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ORIGINAL ARTICLE

Lenalidomide enhances the function of chimeric antigen receptor T cells against the epidermal growth factor receptor variant III by enhancing immune synapses

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The epidermal growth factor receptor variant III (EGFRvIII) is exclusively expressed on the cell surface in ~50% of glioblastoma multiforme (GBM). This variant strongly and persistently activates the phosphatidylinositol 3-kinase-Akt signaling pathway in a ligand-independent manner resulting in enhanced tumorigenicity, cellular motility and resistance to chemoradiotherapy. Our group generated a recombinant single-chain variable fragment (scFv) antibody specific to the EGFRvIII, referred to as 3C10-scFv. In the current study, we constructed a lentiviral vector transducing the chimeric antigen receptor (CAR) that consisted of 3C10-scFv, CD3ζ, CD28 and 4-1BB (3C10-CAR). The 3C10-CAR-transduced peripheral blood mononuclear cells (PBMCs) and $CD3^+$ T cells specifically lysed the glioma cells that express EGFRvIII. Moreover, we demonstrated that CAR $CD3^+$ T cells migrated to the intracranial xenograft of GBM in the mice treated with 3C10-CAR PBMCs. An important and novel finding of our study was that a thalidomide derivative lenalidomide induced 3C10-CAR PBMC proliferation and enhanced the persistent antitumor effect of the cells *in vivo*. Lenalidomide also exhibited enhanced immunological synapses between the effector cells and the target cells as determined by CD11a and F-actin polymerization. Collectively, lentiviral-mediated transduction of CAR effectors targeting the EGFRvIII showed specific efficacy, and lenalidomide even intensified CAR cell therapy by enhanced formation of immunological synapses.

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INTRODUCTION

Glioblastoma multiforme (GBM) is the most common malignant brain tumor in adults and the median overall survival time is < 15 months,[1,2] despite the currently available multimodal therapy. Thus far, a variety of immunotherapeutic approaches including cancer vaccines and antibody-mediated and cell-based therapies have been tested to treat cancers.[3–5] However, these therapies have major drawbacks such as major histocompatibility complex restriction, nonspecific killing and technical difficulties for expansion *ex vivo*.[5]

The epidermal growth factor receptor (EGFR) is amplified and overexpressed in 40–60% of GBM.[6–8] One of the most effective immunotherapeutic antigens is the EGFR variant III (EGFRvIII), with an in-frame deletion of 801 base pairs in the extracellular domain. EGFRvIII is expressed on the cell surface exclusively in GBM, but not in normal tissues.[6–9] This variant of EGFR strongly and persistently activates the phosphatidylinositol 3-kinase-Akt signaling pathway in a ligand-independent manner,[10,11] resulting in enhanced tumorigenicity, cellular motility[8,12–14] and resistance to chemotherapy[15] and/or radiation.[16,17] Our group generated the mouse monoclonal antibody 3C10 and a recombinant single-chain variable fragment (scFv) antibody that specifically recognizes the *de novo* glycine residue of EGFRvIII.[18,19] Further, we constructed human T cells that expressed chimeric antigen receptor (CAR) targeting the EGFRvIII antigen (3C10-CAR).[20,21]

CARs are genetically constructed from scFvs connected by a transmembrane hinge region, the T-cell signaling complex, and costimulatory signaling domains (e.g. CD3ζ, CD28, 4-1BB and/or OX-40).[22,23] Moreover, CARs have theoretical and technical advantages, such as the ability to recognize specific antigens without the need for major histocompatibility complex-restricted presentation, a process that is often downregulated in GBM,[24,25] and production of an adequate quantity of tumor-specific effector cells in a short period of time.[26] Although CARs may provide many benefits, the obstacle posed by cancer cells' immune escape mechanisms remains, and overcoming these mechanisms is extremely important for the optimization of all types of immunotherapy.[27]

Lenalidomide, which is a synthetic derivative of thalidomide, has multifaceted immunomodulatory efficacies; perhaps, the most remarkable of these is the restoration and enforcement of immune synapse formation between T-cell and antigen-presenting cells.

In the present study, we focused on the immunomodulatory abilities of lenalidomide, especially the augmentation of immunological synapses, and investigated the efficacy of the potentiation of EGFRvIII-targeting CAR T-cell therapy for GBM.

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MATERIALS AND METHODS

Cell lines

The human GBM cell line U87-EGFRvIII stably expressing EGFRvIII and its parental U87MG cell lines were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA) and penicillin/streptomycin (Life Technologies) at 37 °C with 5% CO_2. U87-EGFRvIII cells expressing luciferase (U87-EGFRvIII-LUC) stably expressing luciferase were generated by transduction with a recombinant lentivirus coding for the luciferase of *Photinuspyralis*. This lentivirus was kindly provided by Dr Stephen H Throne (University of Pittsburgh, Pittsburgh, PA, USA). Two days after transduction, the cells were selected in blasticidin for 2 weeks and were cloned by limiting dilution thereafter.

Construction of a third-generation 3C10-CAR with a lentiviral vector

The monoclonal antibody 3C10 was originally established using mice immunized against a synthetic 14-amino-acid peptide named Pep3, which was characteristically created at the EGFRvIII-specific fusion junction between amino-acid residues, including a novel glycine residue. Its corresponding scFv antibody was then produced. Gene sequence data for the third-generation CAR was kindly provided by Dr Carl June (University of Pennsylvania, Philadelphia, PA, USA).[21] Using this gene sequence data, 3C10-CAR cDNA was generated by gene synthesis (Genscript, Piscataway, NJ, USA). The mock vector was designed to harbor the scrambled sequence of the scFv portion that has shown no functional activity against glioma, breast cancer, colon cancer and pancreatic cancer cell lines.

Preparation of lentiviral vector

Human embryonic kidney 293T cells (8×10^6) were cultured in 175-cm^2 flasks. At 24 h, self-inactivating vectors, pMDLg/pRRE, pRSV-Rev and pMD2.G, were co-transfected using X-tremeGENE 9 (Roche Applied Science, Roche, Penzberg, Germany). The supernatant was collected at 48 h, mixed with PEG-it Virus Precipitation Solution (5×) (System Biosciences, Mountain View, CA, USA) and incubated for 24 h at 4 °C. The supernatant/PEG-it mixture was then centrifuged at 1500 *g* for 30 min at 4 °C, and the pellet was resuspended in 1/10th of the original volume using cold, sterile medium at 4 °C and stored at − 80 °C.

