

ORIGINAL RESEARCH



Identification of Claudin 6-specific HLA class I- and HLA class II-restricted T cell receptors for cellular immunotherapy in ovarian cancer

Junko Matsuzaki^{a,b,c}, Shashikant Lele^d, Kunle Odunsi^{a,b,c,d}, and Takemasa Tsuji^{a,b,c}

^aCenter for Immunotherapy, Roswell Park Comprehensive Cancer Center, Buffalo, NY, USA; ^bDepartment of Obstetrics and Gynecology, University of Chicago, Chicago, IL, USA; ^cUniversity of Chicago Medicine Comprehensive Cancer Center, Chicago, IL, USA; ^dDepartment of Gynecologic Oncology, Roswell Park Comprehensive Cancer Center, Buffalo, NY, USA

ABSTRACT

Adoptive cell therapy (ACT) is one of promising immunotherapies for cancer patients by providing a large amount of cancer antigen-specific effector T cells that can be manufactured rapidly by ex vivo gene engineering. To provide antigen-specificity to patients' autologous T cells in a short-term culture, T-cell receptors (TCRs) or chimeric antigen receptors (CARs) are transduced to bulk T cells. Because of intra- and inter-tumoral heterogeneity in tumor antigen expression, a repertoire of TCR or CAR genes targeting a wide range of tumor antigens are required for a broad and effective treatment by ACT. Here, we characterized immunogenicity of claudin 6 (CLDN6) in ovarian cancer patients and identified specific TCR genes from CD8⁺ and CD4⁺ T cells. CLDN6 protein was frequently expressed on EpCAM⁺ ovarian cancer cells but not CD45⁺ lymphocytes in tumor ascites of ovarian cancer patients. Spontaneous CLDN6-specific CD4⁺ and CD8⁺ T-cell response was detected in peripheral blood mononuclear cells (PBMCs) from 1 out of 17 ovarian cancer patients. HLA-A*02:01 (A2) and DR*04:04 (DR4)-restricted TCR genes were isolated from CLDN6-specific CD8⁺ and CD4⁺ T cells, respectively. T cells that were engineered with A2-restricted TCR gene recognized and killed A2⁺CLDN6⁺ cancer cells. DR4-restricted TCR-transduced T cells directly recognized DR4⁺CLDN6⁺-overexpressed cancer cells. Our results demonstrate that these CLDN6-specific TCR genes are useful as therapeutic genes for ACT to patients with ovarian and other solid tumors expressing CLDN6.

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Introduction

Adoptive cell therapy (ACT) using tumor antigen-specific T cells is a powerful strategy for the treatment of cancer patients as infusion of a large number of anti-tumor effector T cells provides immediate tumor-debulking effects. Furthermore, a subset of infused T cells is expected to differentiate into memory T cells to provide long-term immunosurveillance and potentially prevent recurrence of disease.^{1,2} Recent gene engineering techniques to transduce target antigen-specific chimeric antigen receptor (CAR) or T-cell receptor (TCR) enable rapid generation of a large number of autologous therapeutic T cells with potent anti-tumor activity.^{3–6} In our previous studies, we have cloned and characterized NY-ESO-1-specific and MHC class I- and class II-restricted TCR genes that are currently tested in a phase I clinical trial (ClinicalTrials.gov Identifier: NCT03691376).^{7,8} Although the safety and efficacy of ACT using NY-ESO-1-specific TCR-transduced cells in cancer patients has been demonstrated,^{9,10} eligible patients for the therapy are limited by NY-ESO-1 expression (30–80% of solid tumors)^{11–13} and patients' HLA types. In addition, NY-ESO-1 frequently shows heterogeneous expression within a tumor,^{14,15} which allows antigen-negative tumor variants to escape immune attack. To develop broadly applicable and more efficient TCR gene-engineered T-cell

therapy for cancer patients, identification of a panel of off-the-shelf TCR genes for shared cancer antigens or shared neoantigens is essential.

Claudin 6 (CLDN6) is a cell surface membrane protein expressed on multiple solid tumor tissues such as ovarian cancer, testicular cancer, and endometrial cancer, while the expression is not observed on normal adult tissues at the transcription and protein levels.^{16–20} Thus, CLDN6 is considered to be a promising target for antibody-based cancer immunotherapies. CLDN6-specific antibodies have been engineered as bi-specific antibodies and cytotoxic-drug conjugates.^{21,22} In addition, Reinhard et al. demonstrated the efficacy of CLDN6-specific CAR-T cells in mouse models,¹⁶ and a phase I/IIa clinical trial using the CAR-T cell is ongoing. Because of the tumor-specific cell surface expression, CLDN6 is also a potential target for other immunotherapies such as cancer vaccines. Although the expression of CLDN6 on solid tumors has been broadly investigated, its immunogenicity in cancer patients has not been evaluated. Here, we investigated spontaneously induced T-cell response against CLDN6 in ovarian cancer patients. By characterizing CLDN6-specific CD8⁺ and CD4⁺ T cells, we have identified novel CLDN6-derived short peptides that bind on HLA-A*02:01 (A2) and -DRB1*04

:04 (DR4). Furthermore, we have cloned HLA-A2 and -DR4-restricted CLDN6-specific TCR genes and characterized cancer cell recognition of TCR gene-transduced T cells for the development of TCR-engineered T-cell therapy.

Materials and methods

Biospecimen processing

Blood and malignant ascites were obtained from ovarian cancer patients under an approved protocol (I 215512) from the institutional review board at Roswell Park Comprehensive Cancer Center (Roswell Park). Patients signed a consent form to approve the use of their specimens for research. Most of specimens that were used in this study were collected at the time of debulking surgery prior to chemotherapy from patients with high-grade serous ovarian cancer. Healthy donor blood samples were obtained from the blood donor center at Roswell Park. Peripheral blood mononuclear cells (PBMCs) and ascites mononuclear cells were isolated using lymphocyte separation media (Corning), cryopreserved in 10% DMSO/90% FBS, and stored in a liquid nitrogen freezer.

ELISPOT assay

PBMC were thawed and $5\text{--}10 \times 10^5$ cells were cultured with a pool of synthetic overlapping peptides for CLDN6 (1 $\mu\text{g}/\text{ml}$, JPT Peptide Technologies), or a pool of 27 peptides of Cytomegalovirus, Epstein-Barr virus, Influenza virus, and Tetanus toxin (CEFT; 0.5 $\mu\text{M}/\text{each}$, GenScript) in RPMI1640 medium (Corning) supplemented with 10% human AB serum (Gemini), $1\times$ MEM non-essential amino acid (Corning), 100 U/ml penicillin (Corning), 100 $\mu\text{g}/\text{ml}$ streptomycin (Corning), 10 U/ml IL-2 (Roche), and 10 ng/ml IL-7 (R&D Systems) in 96-well round-bottom plates. The cells were expanded every 3–4 days. After 13–15 d of culture, cells were harvested and suspended in X-VIVO 15 media (Lonza). Fifty thousand cells were seeded in ELISPOT plate (MultiScreen-HA filter plate, Millipore Sigma) that were pre-coated with anti-IFN- γ antibody (clone 1-D1K, Mabtech) in the presence or absence of the respective peptide mix. The plate was incubated in a 5% CO₂ incubator for 20–24 h, followed by development with biotinylated detection anti-IFN- γ antibody (clone 7-B6-1, Mabtech), streptavidin-alkaline phosphatase (ALP) (Mabtech), and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt/nitro-blue tetrazolium chloride (BCIP/NBT) substrate (Sigma-Aldrich). The numbers of spots were counted using an ImmunoSpot S6 Core analyzer (CTL). The response was considered as positive when the number of spots is ≥ 50 and twice higher than background.²³

CLDN6 expression

Mononuclear cells from ascites were thawed and stained with Fixable Viability Stain 700 (BD Horizon) followed by antibodies against CD45 (clone H130, BioLegend), CD326 (EpCAM; clone 9C4, BioLegend), and CLDN6 (clone

342927, R&D Systems). PA-1 ovarian cancer cell line was obtained from the American Type Culture Collection. An epithelial ovarian cancer (EOC) cell line was established from solid tumor single cell suspension. The tumor cells were injected intraperitoneally into NOD/SCID/IL-2R γ^{null} (NSG) mice under the institutional IACUC approved protocol (1346 M) at Roswell Park. When abdominal circumference was extended, ascites was harvested and the mononuclear cells were cultured in RPMI1640 medium supplemented with 20% FBS, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. EpCAM expression was confirmed by flow cytometry. Cancer cell lines were cultured in the presence or absence of 1,000 U/ml human IFN- γ recombinant protein (PeproTech) for 2 days. The cells were harvested using 0.25% trypsin-EDTA solution (Corning) and stained with antibodies for HLA-A2 (clone BB7.2, BioLegend) and CLDN6. Cells were acquired by the BD Fortessa flow cytometer and analyzed using FlowJo software.

Generation of Epstein-Barr virus (EBV)-transformed B cell lines

PBMC were first stained with biotinylated anti-CD4 (clone OKT4) and anti-CD8 (clone HIT8a) antibodies (BioLegend) and CD4⁺ and CD8⁺ T cells were depleted using Dynabeads Biotin Binder (Thermo Fisher Scientific). One million T-cell-depleted PBMCs were cultured with a culture supernatant of B95-8 cell line in the presence of 2 $\mu\text{g}/\text{ml}$ cyclosporin A (Sigma-Aldrich) in RPMI1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin.²⁴ HLA types of EBV-transformed B (EBV-B) cells were determined by the Immune Analysis Shared Resource (IASR) at Roswell Park.

