ARTICLE

Immunological quality and performance of tumor vessel-targeting CAR-T cells prepared by mRNA-EP for clinical research

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We previously reported that tumor vessel-redirected T cells, which were genetically engineered with chimeric antigen receptor (CAR) specific for vascular endothelial growth factor receptor 2 (VEGFR2), demonstrated significant antitumor effects in various murine solid tumor models. In the present study, we prepared anti-VEGFR2 CAR-T cells by CAR-coding mRNA electroporation (mRNA-EP) and analyzed their immunological characteristics and functions for use in clinical research. The expression of anti-VEGFR2 CAR on murine and human T cells was detected with approximately 100% efficiency for a few days, after peaking 6–12 hours after mRNA-EP. Triple transfer of murine anti-VEGFR2 CAR-T cells into B16BL6 tumor-bearing mice demonstrated an antitumor effect comparable to that for the single transfer of CAR-T cells engineered with retroviral vector. The mRNA-EP did not cause any damage or defects to human T-cell characteristics, as determined by viability, growth, and phenotypic parameters. Additionally, two kinds of human anti-VEGFR2 CAR-T cells, which expressed different CAR construction, differentiated to effector phase with cytokine secretion and cytotoxic activity in antigen-specific manner. These results indicate that our anti-VEGFR2 CAR-T cells prepared by mRNA-EP have the potential in terms of quality and performance to offer the prospect of safety and efficacy in clinical research as cellular medicine.

INTRODUCTION

Adoptive immunotherapy using cytotoxic T lymphocytes (CTLs) has been greatly anticipated as an ideal cancer treatment strategy that is efficacious for not only the regression of primary cancer but also the suppression of metastasis and its recurrence, and it has few side effects for normal tissue.[1,2] However, the inability to prepare CTLs of sufficient number and quality due to immunosuppression in most cancer patients and the lack of transferred CTL accumulation in tumor limit the clinical response of this approach.[3] Chimeric antigen receptor (CAR)-T cell therapy, which has been developed to overcome the issues of CTL adoptive immunotherapy, is advancing toward its clinical application via various protocols proposed by many research groups, particularly in Europe and the United States.[1,2,4–10] These protocols for CAR-T cell therapy are mainly intended for hematologic cancer[9,10] because transferred CAR-T cells can easily contact target cells in blood vessels. On the other hand, this therapy is difficult to demonstrate marked *in vivo* efficacy for solid tumor by some barriers including vessel walls and the stroma before access of transferred CAR-T cells to target malignant cells.[11–13]

Tumor angiogenesis, which controls O_2/CO_2 exchange, nutrient supply, and waste exclusion in tumor tissue, is essential for tumor growth and commonly occurs in solid cancer.[14] Because vascular endothelial cells are far fewer than tumor cells in the tumor tissue,[15,16] we can easily imagine that one endothelial cell controls the survival and proliferation of many tumor cells. In recent years, cancer treatments targeting tumor vessels, which drugs and antibodies can easily access, have attracted attention and have been actively developed.[17–19] To introduce this therapeutic approach to CAR-T cell therapy, we focused on vascular endothelial growth factor receptor 2 (VEGFR2) as a highly desirable target molecule because VEGFR2 abundantly exists on endothelial cells of tumor blood vessels, whereas normal blood vessels express few VEGFR2.[20–22] In our previous work, CAR-T cells, which were transduced with murine VEGFR2 (mVEGFR2)-specific CAR using a retroviral vector (Rv), demonstrated a significant growth inhibitory effect on various solid tumors on the basis of high accumulation in tumor tissue and tumor vessel-specific injury.[23] To realize the clinical application of this promising novel CAR-T cell therapy, we planned clinical research for the verification of safety and efficacy in human. A high level of safety based on rational and scientific evidence is demanded in the clinical research protocol of CAR-T cell therapy. Therefore, we considered that switching from conventional Rv transduction, which

has a genotoxic potential due to the chromosome insertion of the foreign gene, to another technique was desirable for the preparation of CAR-T cells.

In this study, to circumvent the genotoxic issue, we assessed electroporation (EP) of the mRNA encoding CAR as a clinical platform in CAR-T cell preparation. We optimized a mRNA-EP condition for murine and human T cells and demonstrated the efficacy of mVEGFR2-specific CAR-T cell therapy using mRNA-EP in tumor-bearing mice as proof of concept. Furthermore, as a type of cellular medicine, the quality and performance of anti-human VEGFR2 (hVEGFR2) CAR-T cells were confirmed from the perspective of clinical research.

RESULTS

CAR expression profile and antitumor effect of mV/m28/m3z CAR-T cells

CAR mRNA constructions used in the present study were summarized in Figure 1. By using mV/m28/m3z CAR mRNA, the mRNA-EP condition for murine CD8^{+} T cells was optimized to achieve a CAR expression efficiency of almost 100% without cellular damage. EP is composed of poring pulse (P_p) to open the cell membrane and driving pulse (D_p) to introduce mRNA, and so on. Generally, in transfection using EP, it is known that P_p voltage and P_p duration have an impact on the expression efficiency of target proteins. We, therefore, started the optimization study by changing P_p voltage in murine CD8^{+} T cells, but the study clarified that using P_p voltage of over 450 V can cause bumping. For the stable EP, a P_p voltage of 300 V was fixed. We changed the other factors as shown in Figure 2a,b, and the conditions of No. 9 and No. 10 showed higher expression intensity. In consideration of large-scale production, a cell number of 1×10^7 cells was preferable. The increase in the CAR-mRNA quantity did not improve the expression intensity (data not shown). Ultimately, we decided the condition of No. 9 was the optimal EP condition in murine CD8^{+} T cells.
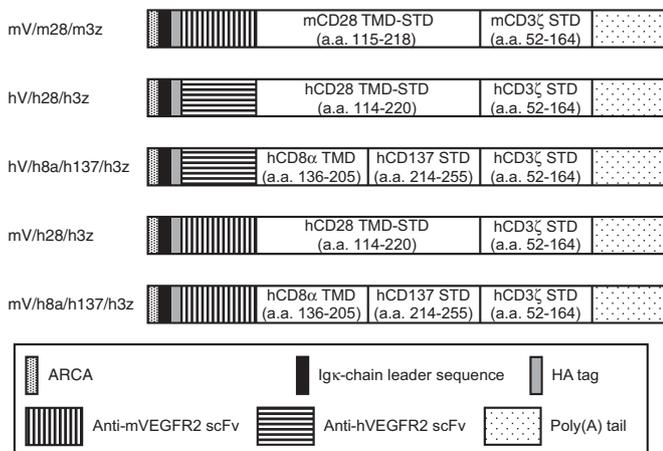
Figure 1 Construction of mRNA encoding VEGFR2-specific chimeric antigen receptor (CAR). mRNA comprised ARCA, an Igκ-chain leader sequence, an HA tag for detecting CAR, mVEGFR2/hVEGFR2-specific CAR, and a poly(A) tail. mV/m28/m3z contains anti-mVEGFR2 scFv, mCD28 from transmembrane domain (TMD) to signal transduction domain (STD) (a.a. 115–218), and mCD3ζ STD (a.a. 52–164). hV/h28/h3z and mV/h28/h3z contain anti-hVEGFR2 scFv or anti-mVEGFR2 scFv, hCD28 from TMD to STD (a.a. 114–220), and hCD3ζ STD (a.a. 52–164). hV/h8a/h137/h3z and mV/h8a/h137/h3z contain anti-hVEGFR2 scFv or anti-mVEGFR2 scFv, hCD8α TMD (a.a. 136–205), hCD137 STD (a.a. 214–255), and hCD3ζ STD (a.a. 52–164).