T-cell transduction

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll (centrifuged at 1000 *g* for 20 min at 20 °C). PBMCs were cultured in AIM-V medium (Life Technologies) supplemented with 10% human serum in the presence of interleukin-2 (IL-2; 50 U ml^{-1}; PeproTech, Rocky Hill, NJ, USA) and anti-CD3 monoclonal antibody (catalog no.16-0037-85, 100 ng ml^{-1}; eBioscience, San Diego, CA, USA) for 24 h. PBMCs were harvested, washed once and resuspended and cultured at a density of 1.0×10^6 cells per ml for 24 h. For transduction, thawed viral supernatant was replaced to the medium and the cells were cultured for 24 h, and the medium was then refreshed and the cells were cultured for a further 48 h.

WST-1 proliferation assay

Lenalidomide was kindly provided from Cellgene (Summit, NJ, USA). T-cell proliferation was quantified spectrophotometrically using the metabolic proliferation reagent WST-1 (Roche Applied Science) according to the manufacturer's instructions. For the WST-1 proliferation assays, 2×10^4 3C10-CAR T cells and the same number of non-transduced T cells were cultured in 96-well plates for 24 h in 100 µl of AIM-V medium with different concentrations of lenalidomide (10, 1, 0.1 and 0 µM). Cells were incubated at 37 °C and 5% CO_2 for 3 days. The WST-1 absorbance of the samples, using a background control as a blank (media), was measured at 450 nm using a Multiskan Ascent microplate enzyme-linked immunosorbent assay reader (Thermo Scientific, Rockford, IL, USA). The WST-1 data were plotted using the results from three independent wells per assay. The assay was repeated three times.

Intracellular interferon-γ staining

The effector (E) cells (3C10-CAR T cells, mock T cells) were incubated with or without lenalidomide (1 µM) for 48 h. Intracellular interferon-γ (IFN-γ) staining was performed using the Cytofix/Cytoperm plus GolgiStop Kit (BD Biosciences, Bedford, MA, USA). The effector cells (1×10^5 cells) were incubated with 0.5×10^6 U87-EGFRvIII cells or U87MG cells in 200 µl AIM-V medium along with GolgiStop in a round-bottomed 96-well plate. Following a 4 h incubation period at 37 °C, the cells were incubated with biotin-SP-AffiniPure F(ab)′2 fragment-specific goat anti-mouse immunoglobulin G (catalog no. 115-066-006; Jackson Immuno Research Laboratories, West Grove, PA, USA) at 4 °C for 30 min. After washing, cells were stained with streptavidin-phycoerythrin (PE) (catalog no. 554061; BD Biosciences) and fluorescein isothiocyanate (FITC) mouse anti-human CD8 (catalog no. 555634; BD Biosciences) and incubated for 30 min at 4 °C. The cells were further washed and fixed by adding fixation/permeabilization solution and then incubated at 4 °C for 20 min. Finally, the cells were stained with allophycocyanin mouse anti-human IFN-γ (catalog no. 554702; BD Biosciences) and incubated at 4 °C for 30 min. After washing, cells suspended in 1% paraformaldehyde (Wako, Osaka, japan) were analyzed by FACSCalibur (BD Biosciences). T cells treated with IL-2 were used as a positive control. After the CD8-FITC-positive population was gated, allophycocyanin and PE positivity was analyzed. The experiment was repeated two times.

Calcein assay

The effector cells (3C10-CAR T cells, mock T cells) were incubated with or without lenalidomide (1 µM) for 48 h. Target (T) cells (U87-EGFRvIII, U87MG) were suspended at a final concentration of 1.0×10^6 cells per ml and incubated with 10 µM calcein-AM (Dojindo, Kumamoto, Japan) for 30 min at 37 °C, with occasional shaking. After two washes, cells were adjusted to a concentration of 1.0×10^5 cells per ml, and 1.0×10^4 cells (100 µl) were placed into a round-bottomed 96-well plate. The effector and target cells were then added to each well at various E:T ratios and incubated at 37 °C for 4 h. The plate was centrifuged at low speed (1000 *g*, 5 min), and 75 µl of supernatant was carefully aspirated and loaded into a lumaplate. The fluorescence was recorded using a 490-nm excitation filter and a 520-nm emission filter. Only target cells in medium with 1.5 µl of 10% sodium dodecyl sulfate were used for maximum release. The percentage of specific lysis was calculated as follows: (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100 (%). To exclude the influence of activation of natural killer (NK) cells, $CD3^+$ cells were selected from the PBMCs using CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Selected $CD3^+$ cells were transduced with viral supernatant to express 3C10-CAR (3C10-CAR-CD3 cells). The effector cells (3C10-CAR-CD3 cells, mock-CD3 cells) were incubated with or without lenalidomide (1 µM) for 48 h, and the calcein assay was performed as mentioned above. The assay was repeated three times.

Intracranial glioma xenograft

NOD/Shi-scid, IL-2Rγ-null (NOG) 5- to 6-week-old female mice (Central Institute for Experimental Animals, Kawasaki, Japan) were used for individual experiments. Animals were handled in the Animal Facility at Nagoya University in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Mice were anesthetized with an intraperitoneal injection of pentobarbital (somnopentyl; 150 – 160 mg kg^{-1} body weight; Kyoritsu, Tokyo, Japan). The mice were shaved and an incision was made in the scalp, and then a burr hole was made in the skull, 3-mm lateral to the midline and 4-mm posterior to the bregma, using an 18-gauge needle. Using a stereotactic apparatus, the U87-EGFRvIII-LUC cells (5.0×10^4 cells) suspended in 5 µl of phosphate-buffered saline (PBS) were injected at a depth of 2 mm below the dura matter. A sterile Hamilton syringe fitted with a 26-gauge needle was used with a microsyringe pump. Mice bearing established tumors were randomly assigned to two different experimental groups. Seven days after the inoculation, 2.0×10^6 3C10-CAR PBMCs (13 mice) or mock PBMC cells (12 mice) were infused into the tail vein. Lenalidomide (5 mg kg^{-1}) (13 mice) or PBS (12 mice) was given intraperitoneally for 35 consecutive days, beginning after the PBMC infusion.[28]

