immunoreceptor tyrosine-based activating motif (ITAM) used, are debated,

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it is thought that the CD3ζ chain is most effective at signaling as an ITAM in CAR.[10] Furthermore, it has also been reported that the existence of a costimulatory signaling domain in CAR may be important, especially for long-lasting T-cell activation in vivo.[11–13] The expected effect was, however, not achieved in adoptive cancer therapy when CAR-bearing CTLs were used alone. However, recent gene therapy studies using CAR have suggested that the combination of Th2 cells expressing CAR and CAR-bearing CTLs effectively rejects a tumor mass in the mouse xenograft model and that natural killer (NK) cells expressing CAR also demonstrated a significant antitumor effect equivalent to that of CAR-bearing CTLs.[14–19] Furthermore, in order to maintain a Th1-dominated immune balance in the cancer microenvironment, the antibody that provides or blocks immune signaling by binding to the target (eg, OX40, 4-1BB, or CTLA-4) helps to control the immune balance in adoptive immunotherapy.[20–22] A novel strategy for integrating and enhancing antitumor activity to effect more efficient and continuous cancer immunotherapy might be as follows: 1) the use of other activation signals, such as cytokine/chemokine signals, in addition to signals 1 and 2; and 2) the use of other immune cells, such as neutrophil or NK cells, in addition to Th1 and Th2 T cells.

Previously, we have generated mouse and human antibodies specific for carcinoembryonic antigen (CEA), followed by a single-chain fragmented antibody (scFv), to investigate their usefulness in cell-based cancer immunotherapy.[23–29] In the present study, we constructed a series of CAR genes with or without a spacer/hinge and a CD28 intracellular domain, in addition to CD3ζ, by using the mouse scFv gene, and investigated the efficacy of these in the activation of CAR transfectants. Subsequently, a fully human CAR gene was generated, based on the construct that provided the best results, by exchanging the mouse scFv gene for its human equivalent. Furthermore, a construct encoding a fusion protein of human scFv and human IL-2 (hIL-2), designated scFv-IL2, was also generated, and its usefulness in combination cancer therapy with CAR-bearing peripheral blood mononuclear cells (PBMCs) was investigated.

Materials and methods
Reagents
CEA or bovine serum albumin (BSA) was labeled using the allophycocyanin (APC) labeling kit-NH$_2$ (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol. Mouse anti-IFN-γ-PE and anti-hIL-2-fluorescein isothiocyanate (FITC) monoclonal antibodies for flow cytometry were purchased from Miltenyi Biotech Inc. (Gladbach, Germany). Mouse anti-His and

anti-hIL-2 antibodies and rabbit anti-c-myc and anti-hIL-2 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other reagents were commercial cell culture grade or analytical grade products.

Cancer cell lines
The acute human T cell leukemia line, Jurkat clone E6.1, and the murine cytotoxic T cell line, CTLL-2, from American Type Culture Collection (ATCC; Manassas, VA, USA) were maintained in RPMI-1640 culture medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Sigma-Aldrich). The human gastric cancer cell line, MKN-45, with or without the luciferase gene, from Cell Bank (Riken BioResource Center, Ibaraki, Japan), was grown in DMEM (Sigma-Aldrich) with supplements, as described above. The MKN-45 cells were detached and harvested after treatment with trypsin-EDTA/PBS (Sigma-Aldrich) for 5 min. The cells were maintained at 37°C in humidified 5% CO_2/air, and the culture medium was changed twice a week.

Gene construction
The human genes encoding CD7 and hIL-2 were obtained from RIKEN gene bank. The CAR genes were cloned into *pcDNA3.1(−)* or *pIRES2-EGFP*, the mammalian expression vector (Thermo Fisher Scientific, Waltham, MA, USA; Figure 1A). The series of CAR genes with CD3ζ and with or without CD7/CD8α as a spacer/hinge, or CD28 as a secondary signal, were designated mCR-0, mCR-1, mCR-2, and mCR-3, respectively, as shown in Figure 1B. The cloning of mCR-2 and hCR-2 genes into *pcDNA3.1(−)* has been described previously (*F39scFv/CIR-2* and *L45scFv-CIR*, respectively).[23,26] The hCR-2 in *pIRES-EGFP* was generated by digesting *mCR-2/pcDNA3.1(−)* with *Bgl*II/*Bam*HI. The purified fragment was then cloned into the *pIRES-EGFP* vector. The antibody-cytokine fusion protein, scFv-IL2, was constructed by splice-overlap extension (SOE)-polymerase chain reaction (PCR) using appropriate primers (Figure 2A and B and Table 1). The purified fragment was digested with *Nhe*I/*Hind*III and cloned into the *pBAD/gIII Escherichia coli* expression vector. The integrity of all plasmid constructs was confirmed with restriction digestion and DNA sequencing.