We analyzed the CAR expression profile on mV/m28/m3z CAR-T cells prepared by the optimized mRNA-EP protocol (Figure 2c). The highest CAR expression level was detected 12 hours after mRNA-EP, after which the expression intensity gradually decreased, disappearing almost completely 48 hours after mRNA-EP. Even the maximum expression level 12 hours after mRNA-EP fell short of the CAR expression intensity of our conventional mV/m28/m3z CAR-T cells using Rv transduction.
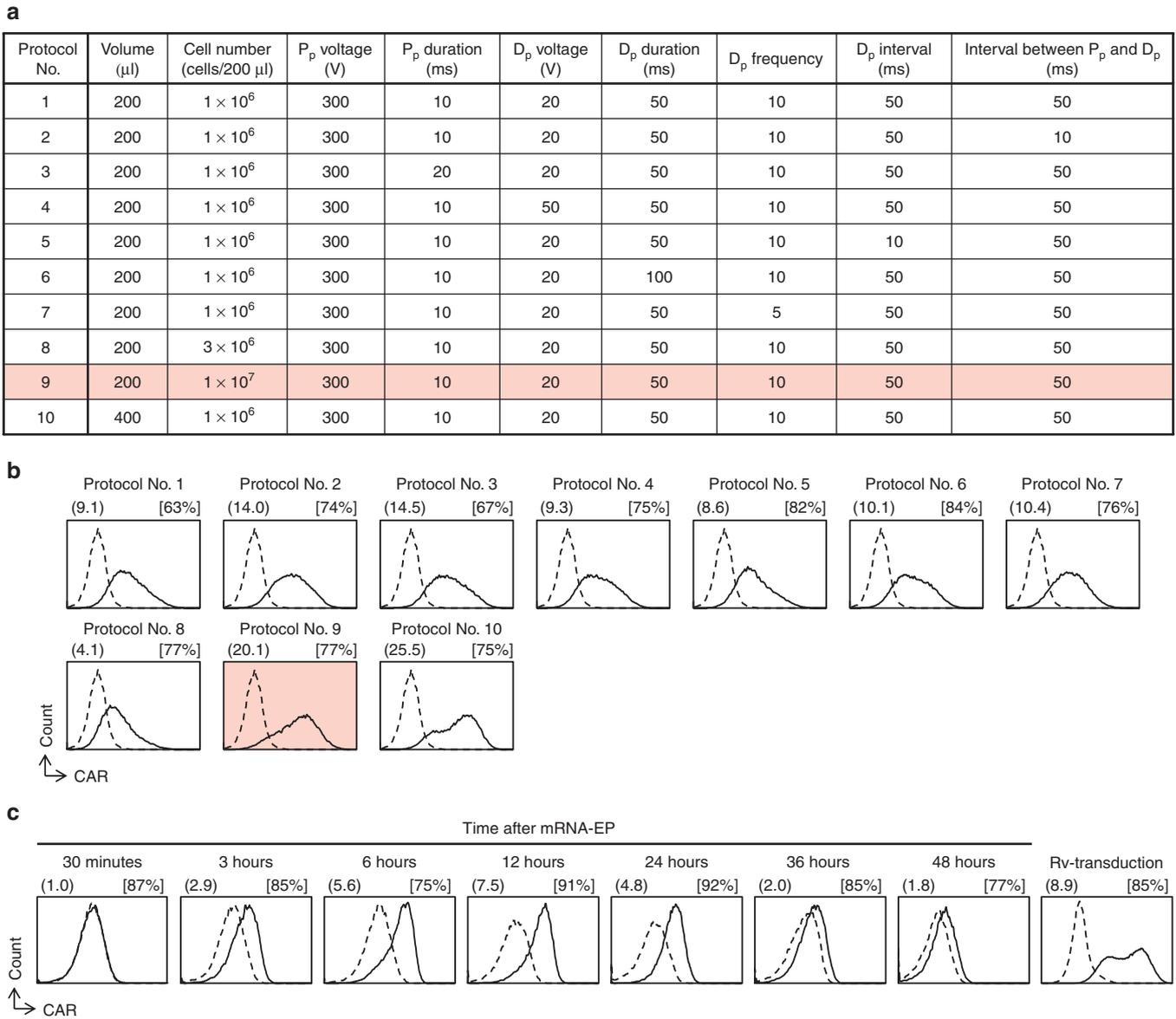
We evaluated the antitumor effect of mV/m28/m3z CAR-T cells 6 hours after mRNA-EP in B16BL6 tumor-bearing mice (Figure 3a). Single-dose transfer of mRNA CAR-T cells significantly suppressed tumor growth compared with that of untransfected T cells. Triple transfer of mRNA CAR-T cells demonstrated a more notable inhibitory effect on tumor growth and this efficacy was comparable to that of single transfer with CAR-T cells prepared by Rv transduction. In addition, single-dose transfer of mRNA CAR-T cells significantly injured tumor vessels compared with the control groups, and the injuring efficacy in triple transfer of mRNA CAR-T cells was comparable to that in single transfer with Rv CAR-T cells (Figure 3b). Therefore, these results indicate that CAR-T cells prepared by mRNA-EP represent a functional *in vivo* cellular medicine capable of injuring tumor vessels and that their multiple transfers can augment the antitumor effect even if their CAR expression is temporary and lower than that of CAR-T cells prepared using Rv transduction.

CAR expression profile and phenotypic parameters of human CAR-T cells

Human peripheral blood mononuclear cells (PBMCs) isolated from volunteers were expanded in the presence of anti-human CD3 (hCD3) monoclonal antibody (mAb) and human interleukin-2 (hIL-2), and human CD8^{+} T cells were purified from the expanded PBMCs. The mRNA-EP condition for human CD8^{+} T cells was optimized to achieve a high CAR expression efficiency without cellular damage (Figure 4a,b). We set each parameter in reference to the results in mice and examined the impact of cell number, P_p voltage, P_p duration, and mRNA amount on CAR expression intensity. The conditions of No. 1 and No. 4 showed higher expression intensity, but the cell viability of No. 4 was the least among the tested 10 conditions. Therefore, we selected No. 1 as the milder and the optimal condition for human CD8^{+} T cells was determined.

We compared the expression profile of four kinds of CAR-T cells prepared by the optimized protocol: hV/h28/h3z, hV/h8a/h137/h3z, mV/h28/h3z, and mV/h8a/h137/h3z (Figure 1). All CAR-T cells showed the same expression tendency, that is that the highest CAR expression level was detected 6 hours after mRNA-EP and then gradually decreased (Figure 4c). While the CAR expression level of hV/h28/h3z CAR-T cells decreased toward the basal level 72 hours after mRNA-EP, hV/h8a/h137/h3z CAR-T cells showed a relatively stable expression, even 72 hours after mRNA-EP. The same phenomenon was observed between mV/h28/h3z and mV/h8a/h137/h3z CAR-T cells, which were replaced with the other scFv, indicating that the CD8α-derived transmembrane domain (TMD) generally stabilizes CAR expression on the plasma membrane of human CD8^{+} T cells to a greater extent than CD28-derived TMD; thereby extending the CAR expression period.

We also assessed the CAR expression of hV/h28/h3z CAR-T cells prepared from the PBMCs of four donors 36 hours after mRNA-EP. The expression efficiency of CAR capable of binding to hVEGFR2-Fc chimera was almost 100% on CD8^{+} T cells from all donors and the expression level was equivalent regardless of donors (Figure 4d), showing the possibility that our optimized mRNA-EP protocol

a

Protocol No.	Volume (μl)	Cell number (cells/200 μl)	P_p voltage (V)	P_p duration (ms)	D_p voltage (V)	D_p duration (ms)	D_p frequency	D_p interval (ms)	Interval between P_p and D_p (ms)
1	200	1×10^6	300	10	20	50	10	50	50
2	200	1×10^6	300	10	20	50	10	50	10
3	200	1×10^6	300	20	20	50	10	50	50
4	200	1×10^6	300	10	50	50	10	50	50
5	200	1×10^6	300	10	20	50	10	10	50
6	200	1×10^6	300	10	20	100	10	50	50
7	200	1×10^6	300	10	20	50	5	50	50
8	200	3×10^6	300	10	20	50	10	50	50
9	200	1×10^7	300	10	20	50	10	50	50
10	400	1×10^6	300	10	20	50	10	50	50

b

c

Figure 2 Optimization of mRNA-EP condition for murine CD8+ T cells and chimeric antigen receptor (CAR) expression profile of mouse mV/m28/m3z CAR-T cells. Murine CD8+ T cells purified from splenocytes and lymph node cells were stimulated with anti-mCD3 and anti-mCD28 mAbs for 96 hours. The activated cells were electroporated with mRNA encoding mV/m28/m3z CAR. (**a**) Summary of mRNA-EP conditions for the optimization study. A CAR-mRNA amount of 100 μg was fixed. (**b**) CAR expression was assessed using immunofluorescent mVEGFR2-Fc chimera and detected by flow cytometric analysis (FCM) at 15 hours after mRNA-EP. The condition of No. 9 was selected as the optimal EP condition for murine CD8+ T cells. (**c**) CAR expression was assessed using immunofluorescent anti-HA antibody and detected by FCM at the indicated time after mRNA-EP under the condition of No. 9. The representative of three experiments is shown. In case of Rv transduction, murine splenocytes and lymph node cells were stimulated with anti-CD3 and anti-CD28 mAbs for 24 hours, and then the activated cells were transduced for 48 hours with Rv containing mV/m28/m3z CAR on a RetroNectin/anti-CD3/CD28 mAbs-coated plate. CAR expression was assessed using immunofluorescent anti-HA antibody and detected by FCM 4 days after Rv transduction. (**b** and **c**) All cells were pregated Aqua⁻ (live) CD8α+ cells. The dotted and solid histograms represent the staining of the mock cells and CAR-T cells, respectively. GMFI ratios were calculated as (GMFI of the CAR-T cells)/(GMFI of the mock cells) and are shown in parentheses at the left upper part of each histogram panel. Cell viabilities were determined by the trypan blue exclusion test and are shown in brackets at the right upper part of each histogram panel.

would have efficiently generated CAR-T cells from the PBMCs of every patient.

Furthermore, the impact of each process during the preparation of CAR-T cells by mRNA-EP on the original characteristics of the T cells was determined using phenotypic parameters. After the expansion of PBMCs containing naive CD8+ T cells expressing CD45RO⁻/CD45RA⁺, almost all CD8+ T cells changed their phenotypic pattern to CD45RO⁺/CD45RA⁻ (Figure 5a). The increase of CD44 expression and decrease of CD62L and CCR7 expression were also detected

after precultivation (Figure 5b), indicating that the expansion in the presence of anti-hCD3 mAb and hIL-2 activated CD8+ T cells from the naive phase to the effector phase. On the other hand, the magnetic-activated cell sorting (MACS) and mRNA-EP procedures did not induce an apparent change in the phenotypic pattern of CD8+ T cells, except for the further decrease in CD62L expression. The distinct increase in expression level of PD-1, the typical immunosuppressive molecule, was not observed in the prepared CAR-T cells compared with naive CD8+ T cells in PBMCs. This indicates the

4

possibility that our mRNA-EP protocol does not make human T cells fall into dysfunction state, such as exhaustion and senescence.