Generation of CLDN6-specific TCR gene-transduced T cells

CLDN6-specific T cells were isolated as previously described using the IFN- γ secretion assay kit (Miltenyi Biotech).²⁵ The patient P01 EBV-B cells (1×10^6 cells) were labeled with CFSE and incubated with 2 μM CLDN6 pooled peptides overnight in a CO₂ incubator. The patient P01 T cells (2×10^6 cells) were incubated with peptide-pulsed CFSE-labeled EBV-B cells in 0.5 ml of X-VIVO medium in a 5 ml round-bottom tube for 4 h. After the incubation period, cells were labeled with an IFN- γ catch reagent, further incubated for 45 min, and stained by anti-IFN- γ detection and anti-CD4 antibodies (clone RPA-T4, BioLegend) according to the manufacturer's instruction. CFSE-negative IFN- γ^+ CD4⁺ and IFN- γ^+ CD4⁻ cells were sorted using the BD FACSAria cell sorter (BD Biosciences). One thousand cells were dissolved in TRI Reagent solution (Invitrogen) for RNA extraction. Total RNA was used for RT-PCR amplification of TCR α - and β -chain genes that were assembled to generate TCR-expressing plasmid vectors as previously described.²⁶ Culture supernatants from high-titer retrovirus producing PG13 clones were harvested and infected to healthy donor PBMC. Transduction efficiency was determined by flow

Table 1. HLA types of patient and EBV-B cells. For each gene, HLA types matched with P01 is shown as bold and underlined. Blank (-) indicates homozygous expression.

Sample	A	B	C	DRB1	DQB1	DPB1
P01	*02	*27	*01	*0404	*0302	*0201
	<u>*2402</u>	-	-	*1401	*0503	*0401
EBVB-1	*01	*0702	*07	*1102	*0301	*0201
	*02	*0801	-	*1401	*0503	-
EBVB-2	*0101	*0801	*0602	*0301	*0201	*0401
	-	*5701	*0701	*0701	*0303	-
EBVB-3	*02	*27	*02	*04	*0201	*0401
	*2301	*44	*04	*07	-	-
EBVB-4	*0201	*0702	*0202	*0404	*0301	*0401
	*0301	*4405	*0702	*1101	*0302	*0401
EBVB-5	*02	*15	*03	*0401	*0301	*0401
	*0301	-	-	*1101	*0302	*0402
EBVB-6	*02	*40	*02	*1101	*03	*0201
	<u>*03</u>	-	*03	*1301	*06	*0401

cytometry using anti-V β 8 (for CD8-TCR; clone JR2, BioLegend) and anti-V β 7.1 (for CD4-TCR; clone ZOE, Beckman Coulter) antibodies.

T-cell functional analysis

CLDN6-specific CD8⁺ T-cell line was established by expanding the sorted CLDN6-specific T cells with 10 μ g/ml phytohemagglutinin in the presence of 30 Gy γ -irradiated healthy donor PBMC, 10 U/ml IL-2, and 10 ng/ml IL-7. For intracellular cytokine staining, T cells were stimulated with peptide-pulsed or -unpulsed P01-derived or HLA-matched EBV-B cells (Table 1) for 6 h in the presence of 5 μ g/ml monensin (Sigma-Aldrich). In some experiments, anti-CD107a (clone H4A3, BD Biosciences) and anti-CD107b (clone H4B4, BD Biosciences) antibodies along with 5 μ g/ml Brefeldin A (Sigma-Aldrich) were added during incubation. Cells were stained for CD3 (clone OKT3, BioLegend), CD4 (clone OKT4, BioLegend), CD8 (clone RPA-T8, BioLegend), V β 7.1, and/or V β 8, followed by fixation with 2% formaldehyde and permeabilization with Permeabilization Medium B (Thermo Fisher) for intracellular staining for IFN- γ (clone B27, BD Biosciences), TNF- α (clone MAb11, BD Biosciences), GM-CSF (clone BVD2-21 C11, BD Biosciences), and/or IL-2 (clone MQ1-17H12, BioLegend). To determine cytokine levels in a culture supernatant, T cells (100,000 cells/well) were cocultured with target cells (50,000 cells/well) for 24 h. To define T-cell epitope, an independent set of 21 CLDN6 20-mer peptides with 10 amino acid overlapping each (EZBiolab) was used (Supplemental Table 1). Cancer cells were cultured for 2 d with 1,000 U/ml IFN- γ (PeproTech) and washed thoroughly before the co-culture with T cells. The culture supernatant was harvested, and IFN- γ level was determined using an ELISA kit (eBioscience) according to the manufacturer's instruction.

Tetramer analysis

HLA-A2-binding epitope of CLDN6 between CLDN6₁₃₁₋₁₄₀ was predicted using SYFPEITHI database.²⁷ TLIPVCWTA (CLDN6₁₃₂₋₁₄₀) peptide was synthesized by EZBiolab with

82% purity. An HLA-A2/peptide monomer was generated with the Flex-T HLA-A*02:01 monomer UVX reagent followed by tetramerization using PE-streptavidin according to the manufacturer's instruction (BioLegend). Optimum staining concentration of the A2/CLDN6₁₃₂₋₁₄₀ tetramer was determined by titration. CD8-TCR- or mock-transduced T cells were first treated with 50 nM dasatinib (Sigma-Aldrich) for 30 min at 37°C and then stained with the tetramer reagent for 30 min on ice. Tetramer-stained cells were further stained with purified anti-PE (clone PE001, BioLegend) and PE goat anti-mouse Ig (multiple adsorption, BD Biosciences) antibodies.²⁸

Retroviral expression of HLA genes

Coding region of HLA-A/B/C genes were amplified by RT-PCR using a tumor tissue as a template and cloned into a murine stem cell virus (MSCV)-based retroviral expression vector. HLA types of cloned genes were determined by Sanger sequencing at the Genomics Shared Resource at Roswell Park. High titer retroviral vectors were produced from PG13 packaging cell lines and were used to transduce K562 and PA-1 cells.

Generation of ovarian cancer cell lines overexpressing CLDN6 and HLA-A*02:01 or HLA-DRB1*04:04

Coding regions of CLDN6, HLA-A2, and -DR4 were amplified by RT-PCR from cDNA of a tumor tissue. CLDN6 gene was genetically fused to the puromycin-resistant gene via P2A translational skipping sequence and DR4 was fused to human CIITA gene⁷ via T2A sequence. CLDN6-Puro and A2 or DR4-CIITA gene were cloned in a piggyBac transposon plasmid, as shown in Supplemental Fig S6. These piggyBac transposon plasmids were electroporated into CLDN6⁻ ovarian cancer cell line, A2780, together with a hyperactive piggyBac transposase (hyPBase)²⁹-expressing plasmid using the Nucleofector 4D instrument. Two days after electroporation, cells were cultured in the presence of 5 μ g/ml puromycin for 10 days. Because a part of cells that were electroporated with the CLDN6 and DR4-expressing plasmid did not express cell surface HLA-class II, HLA-DR⁺ A2780 was isolated using anti-DR antibody (clone L243, BioLegend) and a pan mouse IgG kit (Invitrogen) according to the manufacturer's instruction before co-culturing with TCR gene-transduced T cells.

Cytotoxicity assay

Cytotoxicity was determined by a calcein-AM release assay as previously described.⁸ Briefly, the parental and A2⁺CLDN6⁺ A2780 were labeled with 5 μ M calcein-AM (Sigma-Aldrich) and mixed with CD8-TCR- or mock-transduced T cells for 4 h at E/T ratio, 50, 25, or 12.5. Fluorescence release in cell-free supernatants was measured by the Synergy H1 microplate reader (BioTek) with excitation 485 and emission 528. To induce the maximum release, target cells were treated with 2% Triton-X, whereas the spontaneous release was determined from supernatants

of target cell alone. Cytotoxicity was calculated using the following formula: % cytotoxicity = $100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})]$. To evaluate the induction of apoptotic cell death of cancer cells by CD8-TCR-transduced T cells, the parental and A2⁺CLDN6⁺ A2780 (5×10^5 cells) were cocultured with T cells (1×10^6 cells) for 18 h. Cells were harvested with 0.25% trypsin-EDTA solution and stained with anti-CD3 antibody (clone UCHT1, BioLegend) followed by annexin V and PI according to the manufacturer's instruction (BioLegend). Percentages of Annexin V⁺ and PI⁺ cells were analyzed for CD3⁻ cells.

Statistical analysis

The statistical difference of CLDN6 expression on EpCAM⁺ and CD45⁺ cells was analyzed by GraphPad Prism software using the two-tailed paired *t*-test. The correlation between %EpCAM⁺ and CLDN6⁺ cells was analyzed by GraphPad Prism software using a simple linear regression analysis.

Results

Expression of CLDN6 on EpCAM⁺ cancer cells in ascites of ovarian cancer patients

To investigate the cell surface CLDN6 expression on primary ovarian cancer cells, mononuclear cells from ascites of 32 ovarian cancer patients were stained with antibodies for CLDN6, EpCAM, and CD45 and analyzed by flow cytometry (Figure 1). In most ascites specimens, CD45⁺ cells were highly infiltrated, whereas percentages of EpCAM⁺ cells were relatively low (Supplemental Fig S1). CLDN6 expression on EpCAM⁺ and CD45⁺ cells was determined using a Fluorescence Minus One (FMO) control (Figure 1a). CLDN6 was significantly but variably expressed on EpCAM⁺ cells but not on CD45⁺ cells (Figure 1b). The mean frequency of CLDN6 expression on EpCAM⁺ cells was 30% (range 0.2–80.0%) while it was 0.17% on CD45⁺ cells (0–3.0%). CLDN6 expression on EpCAM⁻CD45⁻ cells was negligible (data not shown). There was no correlation between percentages of EpCAM⁺ cells and CLDN6⁺ expression levels (Figure 1c). The lack of expression in normal tissues,¹⁶ but frequent and tumor-specific expression suggest that CLDN6 is a promising target for immunotherapy for ovarian cancer patients.

Detection of spontaneous T-cell responses against CLDN6 in ovarian cancer patients

To investigate spontaneously induced T-cell response against CLDN6 in ovarian cancer patients, CLDN6-specific response was tested following in vitro stimulation of PBMC with a pool of overlapping peptides. One (P01) out of 17 patients showed a specific response against CLDN6 based on the criteria for a positive response (the number of spots is ≥ 50 and twice higher than background) (Figure 2a). For patient P01, we found that both CD8⁺ and CD4⁺ T cells were reactive to CLDN6 pooled peptides by intracellular cytokine staining (Figure 2b).