Lymphocyte isolation and T cell expansion
PBMCs were obtained from healthy volunteers after obtaining informed consent in accordance with the Helsinki
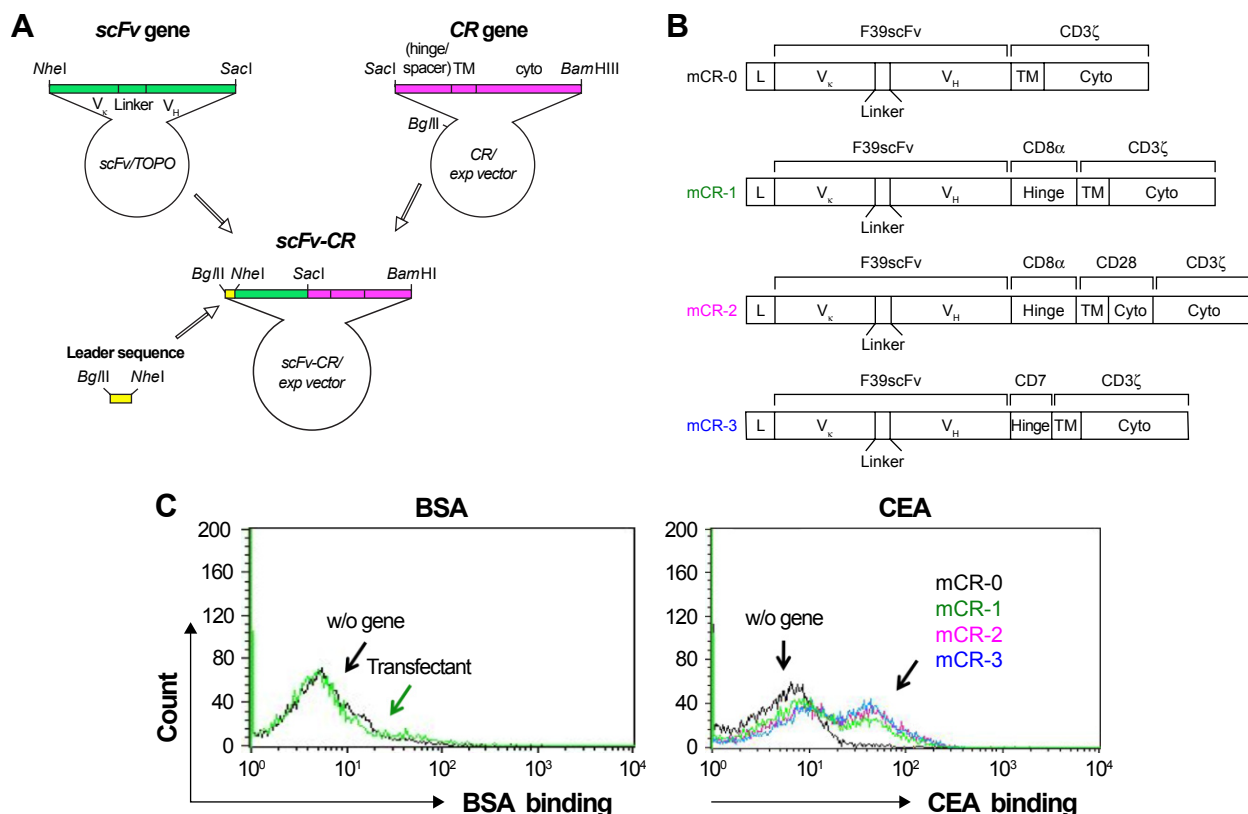
Figure 1 Functional expression of CAR in Jurkat cells.
Notes: (**A**) Schematic representation of the CR gene within a mammalian expression vector. (**B**) A series of mCR genes with or without a hinge domain (CD8α or CD7) and a CD28 intracellular domain. The mouse scFv gene was combined directly with the intracellular domain of CD3ζ (mCR-0) or with a spacer (mCR-1 and mCR-3). The mCR-2 has CD28 between CD8α and CD3ζ. (**C**) The functional and specific binding of a series of mCR to CEA on transfected Jurkat cells were monitored by flow cytometry. Histograms are representative of Jurkat cells transfected with a series of mCR constructs using APC-BSA (left) or APC-CEA (right). Green and black lines on the left represent Jurkat cells with or without mCR-2, respectively. Jurkat cells without mCR-2 could not bind to BSA. Jurkat cells transfected with a series of mCRs could functionally bind to APC-CEA (right).
Abbreviations: APC, allophycocyanin; BSA, bovine serum albumin; CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; w/o, without.

Declaration of 1975 and was approved by the Institutional Review Board of Fukuoka University. The specimens were obtained using LSM® (Cappel, Aurora, OH, USA) according to the manufacturer's protocol. After a 2 h incubation in a 10 cm culture dish, the floating cells were transferred to a new culture dish containing RPMI-1640 culture medium and the human T cell activation/expansion kit (Miltenyi Biotec). After 2 d incubation, 20 IU/mL of hIL-2 was added to the culture medium and the cells were fed twice a week (Figure S1A).

Gene transfection

Jurkat or expanded T cells (1×10^6) were transfected with 6 μg/mL CAR gene construct or pmaxGFP, using the Amaxa Cell Line Nucleofection kit R with program A-23, or the Amaxa Human T Cell Nucleofection kit with program U-14 (Amaxa, Lonza, Switzerland), respectively. Moreover, 1×10^6 expanded T cells and PBMCs were transfected with 10 μg/mL CAR gene construct or *pIRES-EGFP* using a NEPA21 electroporator (NepaGene, Chiba, Japan). After

gene transfection, the cells were immediately placed into RPMI-1640 culture media, and were incubated for 24 h before investigating cell viability, CAR expression on the cellular surface, or the antitumor effect.