U87-EGFRvIII-LUC cells within the brain were monitored noninvasively by bioluminescence imaging using the *in vivo* imaging system, IVIS (Xenogen, Advanced Molecular Vision, Lincolnshire, UK). Mice were injected intraperitoneally with 200 µl of a freshly thawed aqueous solution of D-luciferin potassium salt (15 mg ml^{-1}) (Sigma-Aldrich), anesthetized with isoflurane and imaged for bioluminescence using a 1-min exposure time. Optical images were analyzed using the IVIS living image software package (Summit pharmaceuticals International, Tokyo, Japan). Overall survival following tumor inoculation was monitored.

Evaluation of T-cell migration to tumor

NOG 5- to 6-week-old female mice were stereotactically inoculated with U87-EGFRvIII cells (1.0×10^5 cells per 5 μl PBS) as described above. Seven days after the inoculation, 2.0×10^6 3C10-CAR PBMCs or mock PBMCs were infused into the tail vein. Simultaneously, lenalidomide (5 mg kg^{-1}) or PBS was given intraperitoneally for 20 consecutive days, and then the brain tissue was harvested and embedded in optimum cutting temperature compound (Sakura Finetek, Tokyo, Japan). Six-micrometer-thick frozen sections were prepared with a cryostat (CM3050S; Leica, Wetzlar, Germany). After drying, the sections were heated in a microwave oven for 3 min and fixed with 4% paraformaldehyde (Wako) for 15 min at room temperature. The sections were then dipped in PBS containing 0.1% Triton X-100 (PBST; Sigma-Aldrich) at room temperature for 20 min. Then, the sections were blocked with PBST containing 1.5% normal goat serum (Vector Laboratories, Burlingame, CA, USA) at room temperature for 1 h and were subsequently incubated with rabbit anti-human CD3 antibody (catalog no. RM9107; Thermo Lab Vision, Fremont, CA, USA) at a 1:100 dilution in blocking reagent overnight at 4 °C. After two washes with PBST, the slides were incubated with Alexa Flour 546 goat anti-rabbit immunoglobulin G antibody (catalog no. A11035; Invitrogen, Eugene, OR, USA) at a 1:200 dilution in blocking reagent for 30 min at room temperature, and cell nuclei were counterstained with 2 μg ml^{-1} of DAPI (4',6-diamidino-2-phenylindole) (Dojindo). After three washes, the slides were mounted using Fluorescence Mounting Medium (Dako, Carpinteria, CA, USA). Fluorescence images were acquired using an IX71 inverted microscope with a cooled CCD (Olympus, Tokyo, Japan). Three fields were randomly selected, and the number of $CD3^+$ cells and $DAPI^+$ cells was counted.

Immune synapse staining

Previous to dilution to an appropriate concentration (10 μM) with Dulbecco's modified Eagle's medium, U87-EGFRvIII and U87MG cells were stained with CellTracker Blue CMAC (Life Technologies) to visually distinguish them from T cells, and incubated for 30 min at 37 °C. The solution was exchanged for fresh Dulbecco's modified Eagle's medium and the cells were incubated for a further 30 min at 37 °C.

The glioma cells (1.5×10^5 cells) were collected and cocultured in a round-bottomed 96-well plate for 2 h at 37 °C with an equal number of 3C10-CAR T cells, which had been incubated with lenalidomide (1 μM) in advance. The cells were gently collected and plated onto poly-L-lysine-coated coverslips (BD Biosciences). To visualize the immune synapse, the F-actin Visualization Biochem Kit (Cytoskeleton, Denver, CO, USA) was used, following the manufacturer's instructions. Subsequently, F(ab)'2 fragment-specific goat anti-mouse immunoglobulin G and streptavidin (SA)-FITC, CD11a-FITC were used to stain 3C10-CAR and lymphocyte function-associated antigen-1 (CD11a), respectively.

Cell conjugation and polarization were analyzed using a BZ-X700 (Keyence, Osaka, Japan). This method was repeated three times.

Quantitative image analysis of conjugation and polarization of F-actin

Quantitative images were analyzed, as described previously.[29] The relative recruitment index was calculated using the Hybrid Cell Count software (BZ-H3C; Keyence). Regions of interest were placed around the immune synapse sites and the regions of the T cell not in contact with GBM cells, and in the background.

The relative recruitment index was calculated using the following formula: (mean fluorescence intensity (MFI) at synapse – background)/(mean fluorescence intensity at the T-cell regions not in contact with GBM cells – background). Thirty conjugates that had been incubated with or without lenalidomide were analyzed.

Statistical analyses

All statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). The statistical significance of any differences between the groups was determined using a Student's *t*-test. Differences were considered significant when the *P*-value was < 0.05. A *post hoc* test for the detection of linear trends was used, and we considered $P < 0.05$ to be significant. In the mouse studies, a log-rank test was used to determine significant differences in survival curves among groups.

RESULTS

Lenalidomide increases IFN-γ release by 3C10-CAR T cells and enhances their cytolytic effect on EGFRvIII-expressing glioma cells

Previously, our group showed that the third generation of 3C10-CAR T cells specifically target U87-EGFRvIII cells.[21] First, we evaluated the transduction efficiency of our lentiviral vector that transduces the 3C10-CAR into CD8 T cells by FACS (fluorescence-activated cell sorting) analysis. After CD8 cells were gated by FITC positivity, 3C10-CAR was stained with biotin anti-mouse Fab'2/SA-PE. The transduction efficiency of lentivirus-mediated 3C10-CAR to CD8 T cells was 30 – 40%, whereas that of the mock CAR was 3 – 8% (Figure 1). Next, we stained intracellular IFN-γ in 3C10-CAR PBMCs. CAR PBMCs or mock PBMCs (1×10^5 cells) were incubated with 0.5×10^6 U87-EGFRvIII cells or U87MG cells. PBMCs pretreated with lenalidomide and non-treated PBMCs were used. The proportion of IFN-γ and CAR double-positive cells was 9.84% in 3C10-CAR PBMCs, which were coincubated with U87-EGFRvIII, and 18.82% in lenalidomide-pretreated 3C10-CAR PBMCs (Figure 1). This experiment was repeated two times and similar results were obtained each time (Supplementary Figure 1).