Generation of CLDN6-specific T cells by TCR gene engineering

To clone TCR genes, total RNA was extracted from CLDN6-specific CD4⁺ and CD8⁺ T cells purified by sorting IFN- γ -producing cells from the patient P01 (Figure 2b). The coding regions of TCR α and β chain genes that were amplified by RT-PCR from sorted CD8⁺ or CD4⁺ T cells were assembled as an expression cassette in a retroviral plasmid vector by our TCR-expressing retroviral vector construction method.²⁶ DNA fingerprinting of TCR inserts in randomly selected plasmid clones by restriction enzyme digestion showed that the majority of plasmid clones contained the same TCR inserts, indicating oligoclonal CD4⁺ and CD8⁺ T-cell responses dominated by a single clone against CLDN6 (Supplemental Fig S2). Retroviral particles produced from PG13 virus-producing cells were used to transduce TCR genes to T cells derived from healthy donor PBMC. According to the Sanger sequencing data of dominant TCR-expressing plasmids (Supplemental Data 1), we identified TCR V β subtype of CLDN6-specific CD8⁺ T cells and CD4⁺ T cells as V β 8 and V β 7.1, respectively. TCR-transduced T cells were stained with those TCR V β antibodies and the transduction efficiency was determined to be >85% (Figure 3a). Reactivity of TCR-transduced T cells to CLDN6 pooled peptides was confirmed by intracellular cytokine staining (Figure 3b and Figure 3c). While both CD8⁺ and CD8⁻ (i.e. CD4⁺) T cells transduced with TCR derived from CD4⁺ T cells (CD4-TCR) strongly produced IFN- γ against peptides, CD8⁻ T cells transduced with CD8-TCR showed a weak response, indicating that CD8-TCR requires CD8 co-ligation for the recognition (Figure 3c). Transduction efficiency and reactivity of TCR-transduced T cells were similar in three healthy donor-derived PBMCs (Supplemental Fig S3).

Identification of epitope and HLA restriction for CLDN6-specific CD8⁺ and CD4⁺ T cells

By utilizing TCR-transduced T cells, we characterized a peptide region recognized by CLDN6-specific T cells. By determining reactivity of CD8-TCR- and CD4-TCR-transduced T cells against individual peptides covering the whole CLDN6 protein, the epitope for CD4-TCR was determined to be in the N-terminal CLDN6_{1–20} peptide in a signal peptide region, while the epitope for CD8-TCR is in an overlapping region of peptides CLDN6_{121–140} and CLDN6_{131–150}, as CD8-TCR-transduced T cells similarly recognized these peptides (Figure 4a). The HLA restriction element of CD8-TCR was determined by using K562 cells that were transduced with the patient P01's HLA class I. HLA class I types of the patient P01 were determined to be HLA-A2, -A*24:02, -B*27, and -Cw*01 (Table 1). HLA-negative K562 cells were retrovirally transduced with these HLA genes, pulsed with CLDN6 peptides, and cocultured with CD8-TCR-transduced T cells. CD8-TCR was found to be restricted to A2 as it showed strong reactivity against peptides only when pulsed on A2-transduced K562 cells (Figure 4b). Based on A2 and peptide region recognized by CD8-TCR, A2-binding epitope of CLDN6 was predicted as TLIPVCWTA (CLDN6_{132–140}) using the SYFPEITHI

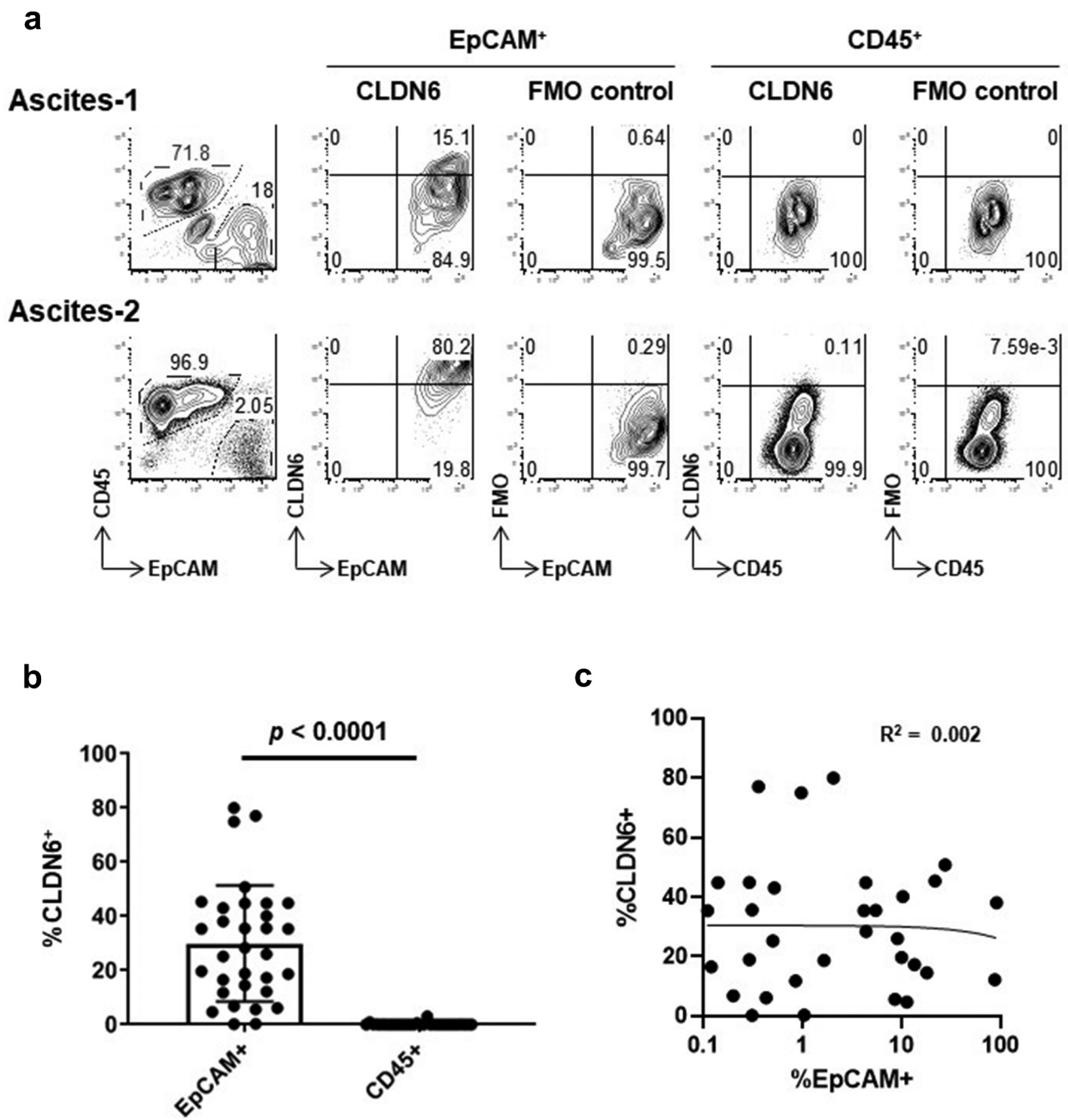


Figure 1. CLDN6 expression on EpCAM⁺ cancer cells and CD45⁺ lymphocytes in ascites fluids of ovarian cancer patients. (a) Flow cytometry analysis of ascites mononuclear cells. CLDN6 expression was analyzed on EpCAM⁺ or CD45⁺ cells. Gating of CLDN6 was determined based on FMO controls. (b) Percentages of CLDN6 expression on EpCAM⁺ or CD45⁺ cells from 32 patients were plotted with mean \pm standard deviation. (c) A relationship between CLDN6 expression levels and percentages of EpCAM⁺ cells in ascites was analyzed by a linear regression analysis.

algorithm.²⁷ TCR binding of the predicted epitope was demonstrated by A2/CLDN6₁₃₂₋₁₄₀ tetramer staining (Figure 4c). Using the CLDN6₁₃₂₋₁₄₀ peptide, we tested TCR avidity against the peptide and found that CD8-TCR-transduced T cells efficiently recognized 1 nM peptide (Figure 4d). The HLA restriction element of CD4-TCR was identified as HLA-DR*04:04 based on the peptide reactivity using a panel of partially HLA-matched EBV-B cells as antigen-presenting cells (Figure 4e and Table 1). The HLA restriction of CD4-TCR was confirmed against CLDN6₁₋₂₀-pulsed DR4-overexpressing A2780 cell line (Supplemental Fig S7).