Flow cytometry

CAR expression on the cells and CEA recognition after gene transfection were detected by flow cytometry using APC-labeled CEA or BSA. APC-labeled proteins (10 μg) were mixed with 5×10^5 of transfected cells and were stained for 1 h on ice for specific binding to CAR. Alternatively, CAR expression on PBMCs was confirmed by detecting EGFP using the *pIRES-EGFP* vector. For intracellular staining of IFN-γ, the transfected T cells were incubated for 24 h with MKN-45 cells and were collected by washing with PBS. The collected T cells were then permeabilized using Inside Fix (Miltenyi Biotech Inc.) and stained with anti-IFN-γ-PE according to the manufacturer's recommendations. For specific binding of scFv-IL2 to CEA, MKN-45 cells were stained with anti-hIL-2-FITC antibody by ensuring
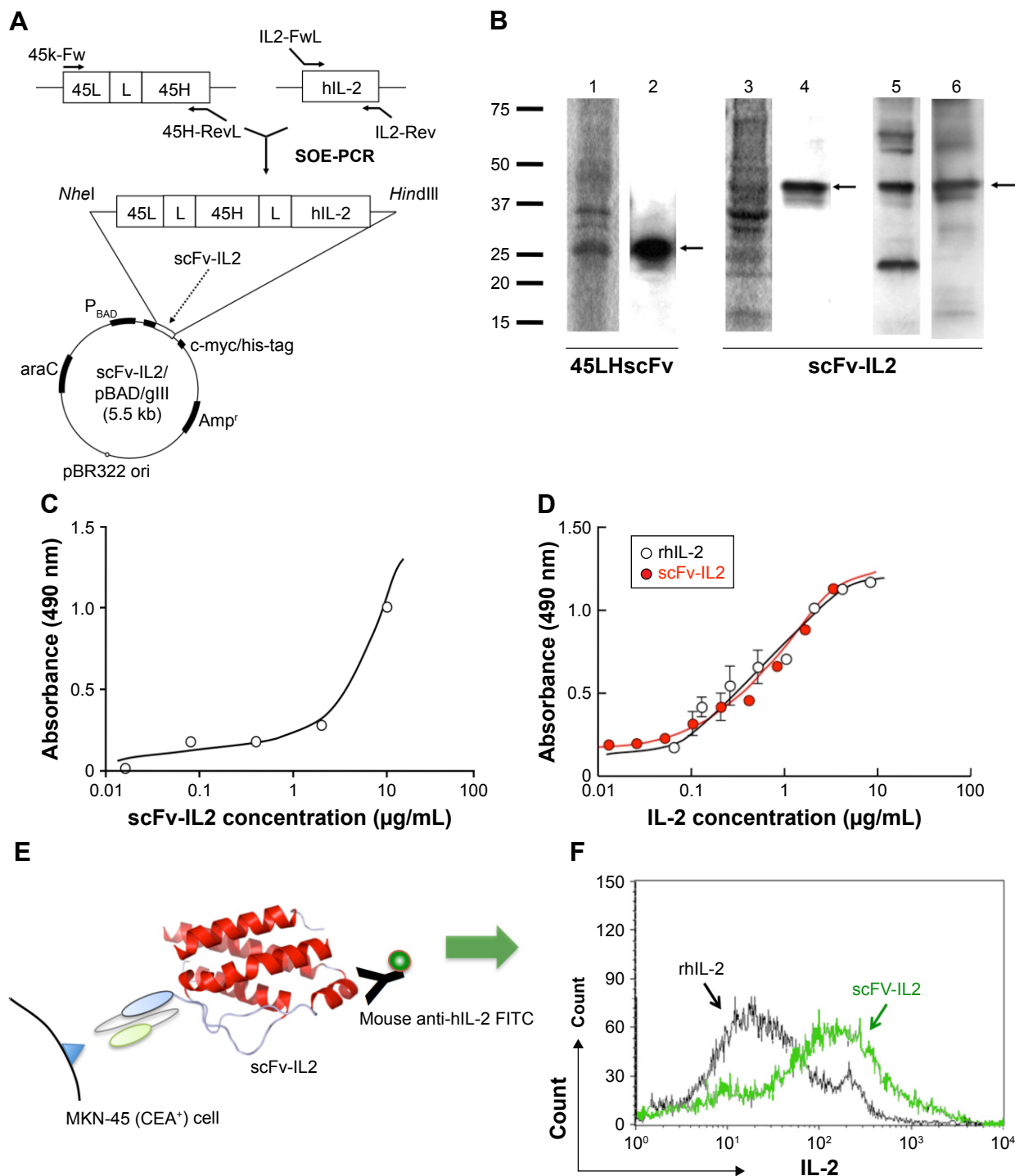
Figure 2 The scFv-IL2 fusion protein maintains the functions of both component proteins.

Notes: (**A**) Schematic representation of the scFv-IL2 gene within the pBAD/gIII expression vector. The 45κHscFv and hIL-2 genes were combined by SOE-PCR. The scFv-IL2 gene was inserted into an *Escherichia coli*. expression vector. (**B**) Detection of scFv-IL2 using CBB staining and Western blotting. The scFv-IL2 and the parental 45κHscFv vectors were transformed into TOP10 cells and were expressed by the addition of D-arabinose. After concentration through an Ni⁺ column, the proteins were detected by CBB staining (lanes 1 and 3) and Western blotting using specific antibodies (lanes 1 and 3, anti-His; lane 5, anti-hIL-2; lane 6, anti-c-myc antibodies), at the expected sizes (28 and 45 kbp, respectively). (**C**) scFv-IL2 was detected by sandwich ELISA using rabbit and mouse anti-hIL-2 antibodies. Various concentrations of scFv-IL2 were incubated in rabbit anti-hIL-2 antibody-coated 96-well microtiter plates, and were then incubated with the mouse anti-hIL-2 antibody, followed by an HRP-conjugated goat anti-mouse antibody. After reacting with the OPD substrate, the absorption at 490 nm was determined using a plate reader. The absorption of scFv-IL2 wells increased in a dose-dependent manner. (**D**) The biological function of IL-2 in the scFv-IL2 fusion protein was determined using a cell proliferation assay. CTLL-2 cells were incubated with various concentrations of IL-2 and the equivalent scFv-IL2. After 24 h incubation, the cells were detected by WST-8 assay. CTLL-2 cells were proliferated by adding scFv-IL2 and IL-2. (**E** and **F**) The biological function of scFv antibody in the scFv-IL2 treated cells was detected by flow cytometry. Schematic representation of functional analysis of scFv antibody in scFv-IL2-treated cells. scFv-IL2 creates a bridge between MKN-45 CEA-positive cells and FITC-labeled anti-hIL-2 antibody (**E**). The fluorescence intensity of FITC was shifted to the right in the presence of scFv-IL2, compared with IL-2 (**F**).

Abbreviations: CBB, Coomassie Brilliant Blue; CEA, carcinoembryonic antigen; FITC, fluorescein isothiocyanate; OPD, o-Phenylenediamine-2HCl; scFv, single-chain fragmented antibody; SOE, splice-overlap extension.

Table 1 The sequences of specific primers used for SOE-PCR of scFv-IL2

Name	Sequences
45k-Fw	5′-CCGCGGTGGCGCGCCTTTGATCTC-3′
45H-RevL	5′-GGAACCACCCCCGCCTGTACCTCCGCCCCCAGACCCGCCTCCACCAGGCCCAACCGGCCA-3′
IL-2-FwL	5′-GGTGGAGGCGGGTCTGGGGGCGGATCAGGCGGGGGTGGTTCCATGGGTGCACCTACT-3′
IL-2-Rev	5′-AAAGCTTCGAGTCAGTGTTGA-3′

Abbreviations: SOE-PCR, splice-overlap extension polymerase chain reaction; scFv, single-chain fragmented antibody.

bridging between cells and the antibody with 1 µg/mL of scFv-IL2 or IL-2. After washing twice with PBS containing 0.5% BSA, the fluorescence intensity of the stained cells was assessed using a FACSCalibur (BD Biosciences, San Jose, CA, USA) and was analyzed using Cell Quest Pro (BD Biosciences).