Then, we examined whether lenalidomide enhanced the killing effect of 3C10-CAR T cells. A calcein assay confirmed that even without lenalidomide treatment, PBMCs transduced with 3C10-CAR lysed ~ 50% of the U87-EGFRvIII cells at an E:T ratio of 25:1, and lysis occurred in an E:T ratio-dependent manner. Mock PBMCs did not demonstrate this killing effect. PBMCs transduced with 3C10-CAR and mock PBMCs were pretreated with 1 μM lenalidomide for 48 h. 3C10-CAR PBMCs pretreated with lenalidomide lysed ~ 80% of the U87-EGFRvIII cells at an E:T ratio of 25:1. Mock PBMCs treated with lenalidomide were not effective, similar to the results for those without lenalidomide (Figure 2, left). $CD3^+$ cells were selectively isolated from PBMCs using CD3 microbeads to exclude the influence of activation of natural killer cells. The selected $CD3^+$ cells were transduced with viral supernatant to express 3C10-CAR (3C10-CAR CD3 T cells). 3C10-CAR CD3 T cells exerted a similar cytotoxicity to that of 3C10-CAR PBMCs. Additionally, lenalidomide enhanced the cytotoxicity of 3C10-CAR CD3 T cells (Figure 2, center). Lenalidomide-pretreated 3C10-CAR PBMCs exhibited no killing effect on the parental U87MGs with no EGFRvIII expression, similar to the results seen for the other effector cells (i.e., original 3C10-CAR cells and mock PBMCs with or without lenalidomide) (Figure 2, right). Collectively, these results show that lenalidomide treatment increases IFN-γ release by 3C10-CAR T cells and enhances their cytolytic effect on EGFRvIII-expressing glioma cells.

Lenalidomide increased the antitumor effect of 3C10-CAR T cells in intracranial glioma xenograft mice

The schema for the animal experiments is summarized in Figure 3a. U87-EGFRvIII-LUC cells (5.0×10^4 cells) were inoculated into the brains of NOG mice. On day 7, 3C10-CAR or mock PBMCs were injected via the tail vein. Starting on the next day, lenalidomide was administered intraperitoneally for 35 consecutive days. Bioluminescence imaging was performed every week, and overall survival time was evaluated. In two mock CAR groups, regardless of the treatment with or without lenalidomide, the tumors grew exponentially, and all mice died by day 20 (Figure 3b and c). In the 3C10-CAR group without lenalidomide, the tumors began to be eradicated from days 14 to 21, but 50% of the animals failed to benefit from the effect of the 3C10-CAR treatment and died by day 20. However, in the 3C10-CAR group with lenalidomide treatment, the tumors began to be eradicated from days 7 to 14, and all mice were eventually tumor-free (Figure 3c).

3C10-CAR T cells migrated to EGFRvIII-expressing glioblastoma

3C10-CAR PBMCs or mock PBMCs (2.0×10^6) were infused into the tail vein of the mice harboring a U87-EGFRvIII tumor. Twenty days after PBMC infusion, the brain tissue of the mice was harvested and embedded in an optimum cutting temperature compound, and the sections were stained with anti-human CD3 antibody. A number of human $CD3^+$ cells were observed in the tumors of the 3C10-CAR-treated mice, but none were seen in the tumors of

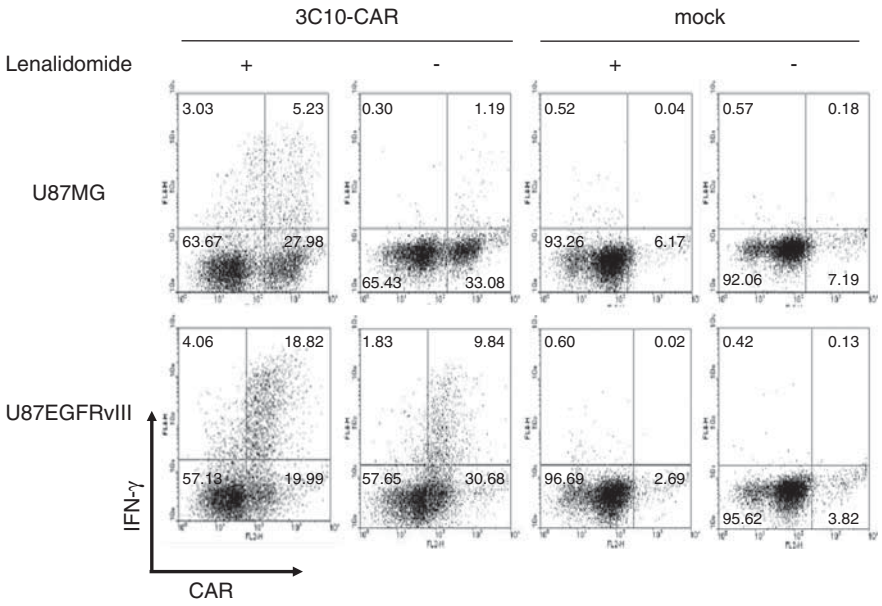

Figure 1. Transduction of lentivirus-mediated 3C10-CAR and intracellular IFN-γ assay. CAR PBMCs or mock PBMCs (1×10^5 cells) were incubated with 0.5×10^6 U87-EGFRvIII cells or U87MGs. PBMCs pretreated with lenalidomide and non-treated PBMCs were used. The approximate transduction efficiency of 3C10-CAR was 30–40%. After gating the CD8-FITC population, the proportion of IFN-γ and CAR double-positive cells was 9.84% in the 3C10-CAR PBMCs that were coincubated with U87-EGFRvIII, and 18.82% in the lenalidomide-pretreated 3C10-CAR PBMCs. The second experiment is shown in Supplementary Figure 1.