Cytokine production from CD4-TCR- and CD8-TCR-transduced CD4⁺ and CD8⁺ T cells

The major role of CD4⁺ T cells is enhancing anti-tumor immunity by producing cytokines. Because CD4-TCR provided function to both CD4⁺ and CD4⁻ T cells, we further characterized cytokine-producing patterns for CD4⁺ and CD8⁺ T cells (Figure 5 and Supplemental Fig S4). CD4-TCR-transduced T cells were stimulated with CLDN6₁₋₂₀-pulsed P01 EBV-B cells and analyzed TNF- α , GM-CSF and IL-2 as well as IFN- γ gated on V β 7.1⁺CD4⁺ or V β 7.1⁺CD8⁺ T cells. As shown in Supplemental Fig S4, cytokines

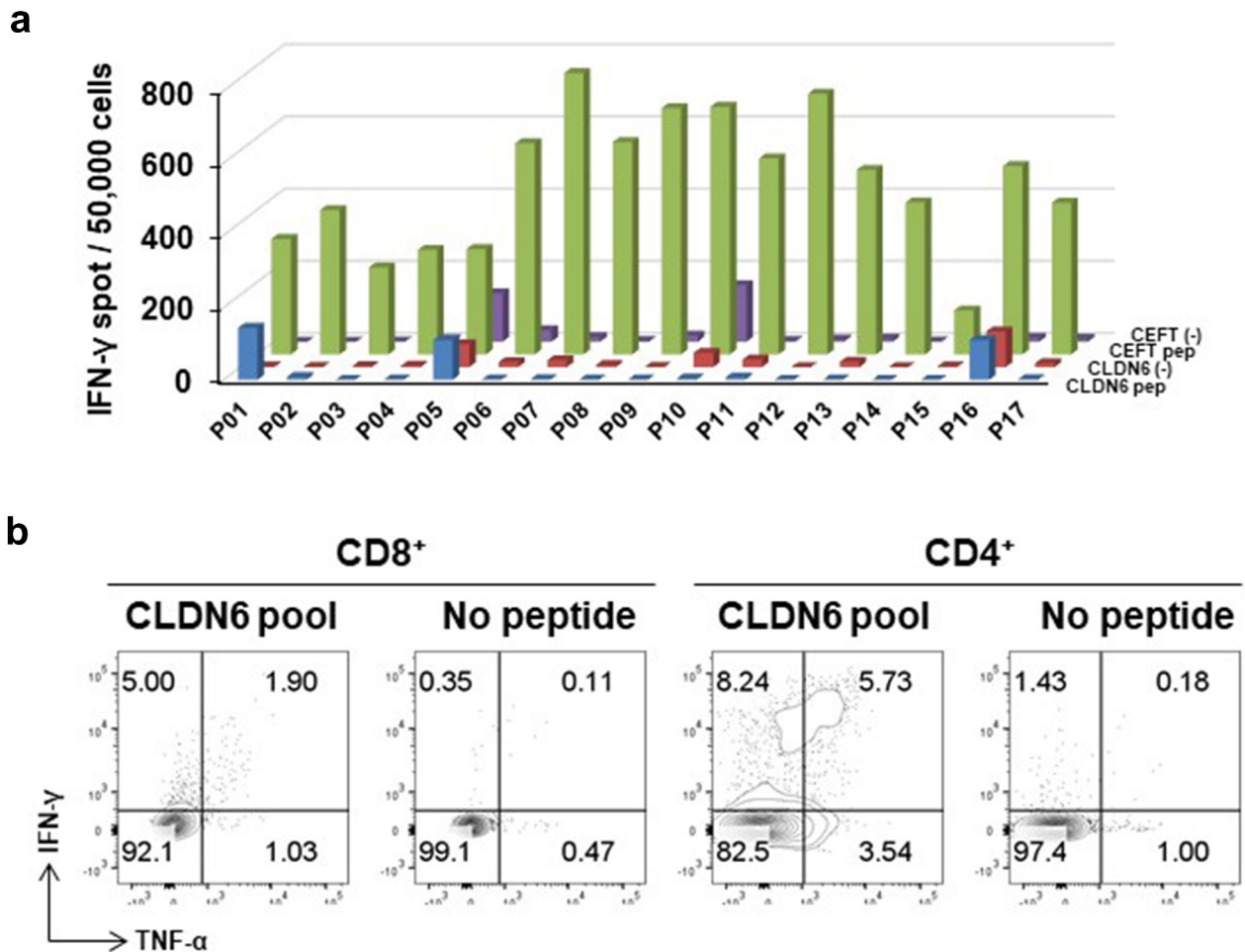


Figure 2. Analysis of CLDN6-specific T-cell response in ovarian cancer patients. (a) CLDN6- or CEFT-specific T-cell responses from 17 patients (P01–P17) were determined by ELISPOT assays. PBMC were cultured with CLDN6 or CEFT peptides for 13–15 d and IFN- γ spots in the presence (pep) or absence (-) of the respective peptide were enumerated. (b) IFN- γ and TNF- α production from CD8⁺ or CD4⁺ T cells from P01 against CLDN6 peptide-pulsed or -unpulsed autologous P01 EBV-B cells was analyzed by intracellular cytokine staining.

were produced from V β 7.1⁺ T cells on both CD4⁺ and CD8⁺ T cells. While 60% of CD4⁺ T cells produced more than three cytokines, only 9% of CD8⁺ T cells showed multi-cytokine production (Figure 5a). CD8-TCR-transduced T cells were also analyzed for multi-cytokine production (Figure 5b). CD8⁺ T cells produced not only IFN- γ but also TNF- α , GM-CSF, and IL-2 similar to CD4⁺ T cells transduced with CD4-TCR. This result suggests that CD4 or CD8 co-ligation is required to produce multiple cytokines.

Cancer cell recognition by CD8-TCR-transduced T cells

We next assessed the reactivity of CD8-TCR-transduced T cells against CLDN6⁺ ovarian cancer cells. To compare the reactivity of TCR-transduced T cells with that of naturally occurring CLDN6-specific CD8⁺ T cells, a part of sorted CLDN6-specific CD8⁺ T cells was polyclonally expanded. As expected, CLDN6-specific CD8⁺ T-cell line expressed TCR V β 8 and showed strong reactivity against CLDN6 pooled peptide-pulsed autologous EBV-B cells (Supplemental Fig S5A and S5B).

We investigated expression of CLDN6 and HLA-A2 on the patient P01-derived EOC cell line and an established ovarian cancer cell line PA-1 that was reported to highly express CLDN6 by flow cytometry.²¹ As PA-1 cells is A2 negative, A2 was retrovirally transduced. We found that both ovarian cancer cell lines express CLDN6 (Figure 6a). Although the patient P01 was A2⁺, the EOC cell line showed marginal cell surface A2 expression (Figure 6a). To upregulate HLA expression, the cancer cells were treated with IFN- γ . IFN- γ treatment upregulated A2 on the EOC and A2-transduced PA-1 cells but did not affect the intensity of CLDN6 expression (Figure 6a). When T cells were cocultured with these cancer cells, the P01 CD8⁺ T-cell line recognized EOC cells only after treatment with IFN- γ (Figure 6b). CD8-TCR-transduced T cells recognized IFN- γ -treated EOC cells with similar efficiency to the P01 CD8⁺ T-cell line (Figure 6c). CD8-TCR-transduced T cells also showed strong reactivity against A2-transduced and IFN- γ -treated PA-1 cells. The results indicate that CD8-TCR transduced T cells can recognize cancer cells expressing CLDN6 and A2.

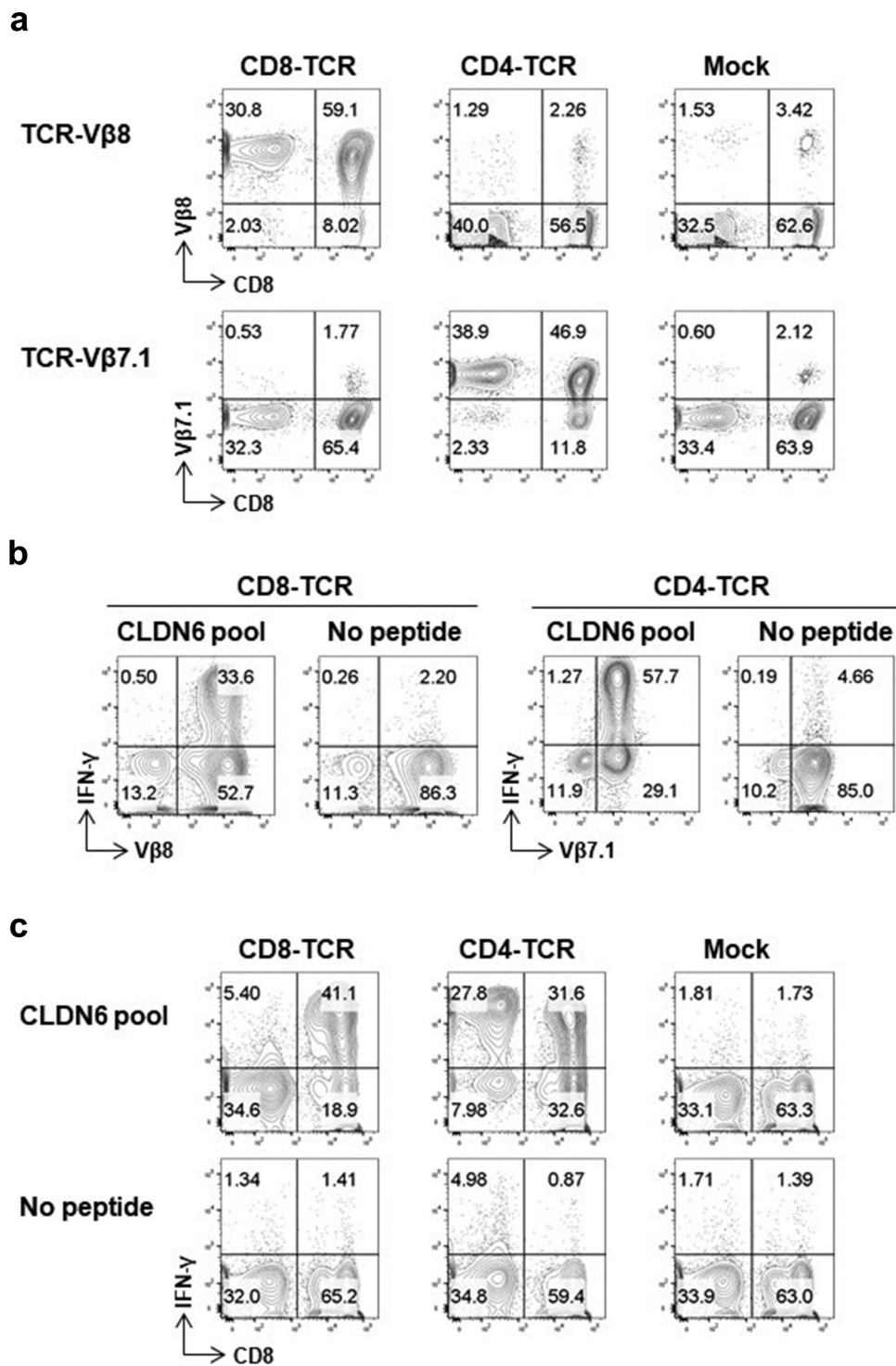


Figure 3. Establishment of CLDN6-specific TCR gene-engineered T cells. Healthy donor PBMC were retrovirally transduced with TCR genes from CLDN6-specific CD8⁺ T cells (CD8-TCR) or CD4⁺ T cells (CD4-TCR) derived from the patient P01 (Figure 2b). (a) Transduction efficiency of TCR- or mock-transduced T cells were analyzed by TCR-Vβ8 or -Vβ7.1 antibody. (b, c) IFN-γ production against CLDN6 pooled peptide-pulsed or -unpulsed P01 EBV-B cells were analyzed by intracellular cytokine staining. IFN-γ production from Vβ8 or Vβ7.1 positive/negative cells (b), and CD8 positive/negative cells (c) is shown.