Microscopic analysis

To investigate the CEA recognition and binding by CAR on the cellular surface of primary T cell transfectants, the cell interaction between CAR-bearing T cells and CEA+ cancer cells was observed using microscopy. One day before observation, a mock or mCR gene was transfected into the primary T cells, and 1×10^5 MKN-45 cells were cultured in a 35 mm diameter dish for 16 h under normal culture conditions. Of these transfected cells, 1×10^6 were then added into the dish, and were co-cultured for an additional 2 h. Before observing cell–cell interaction, cells were washed twice, gently, with PBS.

Immunoblotting

To confirm the expression of scFv-IL2 in *E. coli*, the purified fusion protein was analyzed by Western blotting. After adding 0.02% of L-arabinose in Luria–Bertani (LB) medium, TOP10 cells transfected with the scFv-IL2/pBAD vector were cultured for approximately 24 h at 37°C to express scFv-IL2. After collecting the supernatant of the culture medium, 6× His-tagged scFv-IL2 was purified by AKTAprime™ (GE Healthcare Japan, Tokyo, Japan) using an Ni^{2+} column. The purified scFv-IL2 was analyzed by SDS-PAGE, followed by Western blotting using anti-His, anti-c-myc, and anti-hIL-2 antibodies.

Cell viability

After electroporation, the cell viability of transfected cells was detected using a Countess® Automated Cell Counter (Thermo Fisher Scientific) using trypan blue staining, according to the manufacturer's instructions. Cell viability was calculated as a percentage of the number of living cells versus the total number of cells (living cells and dead cells). In addition, the antitumor effect of the CAR-bearing PBMCs

in combination with scFv-IL2 was evaluated by measuring the intensity of light produced by MKN-45 cells expressing luciferase, using a luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's protocol. MKN-45 cells (1×10^4) were co-cultured with 5×10^4 of PBMCs transfected with the hCR-2 gene construct or PBMCs at an effector to target cell ratio (E:T) of 5:1 in the presence of scFv-IL2 or IL-2, for 24 h. After washing with PBS, 20 µL of lysis reagent was added to each well and, subsequently, the light produced was measured using a MikroWin2000 Advanced II system (Berthold Technologies, Tokyo, Japan) after adding 100 µL of luciferase assay reagent to each well. The cell viability was calculated as a percentage of the light intensity of the treated wells versus that of non-treated wells (n=3–4).

Statistical analysis

Numerical data are presented as the mean ± standard error of the mean (SEM) from at least 3 independent experiments. Significance was assessed with one-way ANOVA followed by Bonferroni's multiple comparison tests; *P*-values <0.05 were considered to be statistically significant.

Results
Various CAR genes transfected by electroporation into Jurkat cells expressed and bound to CEA to the same extent

We first determined whether CAR genes of different sizes and constituent gene types could be functionally expressed in mammalian cells by using the pcDNA3.1(−) expression vector. Four kinds of genes were constructed with or without a spacer/hinge and CD28 intracellular signaling domain, and were designated CR-0 to CR-3 (Figure 1A and B). In this study, the extracellular domains of CD8α (195 bp) and CD7 (96 bp) were assessed as a spacer/hinge because of the difference in their size. Only CR-2 retained the intracellular domain of the secondary signal, CD28, but not the others. Eventually, the size of CAR genes constructed here were 1,201, 1,398, 1,533, and 1,302 bp (CR-0, CR-1, CR-2, and CR-3, respectively). The CAR genes were transfected into

Jurkat cells by electroporation and their functional expression was assessed 24 h after transfection by flow cytometric analysis with an APC-labeled antigen, viz., APC-CEA or APC-BSA (see "Materials and methods" section for details). Interestingly, the expression level of the various CAR genes on Jurkat cells were similar (about 60%) in our experimental conditions, as determined by comparing their binding to CEA and BSA, as a control (Figure 1C).

The CAR gene expressing CD8α, CD28, and CD3ζ most effectively stimulated T cells

To determine which of the 4 CAR genes activated immune cells most effectively and demonstrated the highest anticancer activity, the various CAR genes were transfected into artificially expanded T cells and Jurkat cells and their functional properties were analyzed and compared. T cells from volunteers' PBMCs were sufficiently expanded by over 15-fold over the course of 9 days (Figure S1A). After artificial T cell expansion, the majority of cells became T cells and the ratio of $CD4^+$ T cells and $CD8^+$ T cell was approximately 1.2:1.0 (Figure S1B). The transfection efficacy of CAR genes into these artificially expanded T cells was about 35%, and that of the EGFP control over 70%, as assessed 24 h after transfection and using our experimental conditions (Figure 3A; Table S1).