Taken together, these results indicate that although phenotypic parameters of CAR-T cells prepared by mRNA-EP shift to the effector phase due to the expansion from PBMCs, they maintain a healthy and appropriate quality suitable for use as cellular medicine in clinical research without the damage during the preparation process and the dysfunction or cell death from excess stimulation or activation.

Phenotypic parameters and functions of antigenic stimulated hV/h28/h3z and hV/h8a/h137/h3z CAR-T cells

Antigen-specific phenotypic change was analyzed on hV/h28/h3z and hV/h8a/h137/h3z CAR-T cells. Neither CAR-T cell showed any change in the expression pattern of CD45RO$^+$/CD45RA$^-$, that is, the effector phase, due to stimulation (Figure 6a). hVEGFR2-stimulation increased the activation marker (CD44 and PD-1) expression and memory marker (CD127) expression, as well as anti-hCD3 mAb-stimulation on both CAR-T cells, whereas the apparent change in

phenotypic parameters was not detected for non- or mVEGFR2-stimulation (Figure 6b). These results suggest that hV/h28/h3z and hV/h8a/h137/h3z CAR-T cells prepared by mRNA-EP would demonstrate effector functions in a hVEGFR2-specific manner.

Next, we analyzed the production profile of cytokine and cytotoxic molecules in hV/h28/h3z and hV/h8a/h137/h3z CAR-T cells associated with hVEGFR2-stimulation. On both CAR-T cells, the frequency of cells expressing functional molecules notably increased by hVEGFR2- or anti-hCD3 mAb-stimulation compared with non- or mVEGFR2-stimulation (Figure 7a). Although both cells showed almost the same profile of cytokine and CD107a expression associated with anti-hCD3 mAb-stimulation, hV/h28/h3z CAR-T cells responded more strongly to hVEGFR2-stimulation than did hV/h8a/h137/h3z CAR-T cells. Moreover, by hVEGFR2-stimulation, the frequency of cells co-expressing multiple functional molecules in hV/h28/h3z CAR-T cells was also higher than that in hV/h8a/h137/h3z CAR-T cells. On both CAR-T cells, the frequency of cells producing tumor necrosis factor (TNF)-α, IL-2, and/or CD107a increased
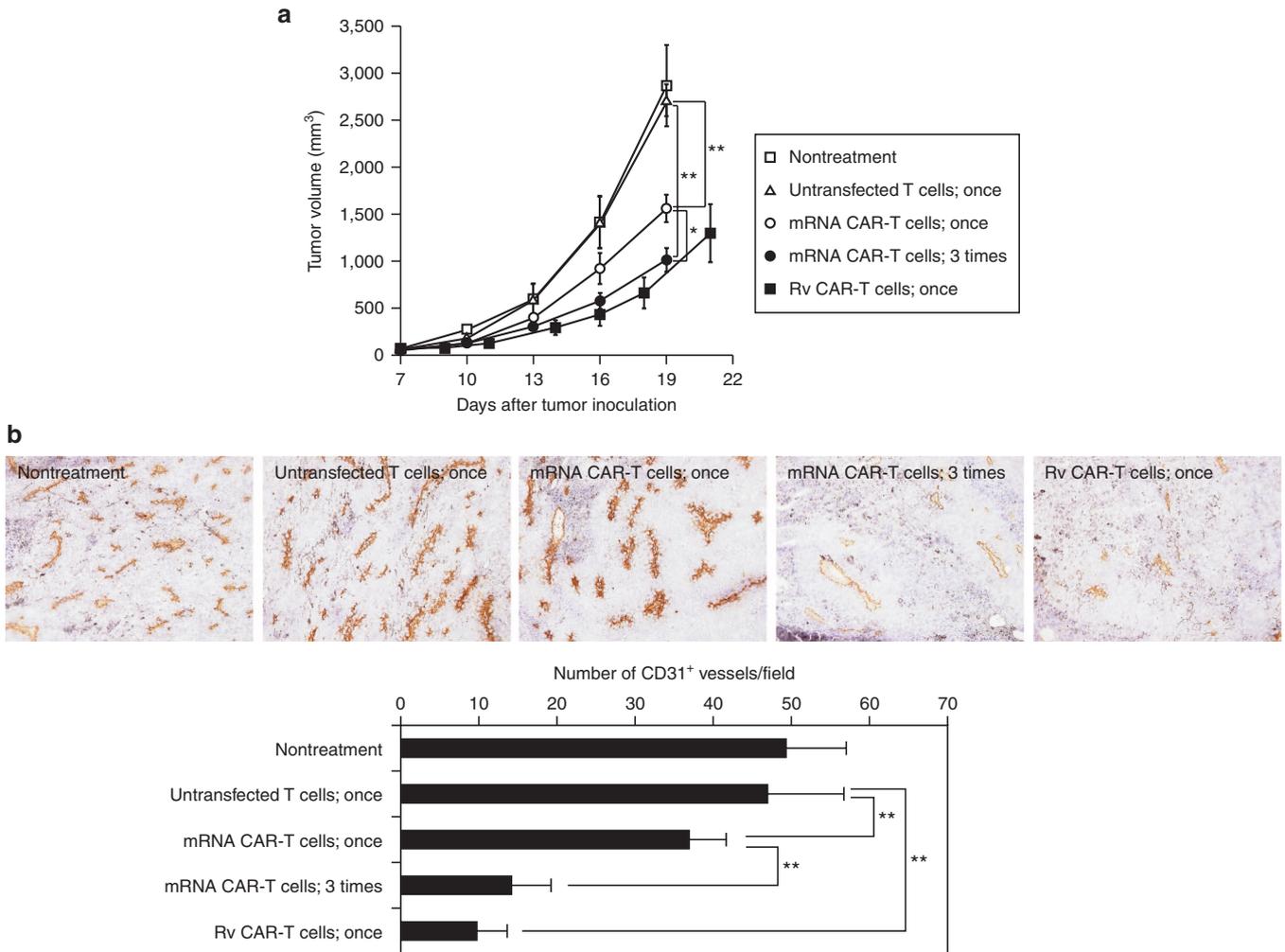
Figure 3 Antitumor effect and tumor vessel reduction by transfer of mV/m28/m3z chimeric antigen receptor (CAR)-T cells. C57BL/6 mice bearing B16BL6 tumors were intravenously injected with mRNA-transfected CAR-T cells, Rv-transduced CAR-T cells, or untransfected T cells at 5×10^6 cells/mouse on day 7 after tumor inoculation. In the case of triple transfer, mice were additionally injected with mRNA-transfected CAR-T cells on days 10 and 13. (**a**) Each point represents the mean ± SE of 3–11 mice (nontreatment: $n = 3$; untransfected T cells once: $n = 4$; mRNA CAR-T cells once: $n = 11$; mRNA CAR-T cells three times: $n = 3$; Rv CAR-T cells once: $n = 7$). Statistical analysis was performed using one-way analysis of variance: **$P < 0.01$, *$P < 0.05$. (**b**) On day 15, immunohistochemical analysis for CD31$^+$ cells in tumor tissue was performed. Original magnifications of each image are ×400. The number of CD31$^+$ vessels in the intratumoral section was assessed by counting five fields per specimen under ×100-magnification. The data represent the mean ± SD of results from three tumors. Statistical analysis was performed using one-way analysis of variance: **$P < 0.01$.

at an early stage (< 6 hours) and then markedly decreased at 12 hours after the antigenic stimulation, whereas the frequency of cells producing interferon (IFN)-γ was detected constantly throughout the assay period. After 24 hours of antigenic stimulation, we measured the concentration of cytokines and granzyme-B in the culture supernatant of hV/h28/h3z and hV/h8a/h137/h3z CAR-T cells. CAR-T cells did not enhance the secretion of cytokines or granzyme-B by mVEGFR2-stimulation, and anti-hCD3 mAb-stimulation increased the secretion of IFN-γ, TNF-α, and granzyme-B in hV/h28/h3z and hV/h8a/h137/h3z CAR-T cells similarly (Figure 7b). hV/h28/h3z CAR-T cells stimulated with hVEGFR2 significantly promoted the secretion of IFN-γ, TNF-α, and IL-2, compared with those stimulated with anti-hCD3 mAb. However, hV/h8a/h137/h3z CAR-T cells showed a similar secretion profile between the hVEGFR2-stimulation and anti-hCD3 mAb-stimulation groups. In particular, the enhancement of IL-2 secretion was observed only in hV/h28/h3z CAR-T cells stimulated with hVEGFR2. Granzyme-B secretion was induced by hVEGFR2-stimulation in both kinds of CAR-T cells at the equivalent level, although anti-hCD3 mAb-stimulation was more effective than hVEGFR2-stimulation. These data reveal that the CD28-derived signal transduction domain (STD) of hV/h28/h3z CAR-T cells is the superior 2nd STD to CD137-derived STD of hV/h8a/h137/h3z CAR-T cells on cytokine secretion elicited by recognition of the target molecule.