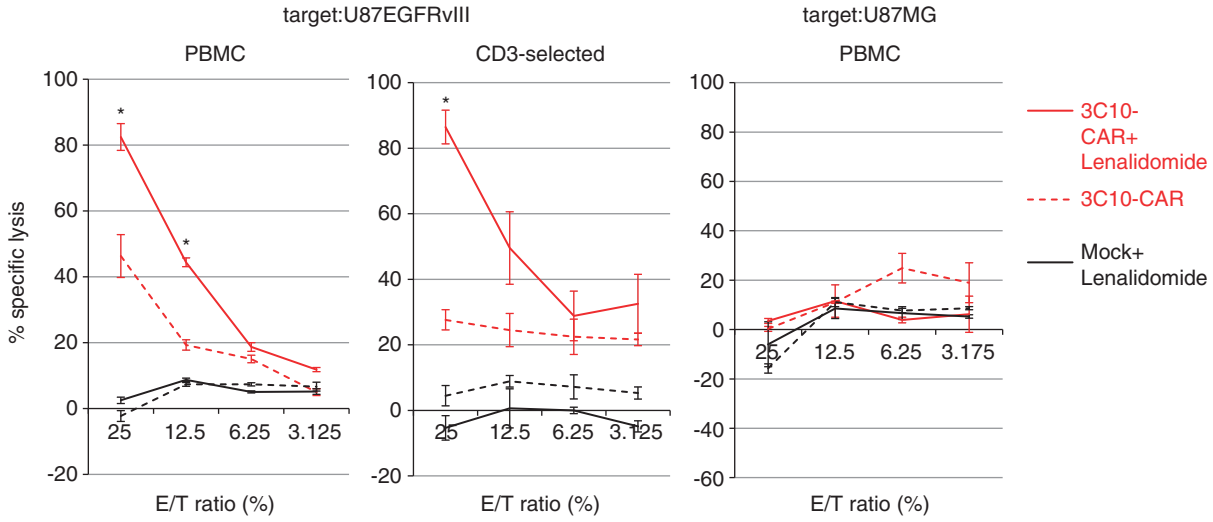

Figure 2. A calcein assay. Even without lenalidomide treatment, PBMCs transduced with 3C10-CAR lysed ~50% of the U87-EGFRvIII cells at an E:T ratio of 25:1, and lysis occurred in an E:T ratio-dependent manner. Mock PBMCs did not demonstrate this killing effect. PBMCs transduced with 3C10-CAR and mock PBMCs were pretreated with 1 μM lenalidomide for 48 h. 3C10-CAR PBMCs pretreated with lenalidomide lysed ~80% of the U87-EGFRvIII cells at an E:T ratio of 25:1. Mock PBMCs treated with lenalidomide were not effective, similar to those without lenalidomide treatment (left). $CD3^+$ cells were selectively separated from PBMCs using CD3 microbeads to exclude the influence of natural killer (NK) cell activation. The selected $CD3^+$ cells were transduced with viral supernatant to express 3C10-CAR (CD3-selected 3C10-CAR), and then a calcein assay was performed. 3C10-CAR CD3 T cells exerted a similar cytotoxicity to 3C10-CAR PBMCs. Additionally, lenalidomide enhanced the cytotoxicity of the 3C10-CAR CD3 T cells (center). Against the parental U87MGs without EGFRvIII expression, lenalidomide-pretreated 3C10-CAR PBMCs exhibited no killing effect, similar to the results for the other effector cells (i.e., original 3C10-CAR cells and mock PBMCs with or without lenalidomide treatment) (right). $^*P < 0.05$. Error bars, s.e.m.