The cell viability of the transfected T cells was about 50% for all 4 CAR genes, as determined by trypan blue staining.
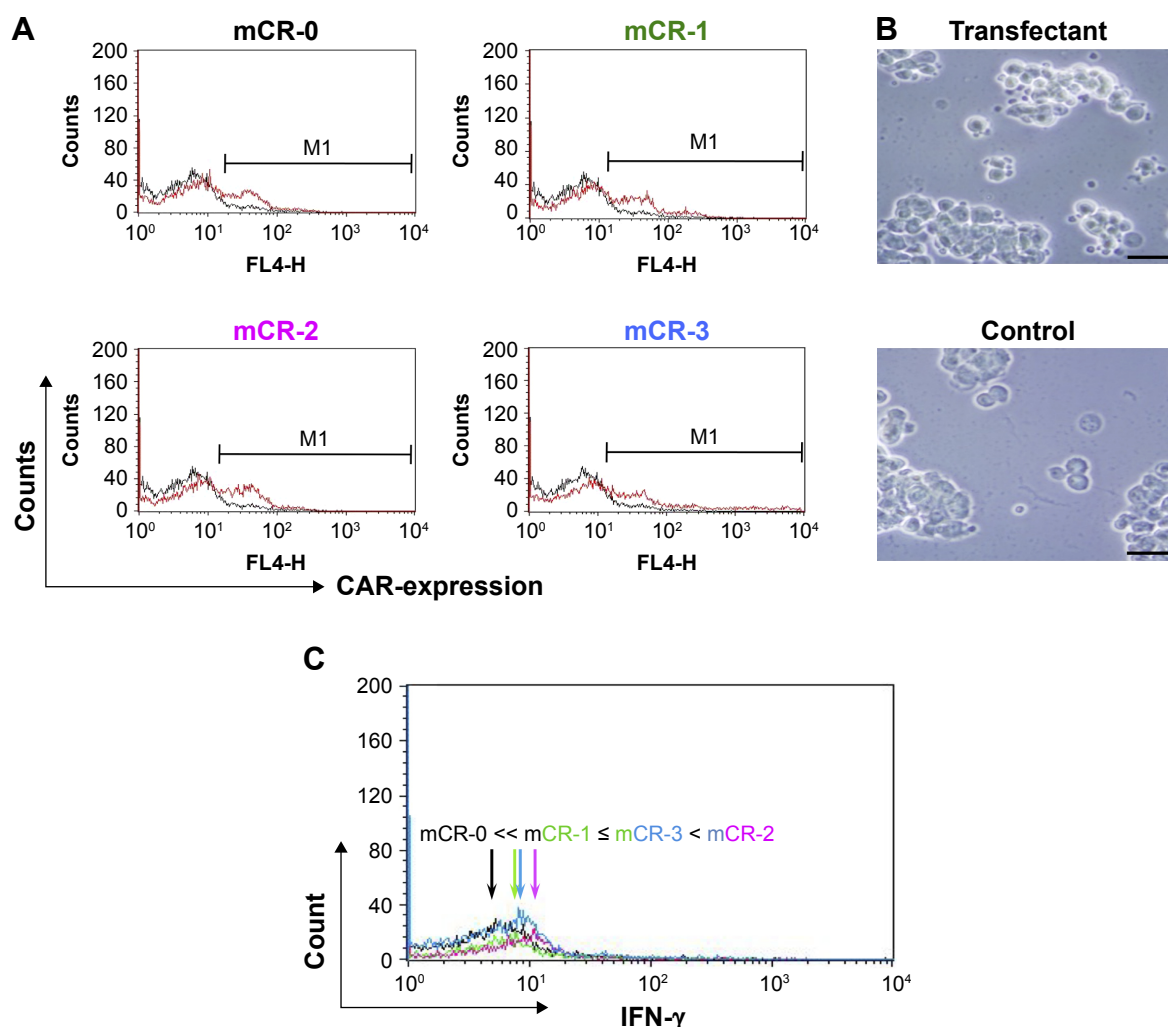

Figure 3 T cells transfected with mCR-2 were most effectively activated by MKN-45 cells.
Notes: (**A**) T-cell transfectants equivalently expressed a functional series of mCR on their surface. Black and purple lines represent APC-BSA and APC-CEA, respectively. (**B**) Expression of IFN-γ by a series of T-cell transfectants was detected by optical microscope after 1 day of incubation with MKN-45 cells, using a specific antibody. Among a series of mCR, mCR-2 could stimulate T cells and express IFN-γ most effectively in T-cell transfectants. Scale bar is 100 μm. (**C**) T cells transfected with mCR-2 bound MKN-45 CEA^+ cells (upper), but not the cells lacking the CR gene (bottom), as well as APC-CEA did.
Abbreviations: APC, allophycocyanin; BSA, bovine serum albumin; CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen.

After the transfected T cells were incubated with CEA^{+} cancer cells (MKN-45) for an additional 24 h, the activation efficacy of CAR-bearing T cells was determined to follow the order: CR-2> CR-3≥ CR-1> CR-0, as assessed by the production of IFN-γ and analyzed using flow cytometry with a specific antibody (Figure 3B). The spacer had no effect on CAR expression on primary T cells or on CEA recognition, but it was critical in efficient activation of genetically engineered cells. After addition of the purified CEA coating onto the bottom of a 24-well plate, recognition and binding of CAR-bearing T cells to CEA expressed on the cellular surface of MKN-45 cells, a human gastric cancer cell line, was confirmed using microscopy. After the T cells transfected with CAR genes were co-cultured with MKN-45 cells for 2 h, they bound to CEA^{+} cancer cells, but not to the control mock gene, as shown in Figure 3C.

The scFv-IL2 fusion protein generated by fully human genes maintains the function of both constituent proteins

The gene encoding the scFv-IL2 fusion protein was generated by SOE-PCR from the human genes by using the primers shown in Figure 2A and Table 1, and was expressed in an *E. coli* protein expression system by adding D-arabinose to the LB medium. The scFv-IL2 and scFv proteins purified from the supernatant of the LB medium using an Ni^{2+} column was confirmed by Western blotting using an anti-hIL-2, anti-c-myc, or anti-His antibody; the appropriate bands were detected at the expected sizes (28 and 45 kbp, respectively; Figure 2B). Furthermore, a sandwich enzyme-linked immunosorbent assay (ELISA) was performed to verify the structure of hIL-2 in scFv-IL2 under physiological conditions (Figure 2C).

As shown in Figure 2D, the cell number of CTLL-2 cells that expressed the IL-2 receptor and proliferated in the presence of hIL-2 increased in a dose-dependent manner after 48 h incubation with scFv-IL2.

The binding properties of svFv-IL2 were investigated by flow cytometry using CEA[+] MKN-45 cells and an FITC-labeled anti-hIL-2 antibody (Figure 2E and F). The fluorescent intensity of FITC increased after 1 h incubation with scFv-IL2, but not with hIL-2, indicating that scFv-IL2 bound to CEA on the cellular surface of MKN-45 cells, whereas hIL-2 did not bind MKN-45 cells (Figure 2F). Taken together, these results indicated that scFv-IL2, the fusion protein of the scFv antibody specific for CEA and hIL-2, could be successfully generated using an *E. coli* protein expression system, and retained their functions.

Combination of CAR-bearing PBMCs and scFv-IL2 efficiently damages MKN-45 cells

Taking into consideration that CAR and the fusion protein may be applied as combination therapy in patients, all genes used for this purpose should be of human origin. In this context, the mouse scFv gene of mCR-2, which most effectively activated primary T cells and produced INF-γ among the 4 CAR genes, was replaced with its human equivalent; this construct was designated as hCR-2, and subsequently the anticancer effect of PBMCs expressing this CAR was investigated in combination with scFv-IL2 (Figure 4A). The expression of the 4 types of CAR genes on Jurkat cells was detected by flow cytometry using APC-CEA and APC-BSA and was compared. The expression level of hCR-2 was marginally higher than that of mCR-2, whereas the different expression vectors, pcDNA3.1(−) and pIRES, did not affect the CAR level on Jurkat cells under our experimental conditions (Figure 4B).