In addition, we investigated the cytotoxic activity of hV/h28/h3z and hV/h8a/h137/h3z CAR-T cells by europium (Eu)-release assay using VEGFR2-transfectants. Both CAR-T cells could injure positive target cells, that is, hVEGFR2hi/NIH3T3 cells, but not negative target cells, that is, mVEGFR2hi/NIH3T3 cells (Figure 8a). These cytotoxic activities were equivalent to one another; that is, the structural difference of CAR did not affect cytotoxicity. The cytotoxic activity of hV/h28/h3z CAR-T cells was enhanced depending on the increase of the hVEGFR2 expression density of target cells, and it was attenuated in the absence of IL-2 (Figure 8b).

Collectively, these results demonstrate that hV/h28/h3z and hV/h8a/h137/h3z CAR-T cells prepared by mRNA-EP are promising for cellular medicine. They can specifically recognize hVEGFR2 and demonstrate effector functions, such as cytokine secretion and cytotoxicity, only after they receive hVEGFR2-stimulation. They are considered of value for clinical research use. Additionally, it was clarified that the difference of the 2nd STD greatly impacted the cytokine secretion level and that the cytotoxic activity caused by the signal of the CD3ζ-derived STD was equivalent in both CAR-T cells.
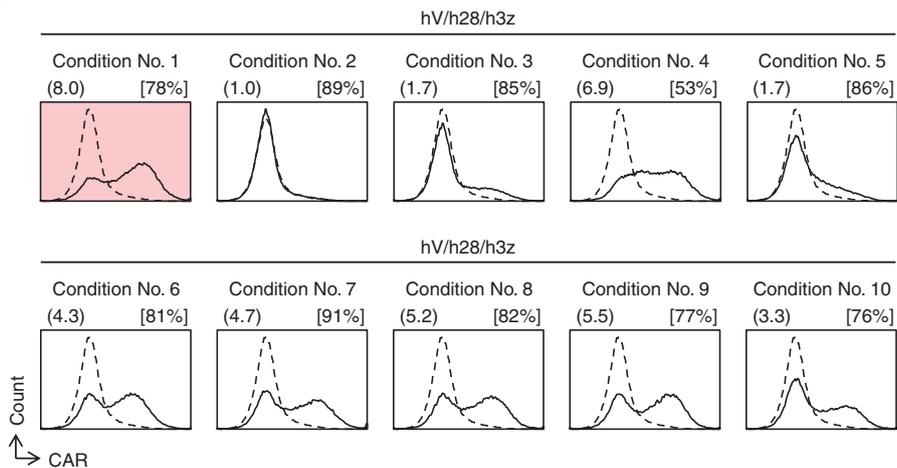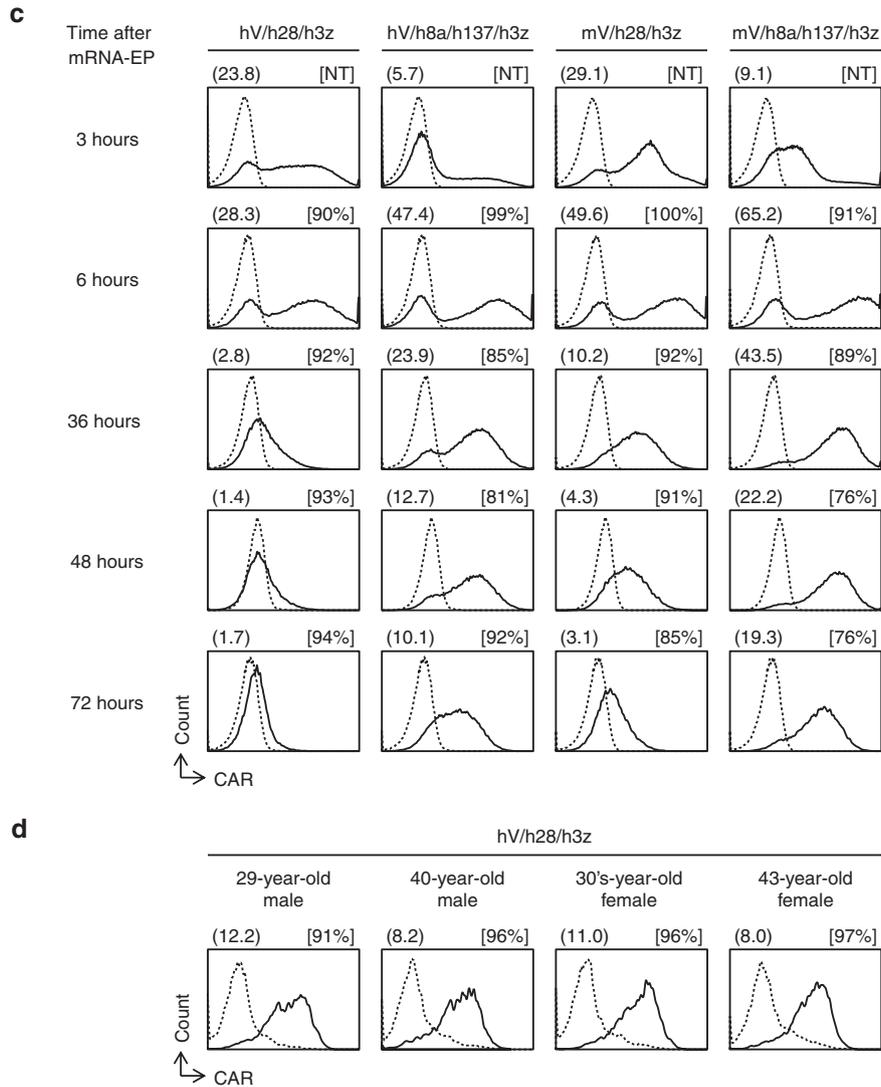
DISCUSSION

Clinical knowledge using CAR-T cells prepared by Rv or lentiviral vector transduction is being gradually accumulated, and CAR-T cell therapy has accomplished drastic development.[9,24,25] In Japan, clinical research in CAR-T cell therapy has been limited because of strict rules on gene therapy and long time periods are required for the toxicity evaluation of viral vectors. Therefore, to progress clinical research in CAR-T cell therapy including the VEGFR2-targeting

a

Condition No.	Volume (μl)	Cell number (cells/200 μl)	P_p voltage (V)	P_p duration (ms)	mRNA amount (μg/200 μl)
1	200	5×10^6	300	10	50
2	200	5×10^6	150	10	50
3	200	5×10^6	225	10	50
4	200	5×10^6	375	10	50
5	200	5×10^6	300	1.5	50
6	200	5×10^6	300	5	50
7	200	1×10^6	300	10	50
8	200	1×10^7	300	10	50
9	400	5×10^6	300	10	50
10	200	5×10^6	300	10	100

b

hV/h28/h3z

Condition No. 1 (8.0) [78%]
Condition No. 2 (1.0) [89%]
Condition No. 3 (1.7) [85%]
Condition No. 4 (6.9) [53%]
Condition No. 5 (1.7) [86%]

hV/h28/h3z

Condition No. 6 (4.3) [81%]
Condition No. 7 (4.7) [91%]
Condition No. 8 (5.2) [82%]
Condition No. 9 (5.5) [77%]
Condition No. 10 (3.3) [76%]

Count
CAR

Figure 4 Optimization of mRNA-EP condition for human CD8+ T cells and chimeric antigen receptor (CAR) expression profile of human CAR-T cells. PBMCs provided by volunteers were cultured with anti-hCD3 mAb-stimulation in media supplemented with recombinant hIL-2 and autologous plasma for 13 days. Human CD8+ T cells purified by MACS were electroporated with mRNA encoding hV/h28/h3z, hV/h8a/h137/h3z, mV/h28/h3z, or mV/h8a/h137/h3z. (**a**) Summary of mRNA-EP conditions for the optimization study. An interval between P_P and D_P of 50 ms, a D_P voltage of 50 V, a D_P frequency of three times, a D_P duration of 50 ms, and a D_P interval of 50 ms were fixed. (**b**) hV/h28/h3z CAR expression was assessed using immunofluorescent anti-HA antibody and detected by flow cytometric analysis (FCM) at 15 hours after mRNA-EP. The condition of No. 1 was selected as the optimal EP condition for human CD8+ T cells. (**c**) CAR expression was assessed using immunofluorescent anti-HA antibody and detected by FCM at the indicated time after mRNA-EP under the condition of No. 1. The representative of three experiments is shown. (**d**) Expression and antigen-binding ability of hV/h28/h3z CAR were assessed using immunofluorescent hVEGFR2-Fc chimera and detected by FCM at 36 hours after mRNA-EP under the condition of No. 1. (**b–d**) All cells were pregated Aqua⁻ (live) CD8α+ cells. The dotted and solid histograms represent the staining of the mock cells and CAR-T cells, respectively. Geometric mean fluorescence intensity (GMFI) ratios were calculated as (GMFI of the CAR-T cells)/(GMFI of the mock cells) and are shown in parentheses at the left upper part of each histogram panel. Cell viabilities were determined by the trypan blue exclusion test and are shown in brackets at the right upper part of each histogram panel. NT, not tested.

strategy in Japan, we must establish a CAR-T cell preparation technique that provides both safety and efficacy. In view of this background, we selected mRNA-EP, which reduces adverse risks, for CAR-T cell preparation because CAR-T cells prepared by this method are expected to time-dependently return to normal T cells, although CAR expression is transient. In the present study, we advance toward clinical research by analyzing the immunological characteristics and functions of tumor vessel-redirected CAR-T cells prepared by mRNA-EP.