Recognition of CLDN6-overexpressing cancer cells by CD4-TCR and CD8-TCR-transduced T cells

To determine if CD4-TCR-transduced T cells directly recognize CLDN6-expressing cancer cells, we generated A2780 cells co-expressing CLDN6, HLA-DR4, and CIITA genes (DR4/CLDN6). For comparison, A2780 cells were similarly

engineered with CLDN6 and HLA-A2 genes (A2/CLDN6) (Figure 7a and Supplemental Fig S6). CD8-TCR-transduced T cells strongly recognized A2/CLDN6-overexpressing cancer cells without IFN-γ treatment (Figure 7b). CD4-TCR-transduced T cells moderately but specifically recognized DR4/CLDN6-overexpressing, but not A2/CLDN6-overexpressing, cancer cells. Because cytotoxic activity by

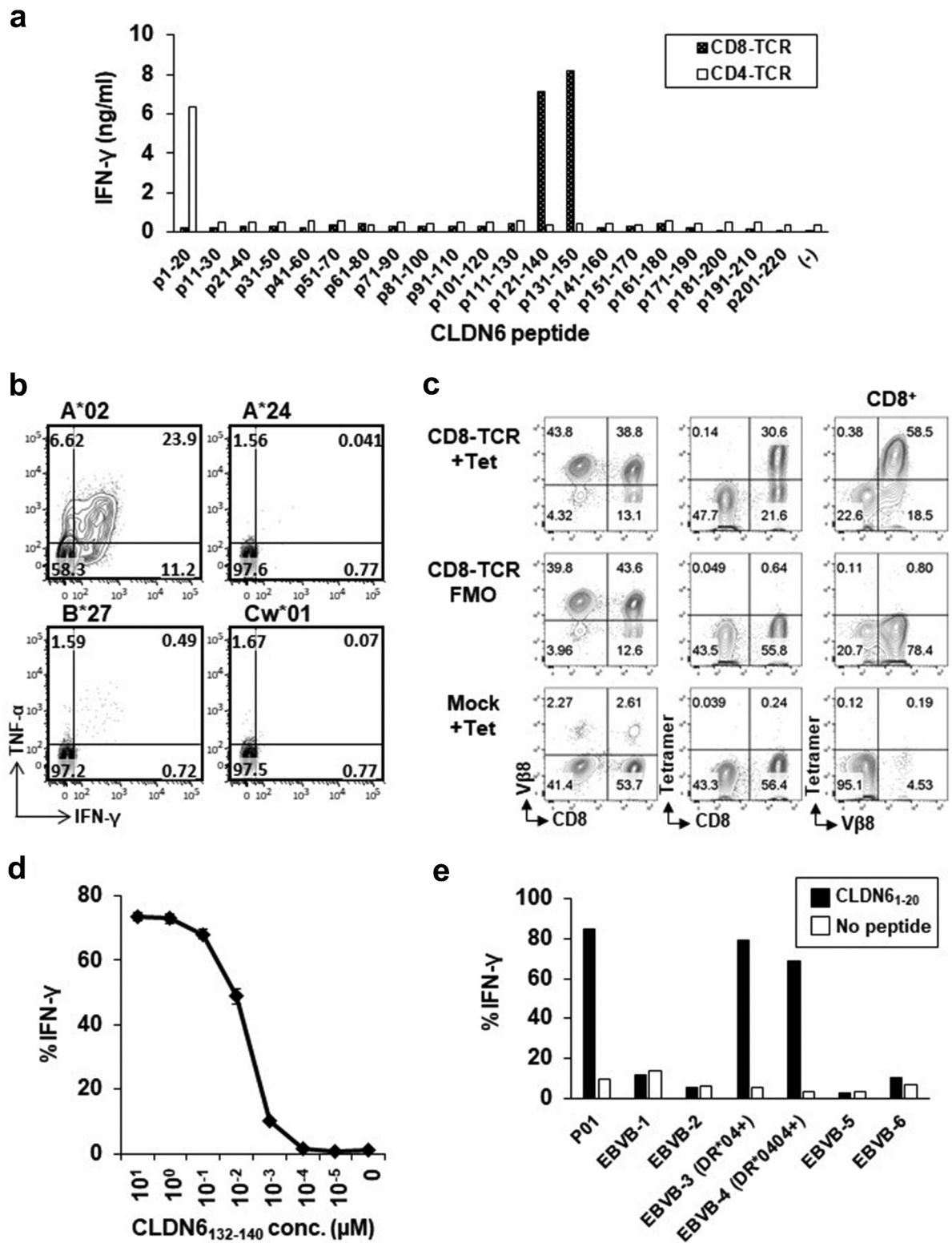


Figure 4. Characterization of CLDN6-specific TCR gene-engineered T cells. (a) TCR gene-engineered T cells were cocultured with 20-mer CLDN6 overlapping peptide-pulsed or -unpulsed P01 EBV-B cells for 24 h. IFN- γ level in the culture supernatant was measured by ELISA. (b) Cytokine production from CD8-TCR-engineered CD8⁺ T cells against CLDN6 peptide-pulsed K562 cells transduced with indicated HLA genes was analyzed by intracellular cytokine staining. (c) CD8-TCR- or mock-transduced T cells were stained with or without HLA-A2/CLDN6₁₃₂₋₁₄₀ tetramer along with anti-V β 8 antibody. (d) IFN- γ production on CD8-TCR-transduced CD8⁺ T cells against different concentration of CLDN6₁₃₂₋₁₄₀ was analyzed by intracellular cytokine staining. Error bars indicate standard deviation of technical duplicates. (e) IFN- γ production on CD4-TCR-transduced CD4⁺ T cells stimulated with CLDN6₁₋₂₀-pulsed or -unpulsed partially HLA-matched EBV-B cells (Table 1) was analyzed by intracellular cytokine staining.

CD8⁺ T cells play a critical role in tumor regression by T-cell therapy, we tested cytotoxicity of CD8-TCR-transduced T cells.

In a 4-h calcein-AM release assay, CD8-TCR-transduced T cells indicated specific cytotoxicity against A2/CLDN6-