Although the efficacy of gene expression in T cells artificially expanded from PBMCs using the Human T cell Activation/Expansion kit (Miltenyi Biotec) reached 40% after electroporation using a Nucleofector™ (Lonza Group Ltd, Basel, Switzerland), the cell viability after a 24 h incubation was, unfortunately, <50% in our experiment. Because we used primary cells to assess the anticancer effect of the combination of scFv in CAR and in the fusion protein, it was important to increase cell viability after gene transfection. We compared 2 electroporation systems, Nucleofector and NEPA21, for transfection efficacy and cell viability: NEPA21 performed better in human T cells, as summarized in Table S1. Therefore, we decided to use NEPA21 for transfection of the hCR-2 gene into PBMCs. In the present study, PBMCs and T cells were used for CAR gene transduction, as we expected synergy in the anticancer effect of immune cells. As shown in Figure 4C the expression of hCR-2 in PBMCs after NEPA21 transfection was about 60% at 24 h after electroporation, as determined by the expression of EGFP by using flow cytometry. Under these experimental conditions, the cell viability of CAR-bearing PBMCs was about 95%.

The PBMCs were then incubated with the luciferase-expressing MKN-45 cells for 24 h in the presence of scFv-IL2 or hIL-2 to investigate the antitumor effect. The cell viability decreased in a dose-dependent manner in the presence of hIL-2 (Figure 4D). The CAR-bearing PBMCs damaged cancer cells more efficiently than did untransfected PBMCs, as expected. Comparison of the antitumor effects
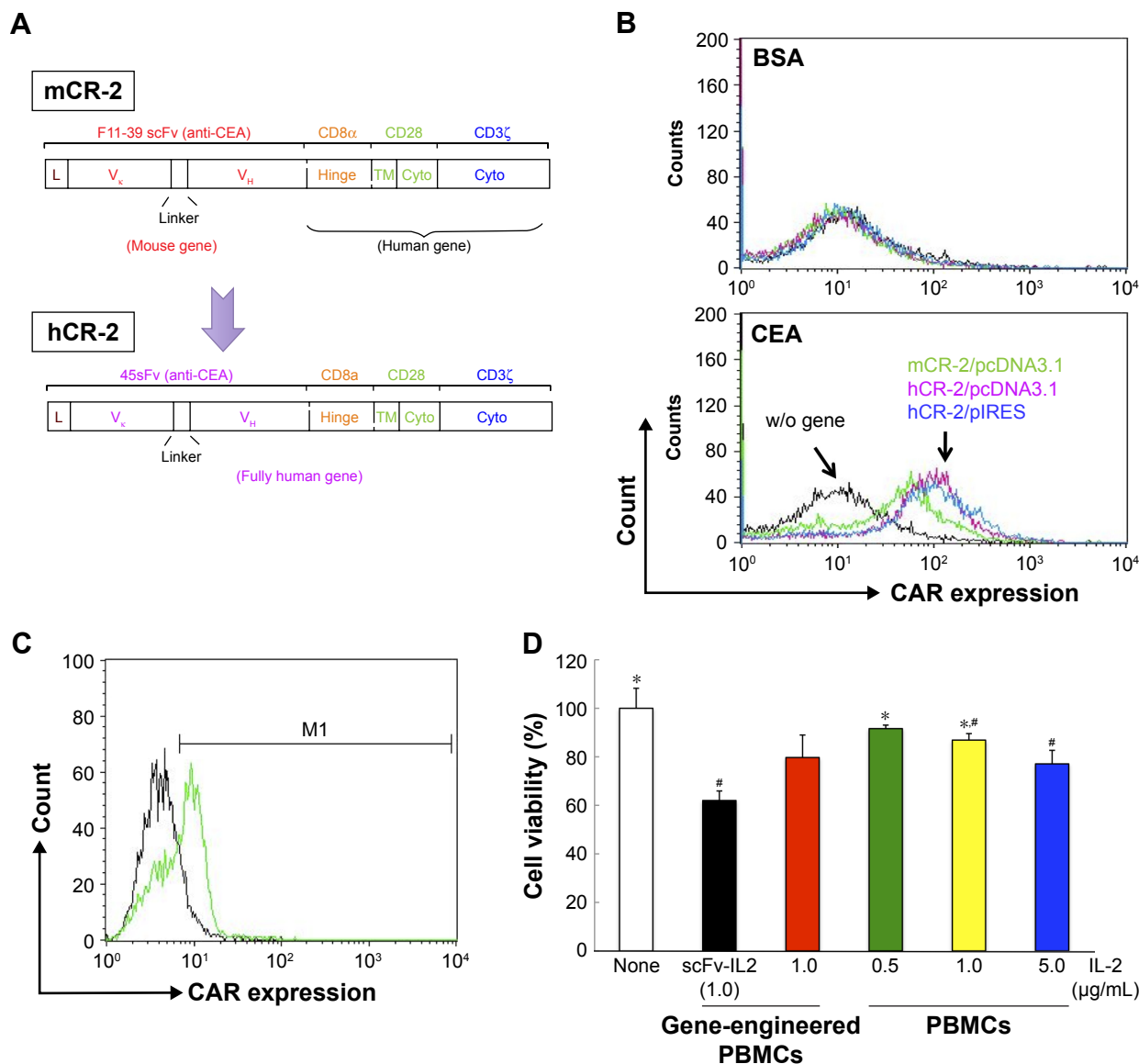
Figure 4 Combination of CAR-bearing PBMCs and scFv-IL2 enhances the antitumor effect on MKN-45 cells.

Notes: (**A**) Exchange of the mouse scFv gene for its human equivalent in the CAR gene construct. The mouse scFv of mCR-2, which was the most effective among 4 CAR constructs, was exchanged for the 45κHscFv, a human scFv antibody, and this fully human CAR gene was designated hCR-2. (**B**) Expression of hCR-2 inserted into different expression vectors, pcDNA3.1(−) or pIRES, in Jurkat cells was detected by flow cytometry using APC-BSA and APC-CEA. Although hCR-2 was expressed at slightly higher levels in Jurkat cells than mCR-2, no other difference could be detected between the 2 expression vectors. (**C**) hCR-2 in PBMCs was detected by flow cytometry, by using EGFP expression. Approximately 60% of PBMCs expressed hCR-2 after transfection of the CAR gene within a pIRES vector using NEPA21. (**D**) PBMCs expressing hCR-2 in combination with scFv-IL2 demonstrated a higher antitumor activity on MKN-45 cells than those expressing IL-2 or PBMCs alone. Cell viability was determined by measuring the light products using a luciferase assay system. Data represent the mean ± standard error of the mean from at least 3 independent experiments. *$P<0.05$ or #$P<0.05$, significantly different from CAR-bearing PBMCs in combination with scFv-IL2 or the control (none) group, respectively.