In CAR-T cell preparation by mRNA-EP, none of the processes impaired T cell function and viability, and the individual differences in PBMCs (the source of CAR-T cells) did not have any influence on CAR expression level and intensity. The decrease in CD62L expression after mRNA-EP suggests that human CD8+ CAR-T cells differentiated to the effector phase, such as effector memory T cells and effector T cells. Although they might be short-lived after transfer to patients, we think that this is not a problem because CAR-T cells only have to show cytotoxicity to target cells, while mRNA CAR-T cells express CAR. The sufficient cytotoxicity during transient CAR expression is preferable to decrease the risk of on-target/off-tumor effects.[26] Importantly, the CAR expression level of murine mV/m28/m3z CAR-T cells prepared by mRNA-EP was sufficient to demonstrate significant antitumor efficacy in tumor-bearing mice. Persistent CAR expression by Rv or lentiviral vector transduction
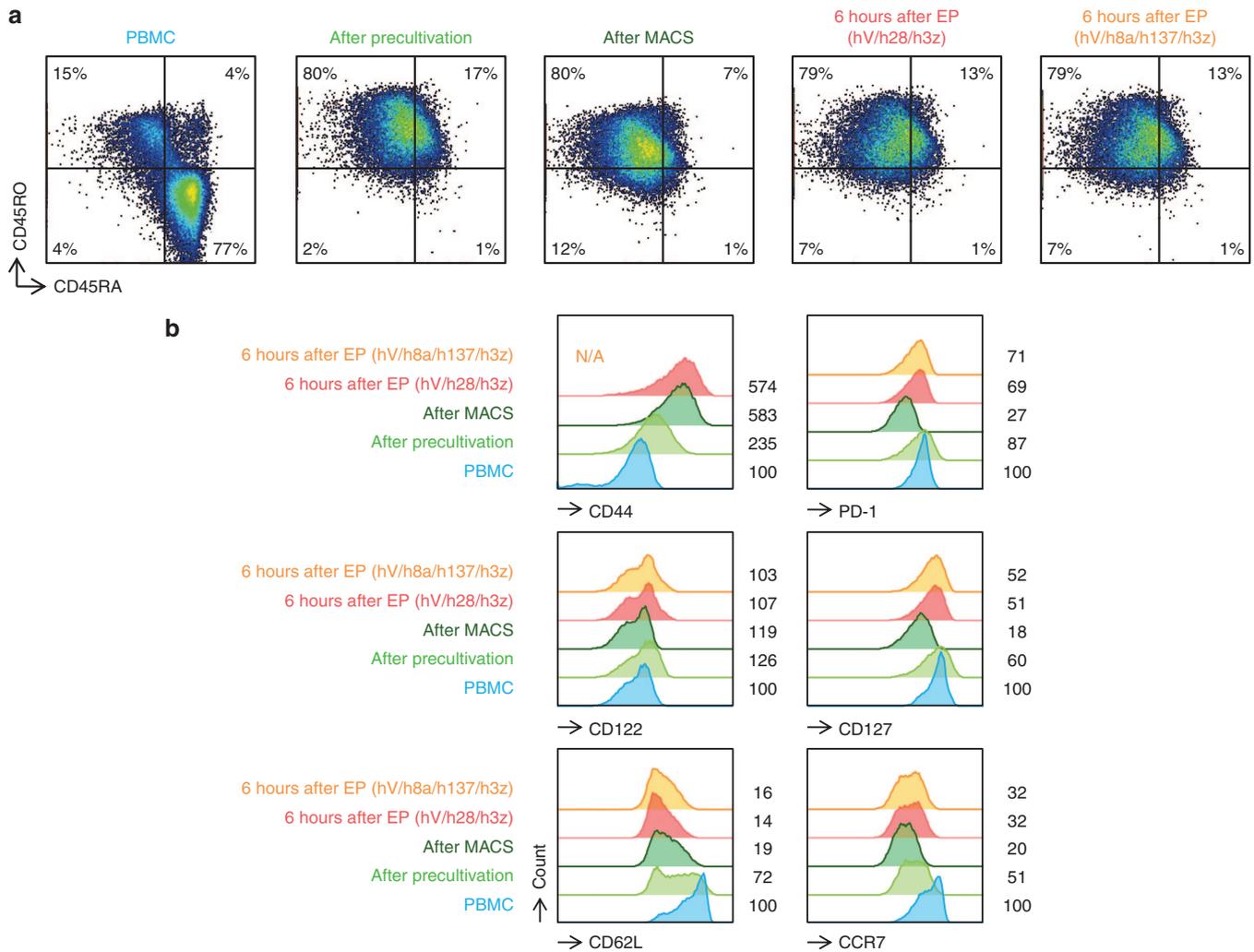
Figure 5 Phenotypic analysis at each step of chimeric antigen receptor (CAR)-T cell preparation using mRNA-EP. PBMCs, expanded PBMCs, MACS-purified CD8$^+$ T cells, and CAR-T cells 6 hours after mRNA-EP were stained with fluorescent immunoreagents for each surface molecule. Immunofluorescence, which was analyzed as the relative log fluorescence of gated Aqua$^-$ (live) CD8α^+ cells, was measured by flow cytometric analysis (FCM). (**a**) The percentage shown in each dot plot represents the quadrant frequency. (**b**) The value shown in the right part of each histogram represents the percentage of geometric mean fluorescence intensity to PBMC group. The representative of three experiments is shown.

requires the safety-net to remove transferred CAR-T cells in case a serious adverse event occurs,[27,28] although long-term functional CAR-T cells have advantages in terms of the reduction of transfer frequency and the exertion of memory function. On the other hand, the time-course loss of CAR expression in CAR-T cells prepared by mRNA-EP is conductive to the advantage that the unexpected on-target/off-tumor effect can be reduced or avoided by halting the transfer of CAR-T cells. Actually, the toxicities such as on-target/off-tumor toxicity and cytokine release syndrome by CAR-T cells prepared by virus vectors were previously reported.[29–31] In these case reports, authors insist the toxicities were caused by the persistent existing of CAR-expressing T cells. They suggested that the dose of CAR-T cells is decreased or the suicide dene is installed in advance to escape these toxicities for the safe clinical study. Because mRNA-EP CAR-T cells express CARs transiently, CAR expression is spontaneously lost time-dependently and CAR-T cells return to natural T cells without the installation of the suicide gene to CAR-T cells. Therefore the decrease of infusion dose is not needed, and the multiple dose infusion is also acceptable. CAR-T cells prepared by mRNA-EP method are expected to ameliorate these toxicities in clinical study. However, because the functional period of

CAR-T cells depends on the CAR expression period, we should establish the relevant mechanisms, such as the stabilization of mRNA[32] and the improvement of the translation efficiency from mRNA,[33,34] to control the CAR expression period in the mRNA-EP protocol. Furthermore, the establishment of a large-scale and stable preparation method that conforms with GMP/GCP standards and the selection of an appropriate T cell source, such as young T cells[35,36] and allo-T cells,[37] are required for the clinical research use of CAR-T cells prepared by mRNA-EP. For example, Krug *et al.* previously reported on the GMP-compliant protocol for the CAR-T cell preparation method by mRNA-EP.[38] The CAR expression profiles in reports of Krug *et al.* or Zhao *et al.*[39,40] were different from those of our mRNA CAR-T cells because their CAR-T cells were prepared using a different CAR construct, a different EP device, and different EP conditions. For feasible, highly reproducible, and GMP-compliant preparation methods for various CAR-T cells with different target molecules or CAR constructs, the establishment of a solid basis for the preparation method is strongly required.