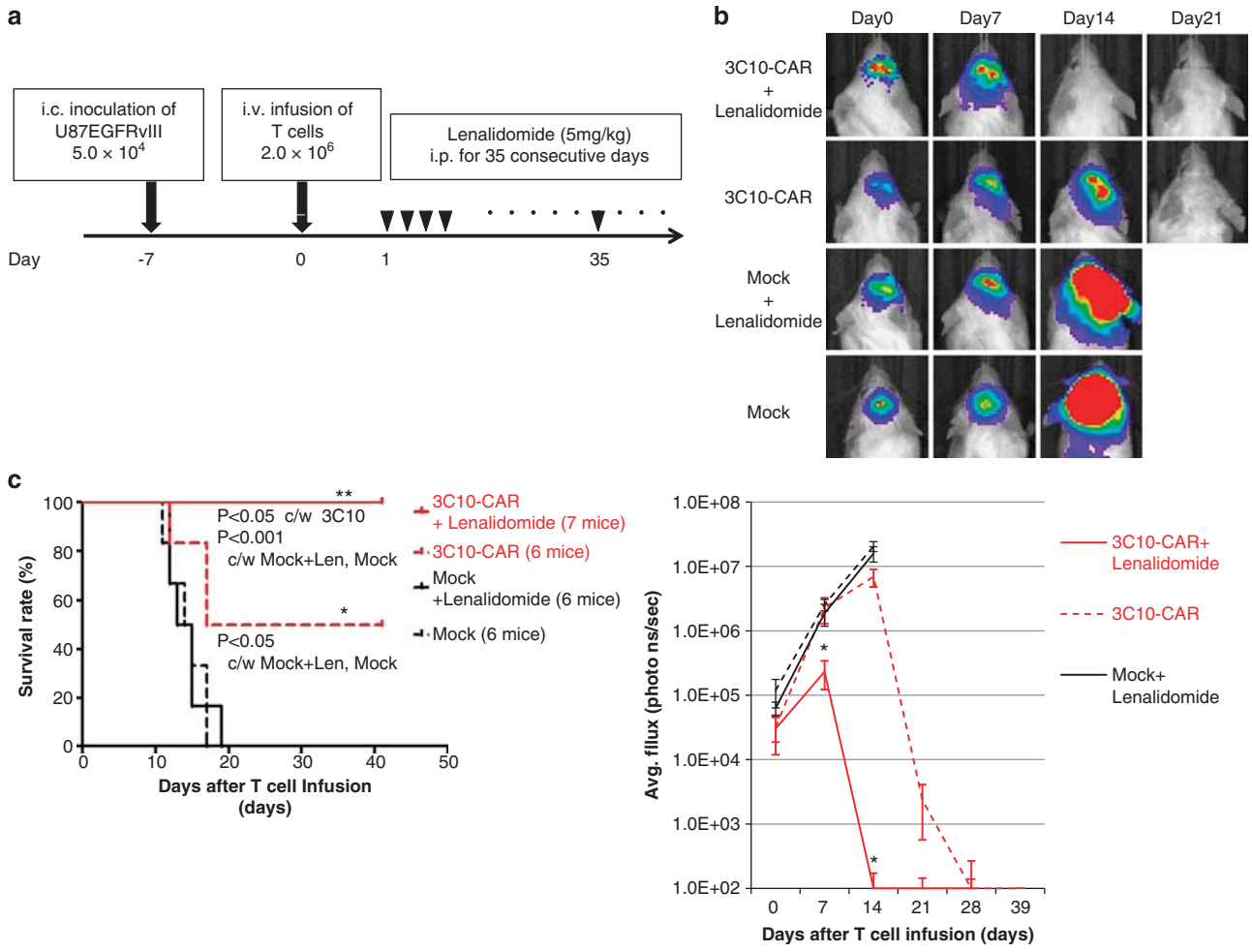

Figure 3. Antitumor effect of 3C10-CAR T cells in intracranial glioma xenograft mice. (**a**) The schema for the animal experiments. (**b**) Bioluminescence imaging. Tumors began to be eradicated from days 7 to 14 in the 3C10-CAR+lenalidomide group, and from days 14 to 21 in the 3C10-CAR group. *Significant difference ($P < 0.05$) between the 3C10-CAR+lenalidomide group and the other three groups. Error bars, s.e.m. (**c**) Survival curve. Although 50% of the mice treated with 3C10-CAR PBMCs died near day 20, all mice treated with 3C10-CAR+lenalidomide were eventually tumor-free. **Significant difference ($P < 0.05$) between the 3C10-CAR+ and 3C10-CAR − lenalidomide groups, *significant difference ($P < 0.05$) between the 3C10-CAR − lenalidomide group and the two mock-treated groups.

mock-CAR-treated mice. Lenalidomide treatment significantly increased the numbers of migrated $CD3^+$ T cells from those in the tumors of the mice treated without lenalidomide (Figure 4).

Lenalidomide increases the number of T cells and enhances immune synapse formation

Lenalidomide is a multifaceted chemotherapeutic agent. In the current study, we focused on its immunological effects. We speculated that lenalidomide might act by increasing the number of effector cells or by enhancing tight connections between effector T cells and target tumor cells.[30] To address the first question, 2×10^4 3C10-CAR T cells and the same number of mock T cells were cultured in 96-well plates for 24 h, and the cell number was indirectly evaluated by a WST-1 assay. As expected, the number of third-generation CAR PBMCs with costimulatory signals for self-proliferation was higher compared with that of the control PBMCs. Of note, lenalidomide treatment increased the growth of both types of PBMCs, regardless of CAR treatment, in a dose-dependent manner (Figure 5a). When effector T cells conjugate with target cells, F-actin polymerizes and CD11a (lymphocyte function-associated antigen-1) accumulates in the junction, which is known as an immune synapse. Figure 5b shows tumor cells labeled with CellTracker Blue CMAC (blue); 3C10-CAR, F-actin and CD11a were colocalized in the connections between the tumor cells and 3C10-CAR PBMCs, suggesting the formation of immune synapses between CAR T cells and tumor cells. To address the question of whether lenalidomide strengthens immune synapses, F-actin polymerization was quantified using an relative recruitment index. The thickness of the F-actin polymers was significantly higher in the lenalidomide (+) group compared with that in the lenalidomide (−) group. To summarize, in our study, lenalidomide enhanced both the extent and speed of the antitumor effect of T cells on antigen-specific glioma.

DISCUSSION

This is the first study to indicate that lenalidomide strengthens CAR T-cell therapy and enhances the formation of immune synapses between target cells and CAR T cells in cancer.

One of the major challenges in immunotherapy is to overcome cancer immunosuppression and immune evasion, but this is very