Abbreviations: APC, allophycocyanin; BSA, bovine serum albumin; CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; PBMCs, peripheral blood mononuclear cells; scFv, single-chain fragmented antibody; w/o, without.

of genetically engineered PBMCs and parental PBMCs revealed a fivefold difference in the concentration of free hIL-2. Interestingly, the efficacy of CAR-bearing PBMCs in combination with scFv-IL2 was double that of treatment with the same amount of free-hIL-2.

Discussion

We have demonstrated the potential of a combination cancer treatment using a human scFv gene in both CAR and a fusion protein of a cytokine in this study. Furthermore, PBMCs, in addition to CLT, CD4^{+}, and CD8^{+} T cells or NK cells, were found to be effective targets for gene engineering along with CARs in adoptive cancer immunotherapy. Very recently, Srivastava et al demonstrated that activated NK cells and dendric cells (DC) interacted and collaborated to enhance antitumor immunity related to CTL.[30] This may be an advantage for the use of PBMCs expressing CAR in patients, without HLA restriction, in adoptive cancer immunotherapy.

To date, many genes from the variable fragment region of an antibody specific for TAA have been reported and applied in the production of CAR or fusion protein in cancer treatment in order to overcome the loss or dysfunction of HLA on the cellular surface of tumor cells.[9] In combination with CAR-bearing PBMCs, the scFv-IL2 fusion protein was twice as effective in damaging MKN-45 cells as compared to the same amount of free hIL-2. This phenomenon may indicate the importance of simultaneous input of a third signal, together with the first and secondary signals for activation of T cells, because the anticancer activity of LAK cells using free hIL-2 alone or the fusion of the scFv antibody and hIL-2 indicated similar effects in adoptive immunotherapy.[31] Artificial expansion of primary T cells from PBMCs may support this hypothesis. In this context, the combination of CAR and the fusion protein containing the scFv specific for TAA seems to be a promising approach for activating immune cells efficiently and maintaining long-lasting activation of cytokine production in the cancer microenvironment, in addition to the advantage of reduction in the dose of exogenous cytokines required for the treatment of the patient. Moreover, the importance of other cytokines (eg, IL-12, IL-15, and IFN-γ) or immunoregulatory antibodies (eg, anti-CTLA-4, anti-PD-1, anti-IL-10, anti-TGF-β, anti-4–1BB, and anti-OX-40) in the microenvironment has also been reported.[22,32–35] Thus, the usefulness of fusion proteins of scFv and these cytokines, or bispecific antibodies, in combination with CAR-bearing PBMCs, as well as the efficacy of these molecules in an in vivo xenograft model, should be assessed in future experiments.[36]

In our results, the spacer between the domains of the cellular arm and the intracellular ITAM was critical for activation of CAR-bearing T cells, whereas the type of spacer may not be as important as the ITAM domain in CAR. Although there is still considerable debate as to the type of ITAM domain used to construct an effective CAR gene in adoptive cancer immunotherapy, recent reports using a third-generation of CAR genes suggest that it may be important to have signal 1 and at least 1 or 2 signal 2-related ITAM domains, with a spacer, to activate T cells expressing CAR and to maintain long-lasting stability in the patient.[13] When comparing the 4 kinds of CAR genes reported here, the CR-2 gene demonstrated the desired results.

Given the efficacy of gene transfection and cell viability after 24 h, electroporation using the NEPA21 system seems to be a promising candidate for use in gene therapy. Interestingly, the hCR-2 gene may be transduced not only into T cells but also into NK cells because PBMCs constitute less than 50% of CD3$^+$ T cells. This may be an explanation for the higher antitumor effect in CAR-bearing PBMCs than in the parental PBMCs with the same concentration of hIL-2.

A concern about using PBMCs in gene-engineered cell-based cancer immunotherapy is that regulatory T (Treg) cells expressing CAR, which activate as well as CTL and NK cells expressing CAR, will suppress the immune response in a cancer microenvironment. Fortunately, because Treg cells can be positively selected from PBMCs using a cell sorter or separator with their specific antibody, PBMCs lacking Treg cells from patients will provide adequate cell-based immunotherapy.

In conclusion, application of the CEA-specific scFv in the combination of CAR and cytokine fusion protein, resulting in the enhancement of anticancer activity of immune cells at the cancer microenvironment might be a promising candidate in adoptive cancer immunotherapy (Figure S2).

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials
Artificial T cell expansion

PBMCs were obtained from healthy volunteers. The specimens were obtained using LSM® (Cappel, Aurora, OH, USA) according to the manufacturer's protocol. After a 2 h incubation in a 10 cm culture dish, the floating cells were transferred to a new culture dish containing RPMI-1640 culture medium and the human T cell activation/expansion kit (Miltenyi Biotech Inc., Gladbach, Germany). After 2 d incubation, 20 IU/mL of hIL-2 was added to the culture medium and the cells were fed twice a week with hIL-2.