After the significant clinical effects of CAR-T cell therapy targeting CD19 for hematological malignancies were reported,[31,41–45] the expectations for this approach continue to rise in cancer
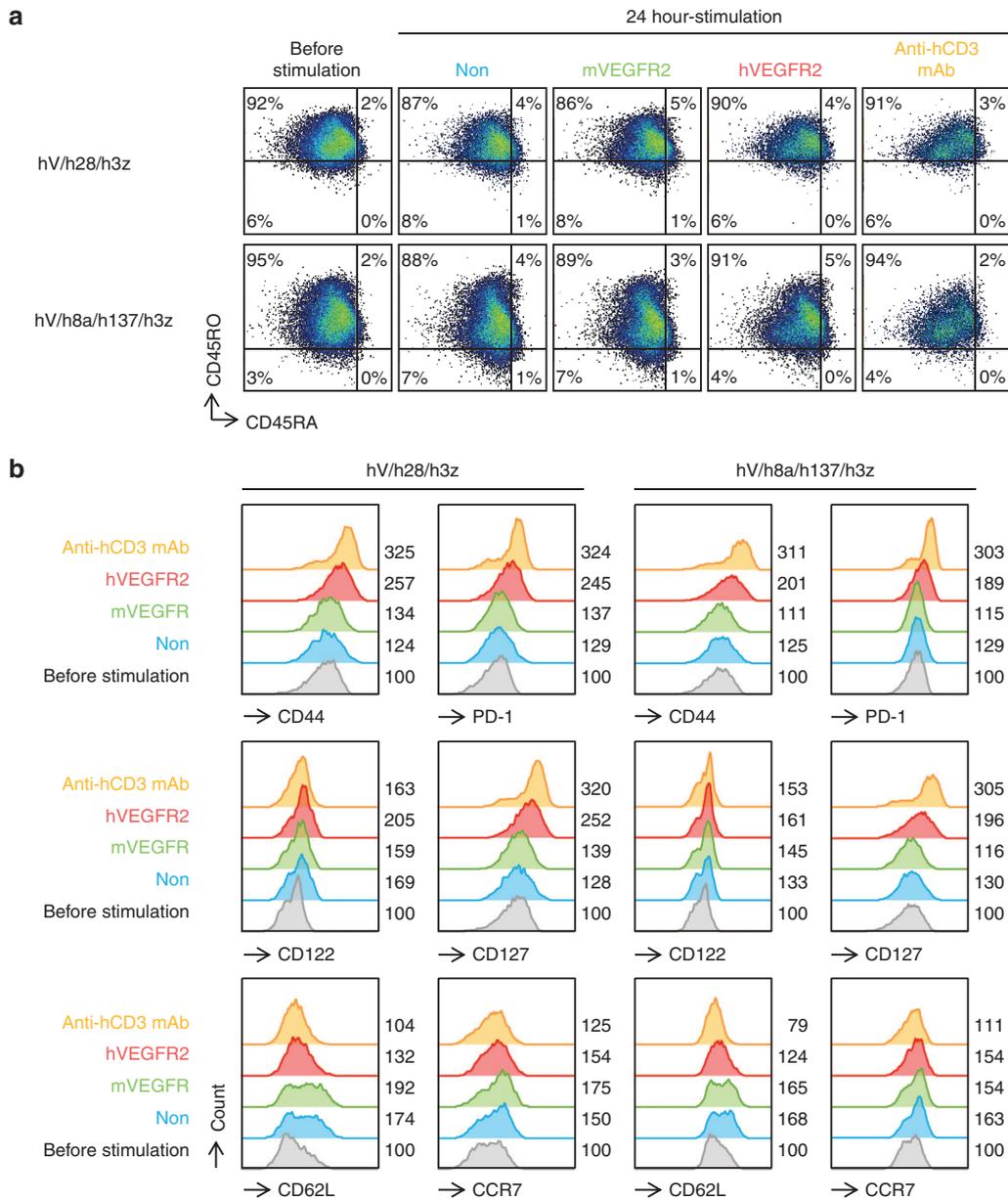
Figure 6 Antigen-specific phenotypic changes of hVEGFR2-specific chimeric antigen receptor (CAR)-T cells prepared by mRNA-EP. hV/h28/h3z or hV/h8a/h137/h3z CAR-T cells 6 hours after mRNA-EP were stimulated with anti-hCD3 mAb, hVEGFR2-Fc chimera, or mVEGFR2-Fc chimera for 24 hours. Cells were stained with fluorescent immunoreagents for each surface molecule. Immunofluorescence, which was analyzed as the relative log fluorescence of gated Aqua⁻ (live) CD8α⁺ cells, was measured by flow cytometric analysis. (**a**) The percentage shown in each dot plot represents the quadrant frequency. (**b**) The value shown in the right part of each histogram represents the percentage of geometric mean fluorescence intensity to the group of before stimulation. The representative of three experiments is shown.

immunotherapy research.⁹ However, in relation to producing optimal CAR capability, it has been recently argued that not only the precise and provident choice of target molecules for CAR, but also the establishment of the methodology for design and tuning,⁴⁶⁻⁵¹ are important to prevent or avoid adverse effects such as on-target/off-tumor toxicity and cytokine release syndrome.³⁰,⁵² By comparing the CAR expression profile on four kinds of CAR derivatives, we found that CAR including CD8α-derived TMD was expressed in human CD8⁺ T cells for a longer period than CAR including CD28-derived TMD. This difference in CAR expression profile might have an influence on the antitumor effect after *in vivo* transfer. In this study, the phenotypic parameters and functions of hV/h28/h3z and hV/h8a/h137/h3z CAR-T cells were compared 6 hours after

mRNA-EP, when both CAR-T cells expressed equivalent CAR on their surfaces. The hVEGFR2-responding cytokine secretion ability of hV/h28/h3z CAR-T cells was obviously superior to that of hV/h8a/h137/h3z CAR-T cells, whereas the cytotoxic activity against hVEGFR2-expressing cells was equivalent in both CAR-T cells. As these results suggest, we can easily predict that the CAR expression stability on a plasma membrane, and the signal strength and function of CAR associated with target recognition, would be greatly affected by the structure modification of CAR. However, in current research on CAR-T cell therapy, the structure analysis approach, which is intended for the optimization of the CAR function, is not often used, and the establishment of methodology to systematically analyze the relationship between CAR structure and function
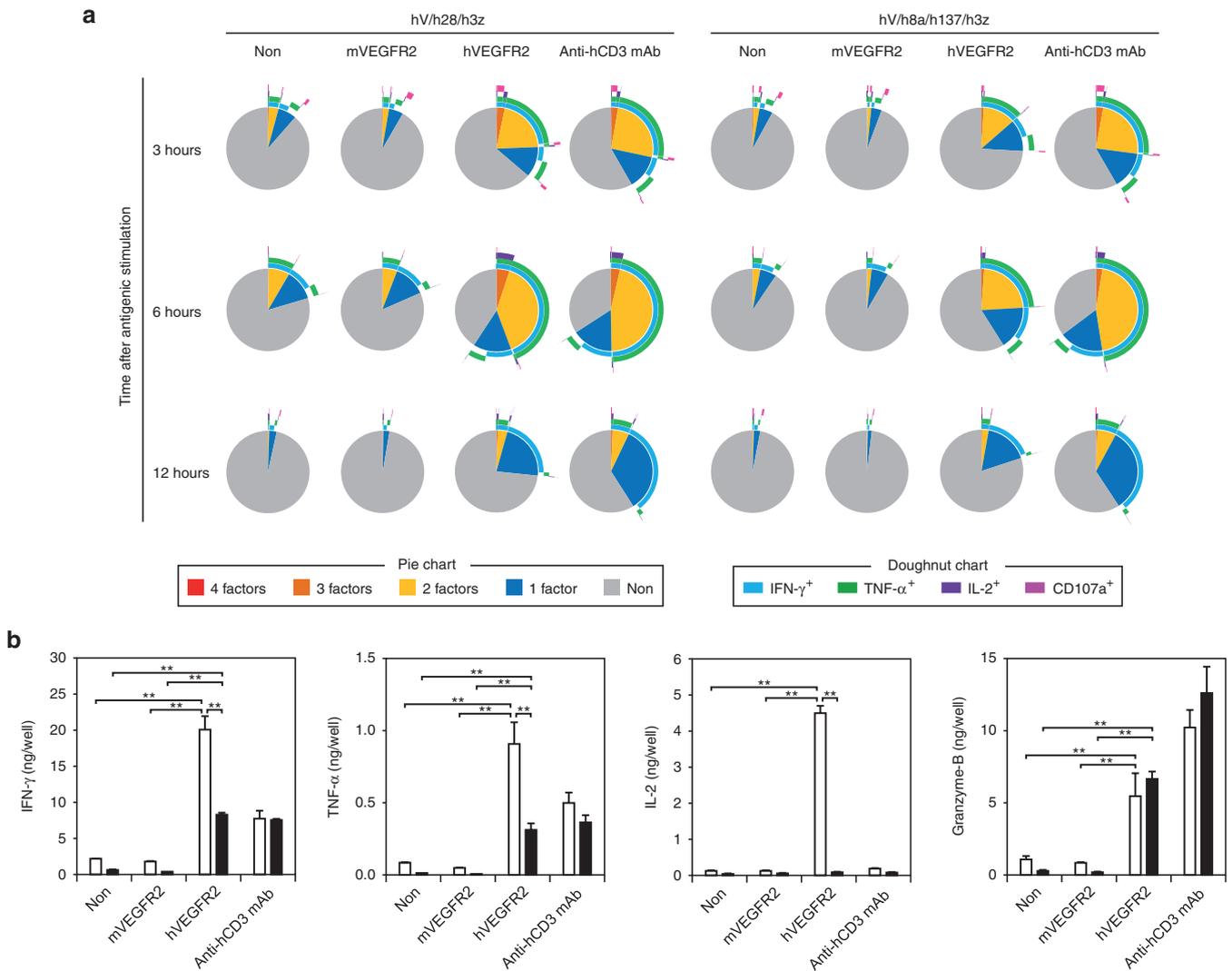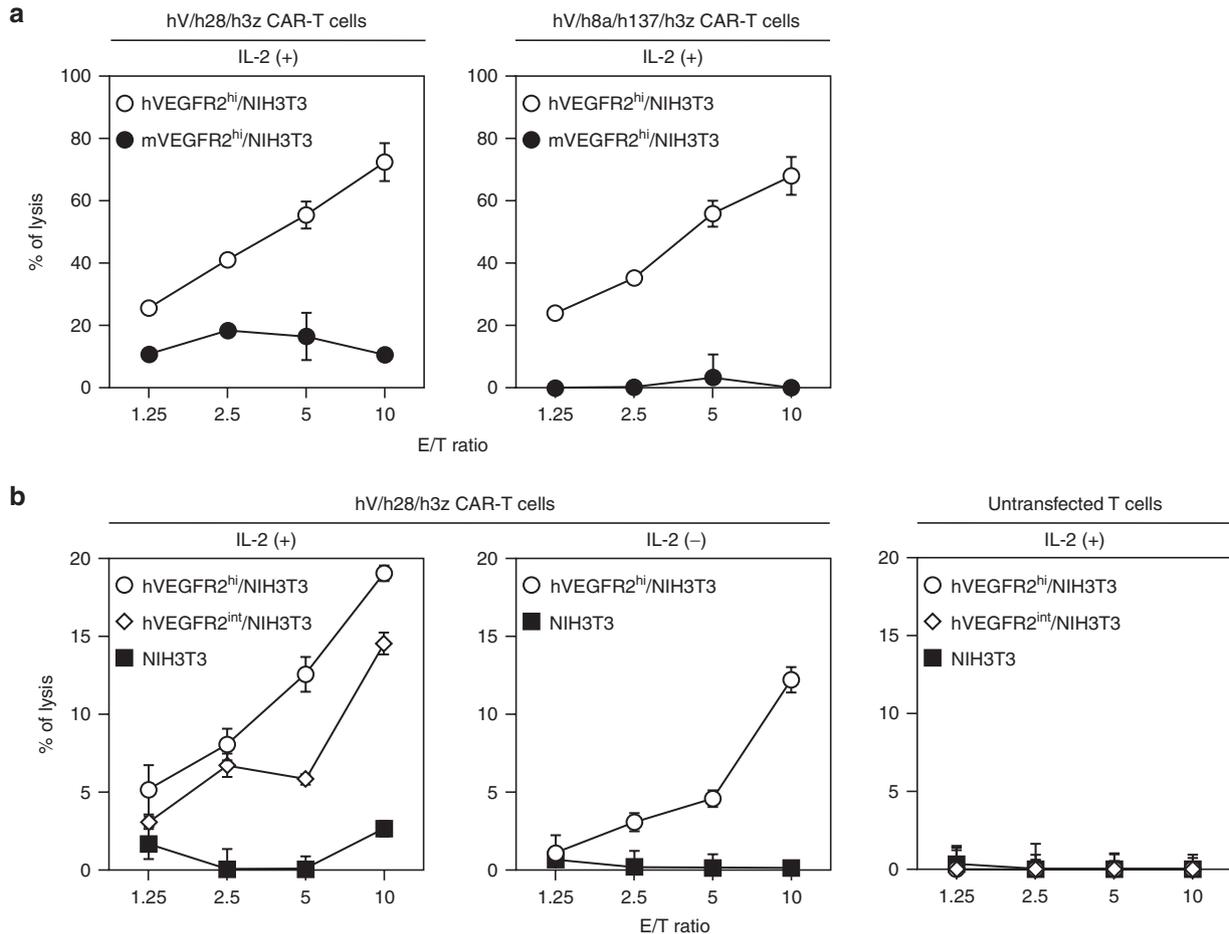
Figure 7 Antigen-specific cytokine production of hVEGFR2-specific chimeric antigen receptor (CAR)-T cells prepared by mRNA-EP. hV/h28/h3z or hV/h8a/h137/h3z CAR-T cells 6 hours after mRNA-EP were stimulated with anti-hCD3 mAb, hVEGFR2-Fc chimera, or mVEGFR2-Fc chimera for the indicated hours (**a**) or 24 hours (**b**). (**a**) Cells were stained with fluorescent immunoreagents for each intracellular cytokine or CD107a. Immunofluorescence, which was analyzed as the relative log fluorescence of gated Aqua$^-$ (live) CD8α^+ cells, was measured by flow cytometric analysis. The pie chart shows the frequency of multifunctional CAR-T cells, and the donut chart shows the frequency of each factor-positive cell. The representative of two experiments is shown. (**b**) The secretion amount of IFN-γ, TNF-α, IL-2, or granzyme-B from hV/h28/h3z (white bar) or hV/h8a/h137/h3z (black bar) CAR-T cells after 24-hour stimulation was determined by ELISA. Data are expressed as the mean ± SD of three cultures. Statistical analysis was performed using one-way analysis of variance: **$P < 0.01$.