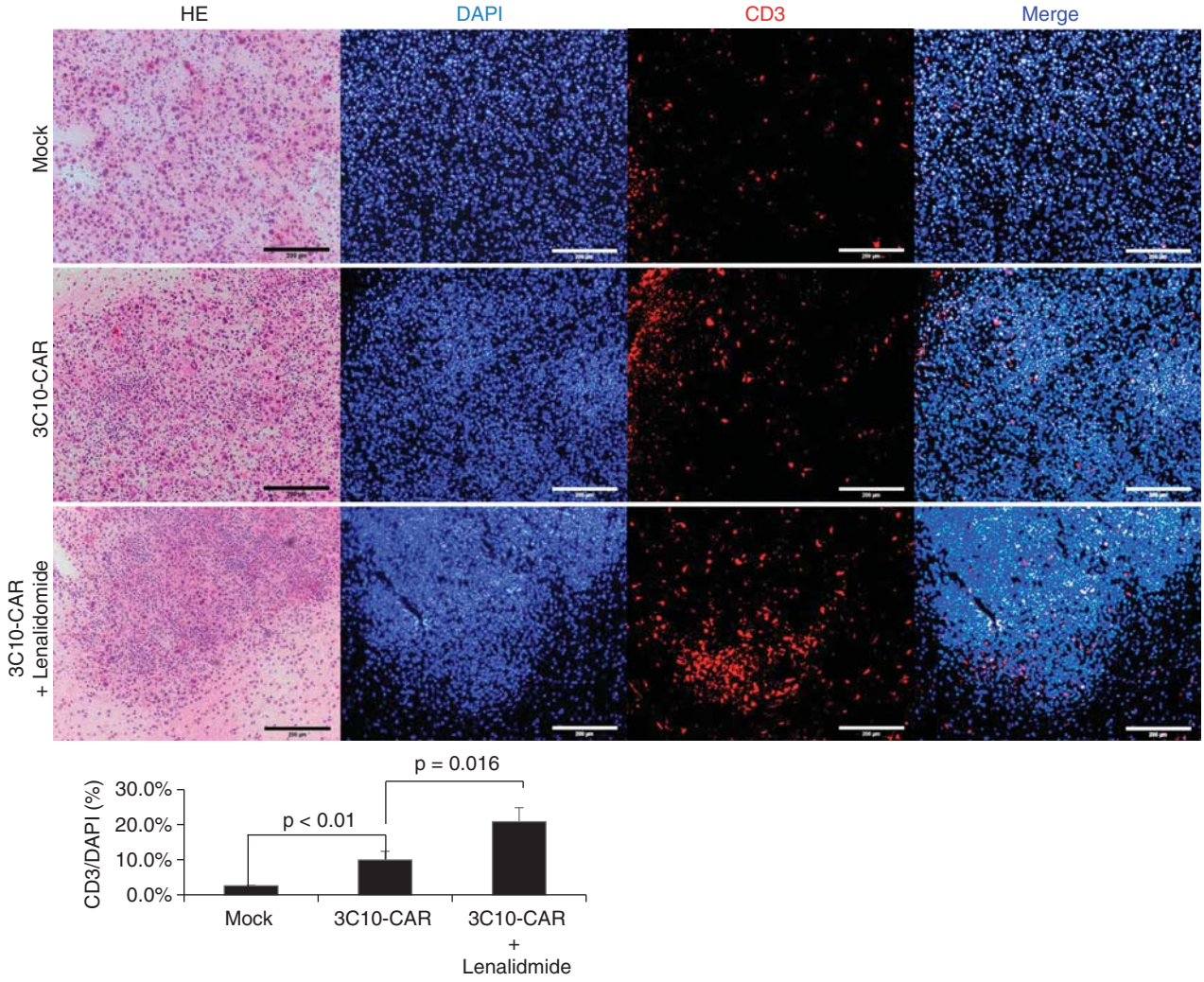

Figure 4. 3C10-CAR T cells migrated to intracranial EGFRvIII-expressing glioblastoma. Seven days after the inoculation of tumor cells, 3C10-CAR PBMCs or mock PBMCs were infused. Simultaneously, lenalidomide or PBS was given for 20 consecutive days. The brain sections were stained with anti-human CD3 antibody. A number of human $CD3^+$ cells were observed in the tumors of 3C10-CAR-treated mice, but none were seen the tumors of mock-CAR-treated mice. The number of $CD3^+$ cells and $DAPI^+$ cells in three fields was counted. Lenalidomide treatment significantly increased the numbers of migrated $CD3^+$ T cells from those in the tumors without lenalidomide treatment. Scale bars, 200 μm. Error bars, s.e.m.

difficult because the intrinsic mechanisms of these events are highly diverse. The expression of HLA is downregulated in malignant tumors, particularly in GBMs, and there is a significant positive correlation between HLA class I antigen loss and tumor grade.[25] This suggests a defect in the antigen presentation of glioma cells and concomitant cytotoxic T-lymphocyte lysis, resulting in a poor response to T-cell-based immunotherapy. Further, many types of cancer cells secrete multiple factors that have been shown to suppress antitumor immune responses, namely tumor growth factor-β, IL-6, IL-10 and vascular endothelial growth factor.[27] These cytokines are associated with the inhibition of differentiation or maturation of dendritic cells and induction of regulatory T cells.

In this study, we investigated a new method to augment CAR T-cell therapy using the immunomodulatory drug, lenalidomide. Lenalidomide shows a diverse range of activities, including multifocal immunomodulatory activities, direct antitumor activity and antiangiogenetic properties. This agent has been applied clinically in combination with dexamethasone in patients with multiple myeloma who have received at least one prior therapy, who have anemia due to low- or intermediate-1-risk myelodysplastic syndrome with a deletion 5q cytogenetic abnormality with or without additional cytogenetic abnormalities, and relapsed or refractory mantle cell lymphoma.[31,32] Importantly, lenalidomide inhibits the production of the proinflammatory cytokines TNF-α, IL-1, IL-6 and IL-12 and elevates the production of the anti-inflammatory cytokine IL-10.[33,34] Lenalidomide was reported to have the capacity to partially overcome immune checkpoints, such as cytotoxic T-lymphocyte-associated antigen 4 Ig blockade,[35] to decrease the expression of the inhibitory marker programmed cell death protein 1,[36] and to act on T cells via a B7-CD28 costimulatory pathway. Although our preliminary experiments showed that lenalidomide treatment did not alter the expression of cytotoxic T-lymphocyte-associated antigen 4 and programmed cell death protein 1 on CAR T cells *in vitro* (Supplementary Figure 2), the agent may modulate these immune checkpoints in the tumor microenvironment. Moreover, lenalidomide also inhibits the proliferation and suppresses the function of regulatory T cells and myeloid-derived suppressor cells.[28,37,38] These actions lead to stimulation of T-cell proliferation, increased secretion of IFN-γ and IL-2 and augmentation of natural killer cell function. These various immunomodulatory effects of