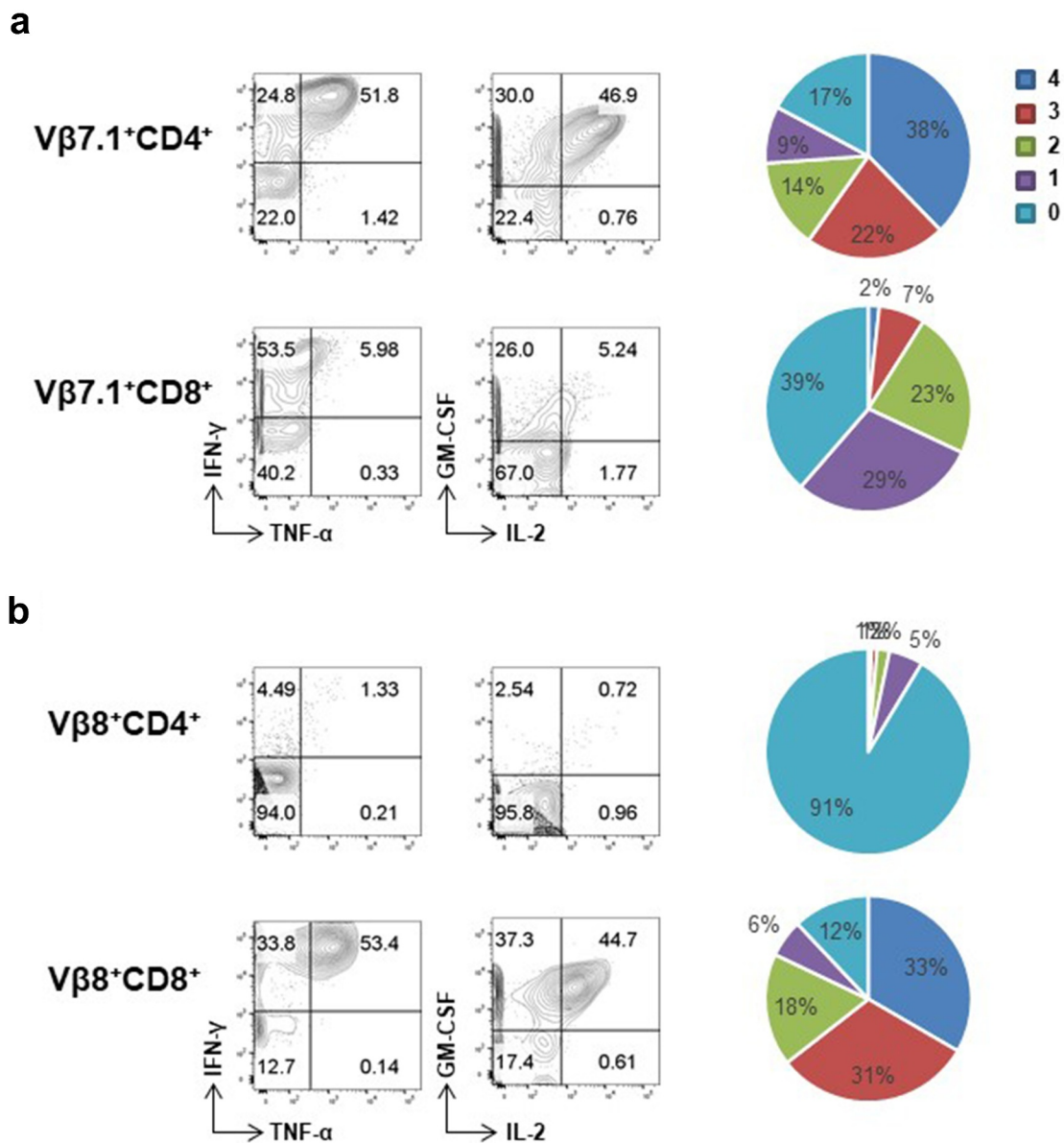


Figure 5. Multi-cytokine production from TCR-transduced CD4⁺ or CD8⁺ T cells. (a) CD4-TCR-transduced T cells was stimulated with a CLDN6_{1–20} peptide pulsed on the patient P01-derived EBV-B cells and cytokine production was analyzed on Vβ7.1⁺CD4⁺ or Vβ7.1⁺CD8⁺ T cells. (b) CD8-TCR-transduced T cells was stimulated with a CLDN6_{132–140} peptide pulsed on the patient P01-derived EBV-B cells and cytokine production was analyzed on Vβ8⁺CD4⁺ or Vβ8⁺CD8⁺ T cells. Pie charts indicate percentages of cells producing 4/3/2/1/0 cytokines.

overexpressing cancer cells (Figure 7c). When CD8-TCR-transduced T cells were co-cultured with cancer cells for 18 h, apoptotic cell death was induced in cancer cells (Figure 7d). These results demonstrate that both epitopes that were recognized by CD4-TCR and CD8-TCR are naturally processed and presented on DR4 and A2, respectively, of CLDN6-expressing cancer cells.

Discussion

Adoptive transfer of tumor antigen-specific T cells is a promising strategy to eradicate malignant cells. Although a large number of antigen-specific T cells can be rapidly generated by genetic engineering of patient-derived T cells

using TCR or CAR genes, this technology is limited by target antigens.^{30,31} To prevent on-target off-tumor toxicity, target antigens should not be expressed on essential normal tissues. As of now, NY-ESO-1 is one of the few promising antigenic targets for TCR-engineered T cells in patients with solid tumors, and ACT using NY-ESO-1-specific TCR-engineered T cells has shown efficacy and safety in clinical trials.^{5,32,33} However, because of heterogeneity in NY-ESO-1 expression and HLA types, eligible patients for the therapy are limited. Extensive efforts are ongoing to identify additional TCR/CAR targets that could provide ACT for a broad range of patients. In this study, we identified TCR genes specific to CLDN6, which is a potential target antigen with frequent expression in cancer patients.

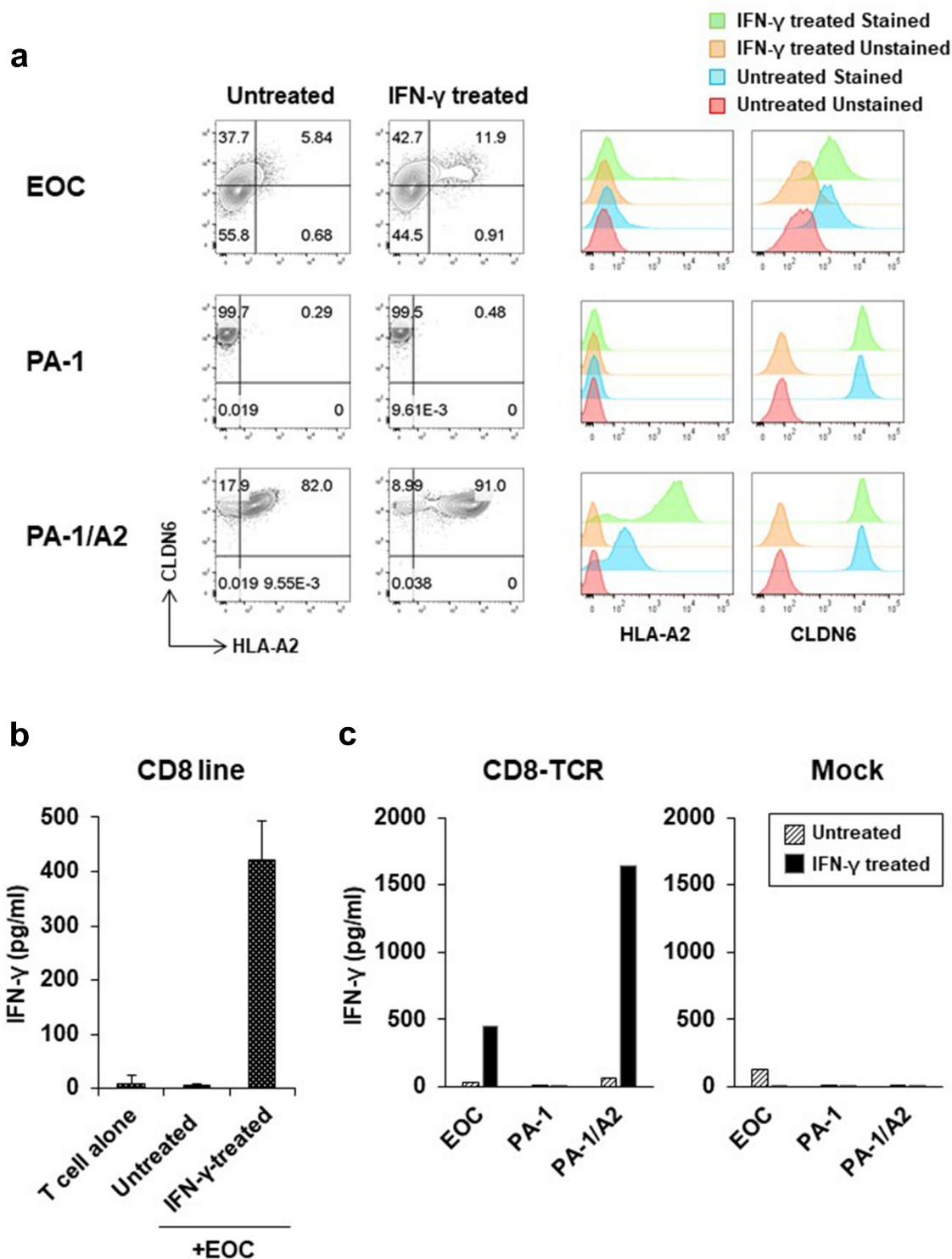


Figure 6. Cancer cell recognition by CLDN6-specific TCR gene-engineered T cells. (a) CLDN6 and HLA-A2 expression on ovarian cancer cells with or without IFN- γ treatment was determined by flow cytometry. Expression was shown as quadrant gating based on the unstained control (left) and histogram including unstained controls (right). PA-1/A2: PA-1 transduced with HLA-A2. (b, c) CLDN6-specific CD8⁺ T-cell line (b), or CD8-TCR- or mock-transduced T cells (c) were cocultured with or without IFN- γ -treated or -untreated cancer cells and IFN- γ level in the culture supernatant was measured by ELISA. Error bars indicate standard deviation of technical duplicates.

CLDN6 is an oncofetal protein expressed on ES cells associated with epithelialization as well as solid cancer cells including ovarian cancer.^{18,19,21} Our flow cytometry analysis of ascites cells demonstrated that the majority (94%, 30 out of

32 samples) of patients' EpCAM⁺ cancer cells express CLDN6 at varied levels. Expression was low (<10%) in 13%, moderate (10–40%) in 53% and strong (>40%) in 33% of 30 samples. CLDN6 expression was observed in 69.4% of ovarian