is not forthcoming. Assay systems capable of analyzing the expression level, affinity, and signal strength of CAR as a linkage with CAR structure are required for designing and generating optimal CAR-T cells with desirable functions such as cytotoxic activity, cytokine release, and *in vivo* persistence. Systematic integration of fundamental knowledge on the CAR structure/function relationship might enable replacement of the past experience-based approach with a rational approach using CAR's structural information for CAR design and construction. This would aid the improvement of efficacy and the reduction of adverse effects.

In the tumor vessel-redirected CAR-T cell therapy using mRNA-EP, it was indicated that frequent transfer of CAR-T cells may be necessary to attain an excellent response rate. Therefore, for framing of the clinical research protocol, we must strictly establish the

cell number, interval, and frequency associated with the transfer of CAR-T cells prepared with mRNA-EP. This is sufficient and optimal for verifying not only safety but also efficacy. Also, to stably supply CAR-T cells for multiple-dose infusion, we require the establishment of an adequate cryopreservation method for PBMCs (the source of CAR-T cells)[53] and CAR-T cells.[54] Furthermore, the progress of strategies such as combination with other therapeutic methods, especially immunotherapy,[55] and the use of multiple-targetable CAR-T cells[23] will be expected to reduce the cell number and infusion frequency in CAR-T cell therapy.

In conclusion, we demonstrated that mRNA-EP was an appropriate method for clinical CAR-T cell preparation in terms of both safety and efficacy, and that tumor vessel-redirected CAR-T cells prepared by mRNA-EP were a promising cellular medicine

Figure 8 Cytotoxic activity of hVEGFR2-specific chimeric antigen receptor (CAR)-T cells prepared by mRNA-EP. hV/h28/h3z CAR-T cells (**a** and **b**), hV/h8a/h137/h3z CAR-T cells (**a**), or untransfected T cells (**b**) 6 hours after mRNA-EP were cocultured with the indicated target cells labeled with Eu for 4 hours at the indicated effector-to-target ratios in ALyS505N-175 [IL-2 (+)] or ALyS505N-0 [IL-2 (-)]. Specific cytolysis was measured by the Eu-release assay. Each point represents the mean ± SD ($n = 3$).

in which quality and performance have the potential. If CAR-T cell medicine that acts on tumor vessel injury is put to practical use following conventional low molecular medicine and antibody-based biopharmaceuticals, we can obtain a new cancer treatment option that is superior in terms of versatility for solid tumors. By incorporating this approach into a multidisciplinary treatment, it could greatly contribute to the improvement of the response rate for refractory cancer.

MATERIALS AND METHODS

Cell line and mice

B16BL6 cells were kindly provided by Mochida Pharmaceutical (Tokyo, Japan) and cultured in minimum essential medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. NIH3T3 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). NIH3T3 cells highly expressing mVEGFR2 or hVEGFR2 (mVEGFR2hi/NIH3T3 cells or hVEGFR2hi/NIH3T3 cells) and intermediately expressing hVEGFR2 (hVEGFR2int/NIH3T3 cells) were established by transduction with Rv carrying mVEGFR2 cDNA (Genbank accession no. BC020530) or hVEGFR2 cDNA (Genbank accession no. NM_002253) and cloning using the limiting dilution method. They were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotics. Female C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan) and maintained in the experimental animal facility at Osaka University. Animal experiments were conducted in accordance with the guidelines provided by the Animal Care and Use Committee of Osaka University.

CAR mRNA preparation

We designed five types of CAR mRNA (Figure 1). These were generated using the mMESSAGE mMACHINE T7 Ultra kit (Ambion, Austin, TX). Each CAR mRNA commonly contains anti-reverse a cap analog (ARCA), an Igκ-chain leader sequence, an HA tag for detecting CAR, and a poly(A) tail. mVEGFR2-specific CAR mRNAs (mV/m28/m3z, mV/h28/h3z, and mV/h8a/h137/h3z) possess anti-mVEGFR2 single-chain variable fragment (scFv) derived from Avas12α1 hybridoma,[23,56] and hVEGFR2-specific CAR mRNAs (hV/h28/h3z and hV/h8a/h137/h3z) include anti-hVEGFR2 scFv created by scFv phage display technology.[57] Murine CD28 (mCD28) TMD-STD and murine CD3ζ (mCD3ζ) STD for mV/m28/m3z CAR mRNA, human CD28 (hCD28) TMD-STD and hCD3ζ STD for hV/h28/h3z and mV/h28/h3z CAR mRNAs, and human CD8α (hCD8α) TMD, human CD137 (hCD137) STD, and hCD3ζ STD for hV/h8a/h137/h3z and mV/h8a/h137/h3z CAR mRNAs were tandemly linked next to scFv. A plasmid encoding the gene fragment with hCD8α TMD and hCD137 STD was kindly provided by Prof. Dario Campana (Department of Paediatrics, National University of Singapore, Singapore).[58]

CAR mRNA-EP into CD8+ T cells

Murine CD8+ T cells, which were purified from splenocytes and lymph node cells by MACS using a murine CD8+ T cell isolation kit and autoMACS (Miltenyi Biotec, Tokyo, Japan) according to the manufacturer's specifications, were activated for 96 hours on a 6-well plate (Sumitomo Bakelite, Tokyo, Japan) coated with anti-mCD3 mAb (5 μg/ml, clone 145.2C11, Bio X Cell, West Lebanon, NH) and anti-mCD28 mAb (2 μg/ml, clone 37.51, eBioscience, San Diego, CA) at 1×10^6 cells/well in RPMI 1640 medium supplemented with 10 U/ml mIL-2 (PeproTech, Rocky Hill, NJ), 10% FBS, 50 μmol/l 2-mercaptoethanol, and a non-essential amino acid solution. This medium was used as cRPMI medium for the cultured murine T cells.