Flow cytometry

The component of CD4+ and CD8+ T cells 9 days after artificial expansion of PBMCs was analyzed using a FACSCalibur system (BD Biosciences, San Jose, CA, USA) with their specific antibodies (CD4-PE and CD8-FITC, respectively; Miltenyi Biotec GmgH, Bergisch Gladbach, Germany), and was analyzed using Cell Quest Pro (BD Biosciences).
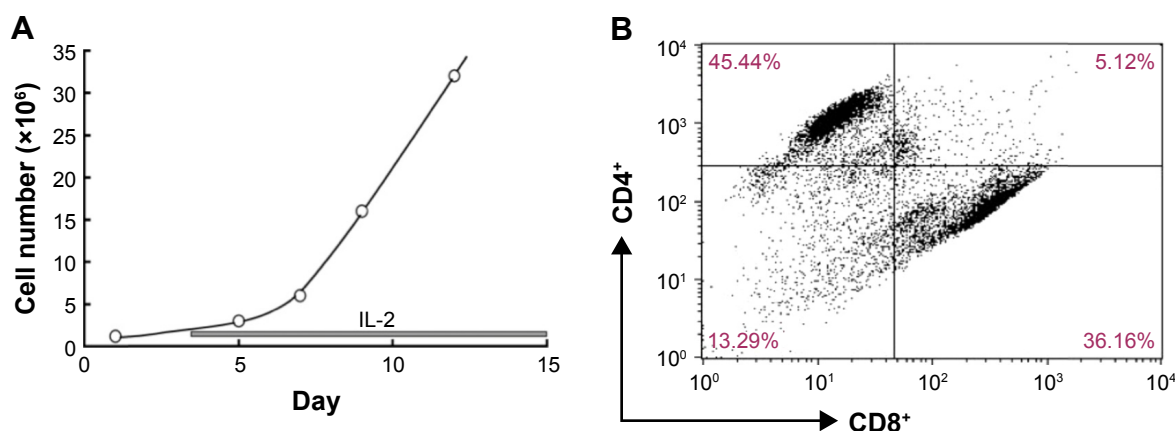
Figure S1 Artificial T-cell expansion (**A**) and the cell ratio of CD4+ and CD8+ T cells after 9-day expansion (**B**).

Table S1 Transfection efficiency of electroporation using Nucleofector™ and NEPA21

Cell type	Electroporation	Gene	Efficacy (%)	Cell viability (%)
Jurkat cells		EGFP	70	50
T cells	Nucleofector™	EGFP	50	60
T cells		CAR	37	<50
T cells		EGFP	>90	>90
PBMCs	NEPA21	EGFP	>60	95
PBMCs		CAR	>60	93

Abbreviations: CAR, chimeric antigen receptor; PBMCs, peripheral blood mononuclear cells.

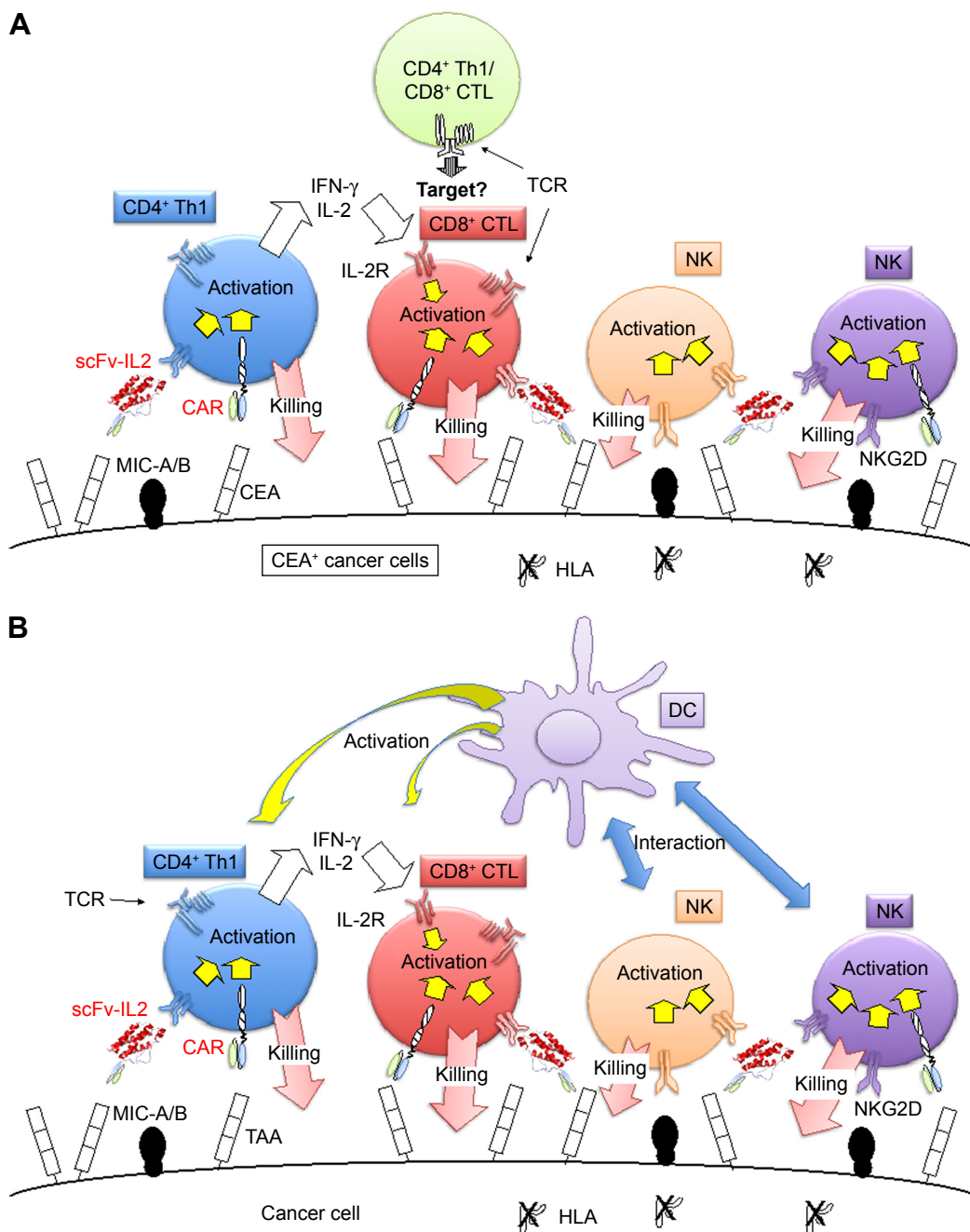**Figure S2** (**A**) Schematic representation of the combination of CAR-bearing PBMCs and scFv-IL2 for enhancing the antitumor activity to CEA$^+$ tumor cells. (**B**) Conceptual representation of CAR-bearing PBMCs, DC, and scFv-IL2 fusion protein for enhancing the antitumor activity to cancer cells.
Abbreviations: CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; CTL, cytotoxic T lymphocytes; DC, dendric cells; NK, natural killer; PBMCs, peripheral blood mononuclear cells; scFv, single-chain fragmented antibody.