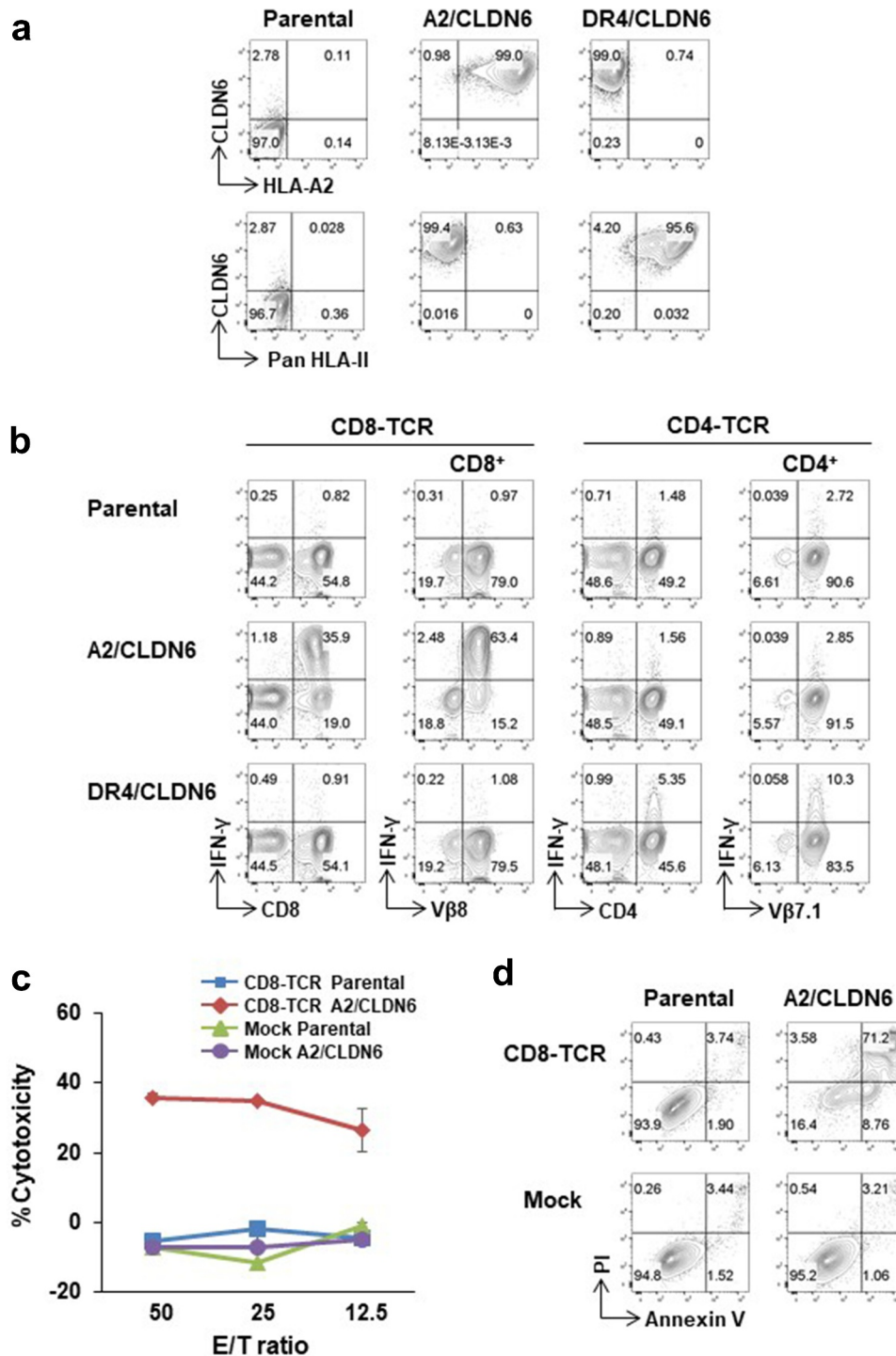


Figure 7. Recognition of CLDN6-overexpressing cancer cells by CD8-TCR- and CD4-TCR-transduced T cells. (a) Expression of CLDN6, HLA-A2, and HLA-class II on the parental, A2/CLDN6-overexpressing, and DR4/CLDN6-overexpressing A2780 ovarian cancer cell line was determined by flow cytometry. (b) IFN- γ production from CD8-TCR or CD4-TCR-transduced T cells against indicated A2780 cell lines was determined by intracellular cytokine staining. (c) Cytotoxicity of CD8-TCR or mock-transduced T cells against indicated A2780 cell lines was determined by a 4-h calcein-AM release assay. (d) Induction of apoptotic cell death in cancer cells by CD8-TCR-transduced T cells was determined by annexin V and PI staining after 18-h coculture.

carcinomas and in 34.6% of ovarian serous adenomas by IHC.¹⁸ Different sensitivities of IHC and flow cytometry can explain higher expression rate in this study. Moreover, CLDN6 expression is reported at a frequency of 7–100% in other solid tumors such as lung,³⁴ endometrial,¹⁷ gastric,^{35–37} and testicular cancers.²⁰

Despite the frequent expression, spontaneous T-cell response against CLDN6 in peripheral blood was detected in only 1 out of 17 (6%) ovarian cancer patients. A patient-derived ovarian cancer cell line from the patient (P01) who showed spontaneous anti-CLDN6 T-cell response expressed significant level of CLDN6 (Figure 6). It is possible that high

level of CLDN6 expression is required to induce spontaneous T-cell response. However, because CLDN6 expression levels of ascitic cells or tumor tissues from patients including P01 who provided PBMC for T-cell analyses were not investigated in this study, further analyses are required to determine the correlation between T-cell response and CLDN6 expression level. In addition, to define the immunogenicity of CLDN6 and anti-tumor role of CLDN6-specific immune response in cancer patients, analyzing more samples including tumor-infiltrating T cells is needed. In our previous study, we were able to detect NY-ESO-1-specific and neoantigen-specific T cells frequently in the tumor microenvironment.^{38,39}

Limited induction of T-cell response against CLDN6 in cancer patients potentially indicates low immunogenicity of the antigen, which can be overcome by ACT of genetically engineered CLDN6-specific T cells. As CLDN6 is expressed on the cell surface of cancer cells, it is a promising target for antibody-based and CAR-T therapies.^{16,21} An early phase clinical trial testing CLDN6-specific CAR-T cells for solid tumors is ongoing [ClinicalTrials.gov Identifier: NCT04503278]. Once safety and efficacy are demonstrated, CLDN6-specific CAR-T cell could be a promising therapy for cancer patients with solid tumors including ovarian cancer because of the frequent antigen expression. In comparison with CAR-T cells, TCR-engineered T cells have several potential advantages including (i) lower immunogenicity of TCR transgenes; (ii) general safety profile because it is derived from naturally occurring T cells responded to tumor or presented by antigen-presenting cells (APCs) in patients; and (iii) reactivation of memory T-cell response through APC,^{40,41} which are critical for long-term in vivo maintenance of engineered T cells for immunosurveillance against recurrent tumors.

A strength of the present study is that we successfully identified TCR genes specific to CLDN6 from both CD8⁺ and CD4⁺ T cells and demonstrated specific reactivity to CLDN6⁺ cancer cells by TCR-transduced T cells. We also identified HLA-A2-binding epitope as CLDN6_{132–140} and CD8-TCR-transduced T cells were stained with tetramer. These CLDN6-specific TCR genes are considered to be safe because they spontaneously occurred and expanded in a patient. In addition, the CD8-TCR exhibited specific cytotoxic activity and is restricted by HLA-A2, which is the most frequent HLA class I allele in the Caucasian population; thus, this TCR could be further developed as off-the-shelf therapeutic TCR gene for clinical use. However, CD8-TCR in this study was found to be CD8-dependent because only TCR-transduced CD8⁺ T cells but not CD4⁺ T cells showed the reactivity against a peptide. T cells with higher affinity TCR could be identified in patients⁸ or affinity enhancement by gene modification⁴² although careful assessment of off-target toxicity is very important before testing in clinical trials. In contrast to CD8-TCR, CD4-TCR is considered as high avidity because the response was observed in a CD4 coreceptor-independent manner. The frequency of HLA-DR*04:04 is about 6% of the Caucasian population in the US (Allele Frequency Net Database). Moreover, because CD4-TCR showed direct recognition of DR4⁺CLDN6⁺ cancer cells,

CD4-TCR-transduced T cells are expected to enhance CD8⁺ T-cell functions.⁷ We also demonstrated that CD4 or CD8 co-ligation is required to obtain multi-cytokine producing ability, which will be considered to co-transduction of those molecules along with TCR genes for cell manufacturing.⁴³

In summary, we for the first time demonstrated naturally occurring CD8⁺ and CD4⁺ T-cell responses against CLDN6 in cancer patients and successful generation of CLDN6-specific TCR-transduced T cells. Together with high expression rate of CLDN6 on cancer cells, our findings suggest that CLDN6 is a promising target for TCR-engineered T-cell therapy for ovarian cancer patients.

Availability of Data and Material

All relevant data is included in the manuscript.

Acknowledgments

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Author contributions

JM and TT designed the study. JM and TT conducted experiments. JM, SL, KO, and TT analyzed and interpreted data. JM, KO, and TT wrote the manuscript. All authors reviewed and approved the manuscript.

Disclosure statement

KO is a co-founder of Tactiva Therapeutics. JM, TT, and KO are inventors of a provisional patent application by Roswell Park for TCR genes. Other authors have no conflict of interest.

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ORCID

Junko Matsuzaki  <http://orcid.org/0000-0002-2555-1234>
Takemasa Tsuji  <http://orcid.org/0000-0001-7563-2211>

References

1. Dossett ML, Teague RM, Schmitt TM, Tan X, Cooper LJ, Pinzon C, Greenberg PD. Adoptive immunotherapy of disseminated leukemia with TCR-transduced, CD8⁺ T cells expressing a known endogenous TCR. *Mol Ther*. 2009;17:742–749. doi:10.1038/mt.2008.300
2. Powell DJ, Dudley ME, Robbins PF, Rosenberg SA. Transition of late-stage effector T cells to CD27⁺ CD28⁺ tumor-reactive effector memory T cells in humans after adoptive cell transfer therapy. *Blood*. 2005;105:241–250. doi:10.1182/blood-2004-06-2482