PBMCs provided by volunteers were cultured for 13 days with an immobilized anti-hCD3 mAb (5 μg/ml, clone OKT3, Janssen Pharmaceutical K.K., Tokyo, Japan) in ALyS505N-175 (Cell Science & Technology Institute, Tokyo, Japan) supplemented with autologous plasma. Human CD8$^+$ T cells were purified from activated PBMCs by a human CD8$^+$ T cell isolation kit (Miltenyi Biotec) and autoMACS according to the manufacturer's specifications. Murine or human activated CD8$^+$ T cells were suspended with Opti-MEM (Thermo Fisher Scientific, Waltham, MA), and the cell suspension and CAR mRNA in nuclease-free water were mixed at the volume ratio of 7:1 in a 4 mm cuvette (BEX, Tokyo, Japan). Then EP was conducted using CUY21Pro-Vitro (Nepagene, Chiba, Japan). As a result of changing the parameter of the EP condition variously, the optimal conditions were the following conditions: a cell number of 1×10^7 cells for murine CD8$^+$ T cells, or 5×10^6 cells for human CD8$^+$ T cells; a CAR-mRNA amount of 100 μg for murine CD8$^+$ T cells, or 50 μg for human CD8$^+$ T cells; a P_p voltage of 300 V; a P_p duration of 10 ms; an interval between P_p and D_p of 50 ms; a D_p voltage of 20 V for murine CD8$^+$ T cells, or 50 V for human CD8$^+$ T cells; a D_p frequency of 3 times; a D_p duration of 50 ms; and a D_p interval of 50 ms. mRNA-transfected CAR-T cells were cultured in cRPMI or ALyS505N-175.

Flow cytometric (FCM) analysis for surface markers and CAR expression

Cells were incubated with anti-murine CD16/CD32 mAb (clone 2.4G2, purified from hybridoma supernatants) or human FcR Blocking Reagent (Miltenyi Biotec), and then stained with Live/Dead Fixable Aqua viability dye (Thermo Fisher Scientific), fluorescent-labeled mAbs for anti-murine CD8α (clone 53-6.7, eBioscience, San Diego, CA), anti-hCD8α (clone OKT-8, eBioscience), anti-human CD45RA (clone HI100, eBioscience), anti-human CD45RO (clone UCHL1, BioLegend, San Diego, CA), anti-human CD44 (clone G44-26, eBioscience), anti-human PD-1 (clone eBioJ105, eBioscience), anti-human CD122 mAb (clone TU27, BioLegend), anti-human CD127 (clone eBioRDR5, eBioscience), anti-human CD62L (clone DREG-56, BioLegend), and anti-human CCR7 (clone 150503, BD Biosciences, San Jose, CA) in phosphate buffer saline (PBS) containing 0.5% FBS and 0.05% sodium azide. CAR expression was evaluated using anti-HA antibody (Miltenyi Biotec). In some cases, CAR was stained with recombinant mVEGFR2- or hVEGFR2-Fc chimera (R&D Systems, Minneapolis, MN), biotin-labeled anti-human IgG Fc antibody (eBioscience), and fluorescent-labeled streptavidin (BD Biosciences). Immunofluorescence, which was analyzed as the relative log fluorescence of gated Aqua$^-$ (live) CD8α$^+$ cells, was measured using a Gallios flow cytometer (Beckman Coulter, Tokyo, Japan), and data were analyzed using the FlowJo software (TreeStar, Ashland, OR). The CAR expression level was expressed in GMFI (geometric mean fluorescence intensity) ratio, calculated according to the following formula: GMFI ratio = GMFI of CAR-T cells / GMFI of mock CD8$^+$ T cells. The expression intensity of surface markers was expressed as the percentage of GMFI to control cells.

Evaluation of anti-tumor efficacy and tumor vessel attack of murine CAR-T cells

C57BL/6 mice were intradermally inoculated with 3×10^5 B16BL6 cells into the flank. Seven days later, mice bearing tumors with diameters of 5.0–6.0 mm were intravenously injected with 5×10^6 CAR-T cells that were generated by EP of mV/m28/m3z CAR and then cultured for 6 hours. In cases of triple infusion, mice were additionally injected with mRNA-transfected CAR-T cells on days 10 and 13. Similarly, CAR-T cells prepared by Rv transduction, as previously described,[23,59] or untransfected T cells were intravenously injected into tumor-bearing mice on day 7. Tumor growth was monitored by measuring the major and minor axes of the tumors using microcalipers, and tumor volume was calculated according to the following formula: tumor volume; mm^3 = major axis; mm $\times$ (minor axis; mm)$^2 \times 0.5236$. Mice bearing tumors with diameters over 20 mm were euthanized.

Immunohistochemical analysis was utilized to determine CD31$^+$ endothelial cells in B16BL6 tumors of treated mice. On day 15, the tumor nodules were harvested, embedded in Tissue-Tek O.T.C. Compound (Sakura Finetek Japan, Tokyo, Japan), and stored at −80 °C. Frozen thin sections (6 μm in thickness) of the nodules were fixed in 4% paraformaldehyde in PBS and washed with PBS, and then were incubated with 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature to block endogenous peroxidase activity. The sections were preincubated with 5% bovine serum albumin in PBS and sequentially incubated with optimal dilution of primary antibody, rat

anti-murine CD31 mAb (clone MEC 13.3; BD Biosciences, Franklin Lakes, NJ). The primary antibody bound was detected with horseradish peroxidase-conjugated goat anti-rat Ig polyclonal antibody (Santa Cruz Biotechnology Dallas, TX). Each of the incubation lasted for 15 minutes at room temperature and was followed by three times repeat of a 15-minute wash in Tris-buffered saline (pH 7.6). Subsequently, catalytic signals were amplified with CSA II, Biotin-Free Catalyzed Amplification System (Dako Japan, Tokyo, Japan). The sections were stained with 3,3-diaminobenzidine tetrahydrochloride solution, and finally counterstained with hematoxylin. The number of immunostained vessels was counted under a light microscope with ×100 magnification. For counting the CD31$^+$ cell number, five fields per section were randomly selected.

Antigenic stimulation of human CAR-T cells

The mRNA-transfected human CAR-T cells, which were cultured for 6 hours after mRNA-EP, were stimulated on a 24-well plate coated with anti-hCD3 mAb (2 μg/ml, clone OKT3), hVEGFR2-Fc chimera (2 μg/ml), or mVEGFR2-Fc chimera (2 μg/ml) at 5×10^5 cells/well in ALyS505N-0 (Cell Science & Technology Institute) or ALyS505N-175.

Expression analysis of cytokines and cytotoxic factors

The mRNA-transfected human CAR-T cells, which were stimulated with antigenic proteins for 3, 6, or 12 hours, were pulsed with BD Golgiplug (BD Biosciences) and BD Golgistop (BD Biosciences) for the last 3–6 hours of antigenic stimulation in ALyS505N-175 and then fixed with BD Cytofix/Cytoperm (BD Biosciences) overnight at 4 °C. Cells were stained with Live/Dead Fixable Aqua viability dye, fluorescent-labeled mAbs for anti-human IFN-γ (clone 4S.B3, eBioscience), anti-human TNF-α (clone MAb11, eBioscience), anti-hIL-2 (clone MQ1-17H12, eBioscience), and anti-human CD107a (hCD107a) (clone H4A3, eBioscience). The frequency of each factor-positive cell in the gated Aqua$^-$ CD8α$^+$ cells was analyzed by FCM, as described above. The amount of IFN-γ, TNF-α, IL-2, and granzyme-B secreted from the mRNA-transfected human CAR-T cells after 24 hours stimulation in ALyS505N-0 was determined by the Human IFN-γ ELISA Set (BD Biosciences), the Human TNF ELISA Set (BD Biosciences), the Human IL-2 ELISA Kit II (BD Biosciences), and the Human Granzyme-B ELISA development kit (Mabtech, Cincinnati, OH).

Evaluation of cytotoxic activity

The mRNA-transfected human CAR-T cells, which were cultured for 6 hours after mRNA-EP, were harvested as effector cells. The mVEGFR2^{hi}/NIH3T3 cells, hVEGFR2^{hi}/NIH3T3 cells, hVEGFR2^{int}/NIH3T3 cells, and NIH3T3 cells were target cells and were labeled with Eu. The effector cells were cocultured with 1×10^4 target cells in ALyS505N-0 or ALyS505N-175 on a V-bottom 96-well plate (Thermo Fisher Scientific) at various effector-to-target ratios for 4 hours. After co-culture, 50 μl of supernatant was mixed with 150 μl of DELFIA enhancement solution (Perkin Elmer, Wellesley, MA), and time-resolved fluorescence was subsequently measured. The percentage of lysis was calculated using the following formula: (experimental release − spontaneous release)/(maximal release − spontaneous release) × 100, where spontaneous release indicates Eu released from Eu-labeled target cells co-cultured with no effector cells, and maximal release indicates Eu released from Eu-labeled target cells lysed using 0.5% Triton-X.

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