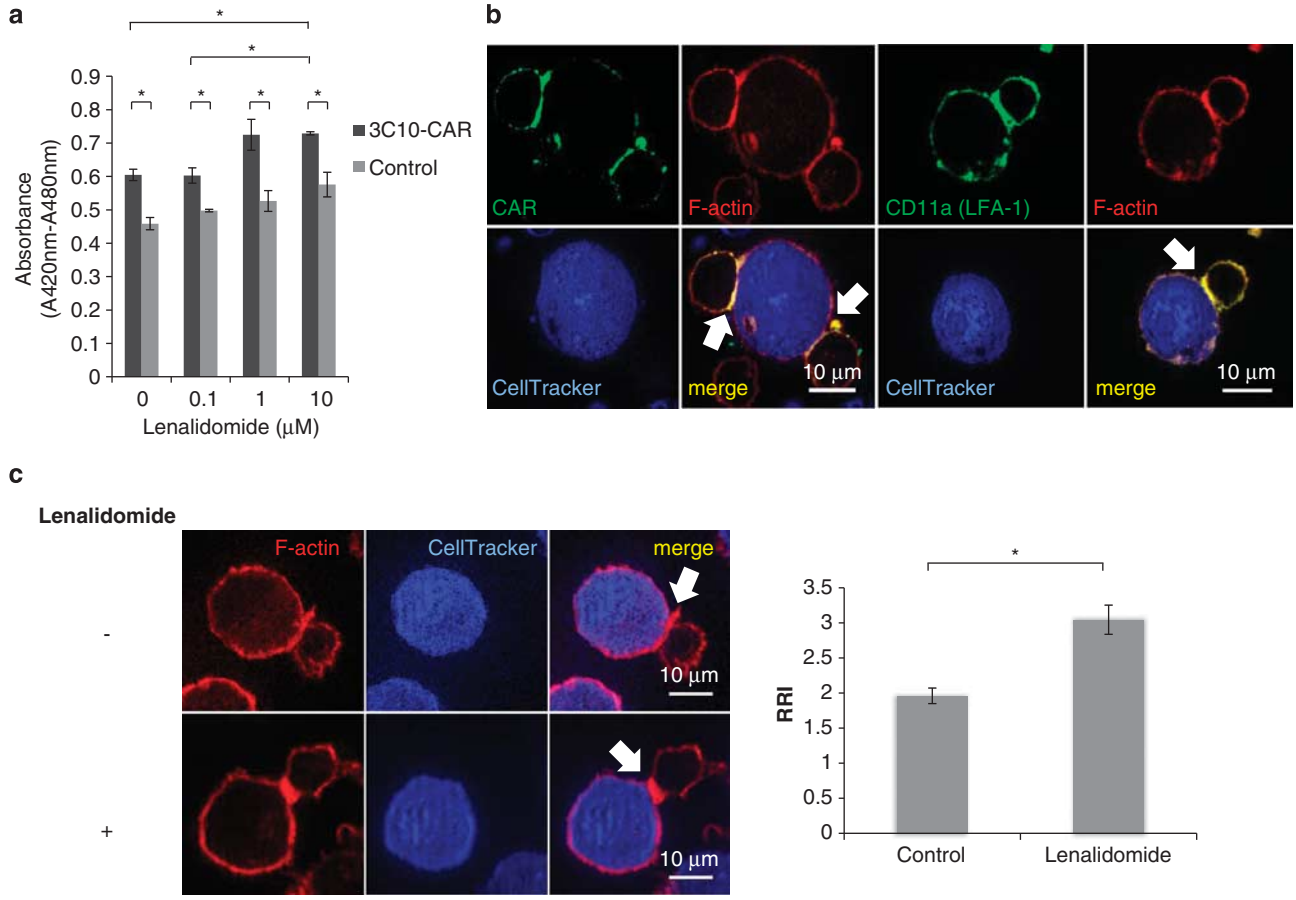

Figure 5. Immune synapse formation. (**a**) WST-1 assay. Lenalidomide increased the growth of both types of PBMCs, regardless of CAR transduction, in a dose-dependent manner. (**b**) Immunofluorescence of CD11a and F-actin in the junction of CAR T cells and tumor cells. Tumor cells labeled with CellTracker Blue CMAC (blue); 3C10-CAR, F-actin and CD11a were colocalized in the connection between the tumor cells and 3C10-CAR PBMCs. (**c**) F-actin polymerization was quantified using a relative recruitment index (RRI). The thickness of the F-actin polymers was significantly higher in the lenalidomide (+) group compared with that in the lenalidomide (−) group. Arrows indicate immune synapses. $*P < 0.05$, Error bars, s.e.m.

lenalidomide raise potential interest in its use in combination with cell-based immunotherapies.

In the current study, we showed a marked improvement in the survival time of the mice when they were cotreated with 3C10-CAR T cells and lenalidomide *in vivo,* resulting in a tumor-free rate of 100%. One possible mechanism of action regarding this result is that lenalidomide can activate the CD28 costimulatory pathway, which was integrated with our CAR. Indeed, lenalidomide augmented the absolute number of 3C10-CAR T cells (Figure 4), resulting in enhancement of their cytolytic effects and IFN-γ release, previously demonstrated *in vitro*. As a more intriguing and novel finding, immune synapse formation was induced between a 3C10-CAR T-cell and U87-EGFRvIII cells. Immune synapses are molecular clusters at the contact site between T-cell receptors and peptide-loaded major histocompatibility complex molecules on the surface of allophycocyanins that are enriched in F-actin.[39] A mature immune synapse forms a specific pattern of receptor segregation, with a central cluster of T-cell receptors surrounded by a ring of integrin family adhesion molecules such as lymphocyte function-associated antigen-1.[40] Ramsay *et al.*[29,41] reported that lenalidomide repaired and enforced immune synapse formation in patients with hematological malignancies. Here, we showed for the first time that immune synapses formed between 3C10-CAR T cells and U87-EGFRvIII cells via the display of F-actin, lymphocyte function-associated antigen-1 and CAR polarization at the contact site, and illustrated that lenalidomide enhanced immune synapse formation, by pictorial means, in accordance with previous studies.[29,41,42] Yokosuka *et al.*[43] reported a method for the visualization of the detailed structure of immune synapses using artificial planar bilayers; further investigations will be needed to reveal the mechanism by which immune synapses are formed between CAR T cells and target cells.

Thalidomide failed to show sufficient efficacy in the treatment of patients with high-grade gliomas.[44,45] However, lenalidomide, a thalidomide derivative with theoretically greater efficacy and fewer side effects, may be efficacious when used in combination with CAR T-cell therapy, given its multifaceted immunomodulatory efficacies.

In summary, we demonstrated that lenalidomide can act as an immune adjuvant when used in combination with CAR T-cell therapy. Our study is the first to show the augmentation of immune synapse formation between CAR T cells and target cells. Although the blockade of immune checkpoints may be a practical approach to cancer immunotherapy, lenalidomide also has the potential to intensify immunotherapies, including CAR T-cell therapy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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