3. Clay TM, Custer MC, Sachs J, Hwu P, Rosenberg SA, Nishimura MI. Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity. *J Immunol.* 1999;163:507–513.
4. Kuwana Y, Asakura Y, Utsunomiya N, Nakanishi M, Arata Y, Itoh S, Nagase F, Kurosawa Y. Expression of chimeric receptor composed of immunoglobulin-derived V regions and T-cell receptor-derived C regions. *Biochem Biophys Res Commun.* 1987;149:960–968. doi:10.1016/0006-291x(87)90502-x
5. Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME, Wunderlich JR, Nahvi AV, Helman LJ, Mackall CL, et al. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol.* 2011;29:917–924. doi:10.1200/JCO.2010.32.2537
6. Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, June CH. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med.* 2011;3:95ra73. doi:10.1126/scitranslmed.3002842
7. Matsuzaki J, Tsuji T, Luescher IF, Shiku H, Mineno J, Okamoto S, Old LJ, Shrikant P, Gnjatich S, Odunsi K. Direct tumor recognition by a human CD4(+) T-cell subset potentially mediates tumor growth inhibition and orchestrates anti-tumor immune responses. *Sci Rep.* 2015;5:14896. doi:10.1038/srep14896
8. Matsuzaki J, Tsuji T, Chodon T, Ryan C, Koya RC, Odunsi K. A rare population of tumor antigen-specific CD4(+)CD8(+) double-positive alphabeta T lymphocytes uniquely provide CD8-independent TCR genes for engineering therapeutic T cells. *J Immunother Cancer.* 2019;7:7. doi:10.1186/s40425-018-0467-y
9. Stadtmauer EA, Fajt TH, Lowther DE, Badros AZ, Chagin K, Dengel K, Iyengar M, Melchiori L, Navenot J, Norry E, et al. Long-term safety and activity of NY-ESO-1 SPEAR T cells after autologous stem cell transplant for myeloma. *Blood Adv.* 2019;3:2022–2034. doi:10.1182/bloodadvances.2019000194
10. Rapoport AP, Stadtmauer EA, Binder-Scholl GK, Golubeva O, Vogl DT, Lacey SF, Badros AZ, Garfall A, Weiss B, Finklestein J, et al. NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. *Nat Med.* 2015;21:914–921. doi:10.1038/nm.3910
11. Chen YT, Scanlan MJ, Sahin U, Tureci O, Gure AO, Tsang S, Williamson B, Stockert E, Pfreundschuh M, Old LJ, et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci U S A.* 1997;94:1914–1918. doi:10.1073/pnas.94.5.1914
12. Odunsi K, Jungbluth AA, Stockert E, Qian F, Gnjatich S, Tammela J, Intengan M, Beck A, Keitz B, Santiago D, et al. NY-ESO-1 and LAGE-1 cancer-testis antigens are potential targets for immunotherapy in epithelial ovarian cancer. *Cancer Res.* 2003;63:6076–6083.
13. Gnjatich S, Nishikawa H, Jungbluth AA, Gure AO, Ritter G, Jager E, Knuth A, Chen YT, Old LJ. NY-ESO-1: review of an immunogenic tumor antigen. *Adv Cancer Res.* 2006;95:1–30. doi:10.1016/S0065-230X(06)95001-5
14. Woloszynska-Read A, Mhawech-Fauceglia P, Yu J, Odunsi K, Karpf AR. Intertumor and intratumor NY-ESO-1 expression heterogeneity is associated with promoter-specific and global DNA methylation status in ovarian cancer. *Clin Cancer Res.* 2008;14:3283–3290. doi:10.1158/1078-0432.CCR-07-5279
15. Thomas R, Al-Khadairi G, Roelands J, Hendrickx W, Dermime S, Bedognetti D, Decock J. NY-ESO-1 Based Immunotherapy of Cancer: current Perspectives. *Front Immunol.* 2018;9:947. doi:10.3389/fimmu.2018.00947
16. Reinhard K, Rengstl B, Oehm P, Michel K, Billmeier A, Hayduk N, Klein O, Kuna K, Ouchan Y, Wöll S, et al. An RNA vaccine drives expansion and efficacy of claudin-CAR-T cells against solid tumors. *Science.* 2020;367:446–453. doi:10.1126/science.aay5967
17. Kojima M, Sugimoto K, Tanaka M, Endo Y, Kato H, Honda T, Furukawa S, Nishiyama H, Watanabe T, Soeda S, et al. Prognostic significance of aberrant claudin-6 expression in endometrial cancer. *Cancers (Basel).* 2020;12:2748. doi:10.3390/cancers12102748
18. Wang L, Jin X, Lin D, Liu Z, Zhang X, Lu Y, Liu Y, Wang M, Yang M, Li J, et al. Clinicopathologic significance of claudin-6, occludin, and matrix metalloproteinases -2 expression in ovarian carcinoma. *Diagn Pathol.* 2013;8:190. doi:10.1186/1746-1596-8-190
19. Turksen K, Troy TC. Claudin-6: a novel tight junction molecule is developmentally regulated in mouse embryonic epithelium. *Dev Dyn.* 2001;222:292–300. doi:10.1002/dvdy.1174
20. Ushiku T, Shinozaki-Ushiku A, Maeda D, Morita S, Fukayama M. Distinct expression pattern of claudin-6, a primitive phenotypic tight junction molecule, in germ cell tumours and visceral carcinomas. *Histopathology.* 2012;61:1043–1056. doi:10.1111/j.1365-2559.2012.04314.x
21. Stadler CR, Bahr-Mahmud H, Plum LM, Schmoltd K, Kolsch AC, Tureci O, Sahin U. Characterization of the first-in-class T-cell-engaging bispecific single-chain antibody for targeted immunotherapy of solid tumors expressing the oncofetal protein claudin 6. *Oncoimmunology.* 2016;5:e1091555. doi:10.1080/2162402X.2015.1091555
22. Kong FE, Li GM, Tang YQ, Xi S-Y, Loong JHC, Li -M-M, Li H-L, Cheng W, Zhu W-J, Mo J-Q, et al. Targeting tumor lineage plasticity in hepatocellular carcinoma using an anti-CLDN6 antibody-drug conjugate. *Sci Transl Med.* 2021;13. doi:10.1126/scitranslmed.abb6282
23. Mahipal A, Ejadi S, Gnjatich S, Kim-Schulze S, Lu H, Ter Meulen JH, Kenney R, Odunsi K. First-in-human phase I dose-escalating trial of G305 in patients with advanced solid tumors expressing NY-ESO-1. *Cancer Immunol Immunother.* 2019;68:1211–1222. doi:10.1007/s00262-019-02331-x
24. Tsuji T, Sabbatini P, Jungbluth AA, Ritter E, Pan L, Ritter G, Ferran L, Spriggs D, Salazar AM, Gnjatich S, et al. Effect of montanide and poly-ICLC adjuvant on human self/tumor antigen-specific CD4 + T cells in phase I overlapping long peptide vaccine trial. *Cancer Immunol Res.* 2013;1:340–350. doi:10.1158/2326-6066.CIR-13-0089
25. Tsuji T, Matsuzaki J, Kelly MP, Ramakrishna V, Vitale L, He L-Z, Keler T, Odunsi K, Old LJ, Ritter G, et al. Antibody-targeted NY-ESO-1 to mannose receptor or DEC-205 in vitro elicits dual human CD8 + and CD4 + T cell responses with broad antigen specificity. *J Immunol.* 2011;186:1218–1227. doi:10.4049/jimmunol.1000808
26. Tsuji T, Yoneda A, Matsuzaki J, Miliotto A, Ryan C, Koya RC, Odunsi K. Rapid construction of antitumor T-cell receptor vectors from frozen tumors for engineered T-cell therapy. *Cancer Immunol Res.* 2018;6:594–604. doi:10.1158/2326-6066.CIR-17-0434
27. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics.* 1999;50:213–219. doi:10.1007/s002510050595
28. Dolton G, Tungatt K, Lloyd A, Bianchi V, Theaker SM, Trimby A, Holland CJ, Donia M, Godkin AJ, Cole DK, et al. More tricks with tetramers: a practical guide to staining T cells with peptide-MHC multimers. *Immunology.* 2015;146:11–22. doi:10.1111/imm.12499
29. Yusa K, Zhou L, Li MA, Bradley A, Craig NL. A hyperactive piggyBac transposase for mammalian applications. *Proc Natl Acad Sci U S A.* 2011;108:1531–1536. doi:10.1073/pnas.1008322108
30. Houot R, Schultz LM, Marabelle A, Kohrt H. T-cell-based immunotherapy: adoptive cell transfer and checkpoint inhibition. *Cancer Immunol Res.* 2015;3:1115–1122. doi:10.1158/2326-6066.CIR-15-0190
31. Rosenberg SA, Restifo NP. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science.* 2015;348:62–68. doi:10.1126/science.aaa4967

32. D'Angelo SP, Melchiori L, Merchant MS, Bernstein D, Glod J, Kaplan R, Grupp S, Tap WD, Chagin K, Binder GK, et al. Antitumor activity associated with prolonged persistence of adoptively transferred NY-ESO-1 c259 T cells in synovial sarcoma. *Cancer Discov.* 2018;8:944–957. doi:10.1158/2159-8290.Cd-17-1417
33. Ramachandran I, Lowther DE, Dryer-Minnerly R, Wang R, Fayngerts S, Nunez D, Betts G, Bath N, Tipping AJ, Melchiori L, et al. Systemic and local immunity following adoptive transfer of NY-ESO-1 SPEAR T cells in synovial sarcoma. *J Immunother Cancer.* 2019;7:276. doi:10.1186/s40425-019-0762-2
34. Micke P, Mattsson JS, Edlund K, Lohr M, Jirstrom K, Berglund A, Botling J, Rahnenfuehrer J, Marincevic M, Pontén F, et al. Aberrantly activated claudin 6 and 18.2 as potential therapy targets in non-small-cell lung cancer. *Int J Cancer.* 2014;135:2206–2214. doi:10.1002/ijc.28857
35. Lin Z, Zhang X, Liu Z, Liu Q, Wang L, Lu Y, Liu Y, Wang M, Yang M, Jin X, et al. The distinct expression patterns of claudin-2, -6, and -11 between human gastric neoplasms and adjacent non-neoplastic tissues. *Diagn Pathol.* 2013;8:133. doi:10.1186/1746-1596-8-133
36. Kohmoto T, Masuda K, Shoda K, Takahashi R, Ujiro S, Tange S, Ichikawa D, Otsuji E, Imoto I. Claudin-6 is a single prognostic marker and functions as a tumor-promoting gene in a subgroup of intestinal type gastric cancer. *Gastric Cancer.* 2020;23:403–417. doi:10.1007/s10120-019-01014-x
37. Rendon-Huerta E, Teresa F, Teresa GM, Xochitl GS, Georgina AF, Veronica ZZ, Montano LF. Distribution and expression pattern of claudins 6, 7, and 9 in diffuse- and intestinal-type gastric adenocarcinomas. *J Gastrointest Cancer.* 2010;41:52–59. doi:10.1007/s12029-009-9110-y
38. Matsuzaki J, Gnjatic S, Mhawech-Fauceglia P, Beck A, Miller A, Tsuji T, Eppolito C, Qian F, Lele S, Shrikant P, et al. Tumor-infiltrating NY-ESO-1-specific CD8 + T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer. *Proc Natl Acad Sci U S A.* 2010;107:7875–7880. doi:10.1073/pnas.1003345107
39. Liu S, Matsuzaki J, Wei L, Tsuji T, Battaglia S, Hu Q, Cortes E, Wong L, Yan L, Long M, et al. Efficient identification of neoantigen-specific T-cell responses in advanced human ovarian cancer. *J Immunother Cancer.* 2019;7:156. doi:10.1186/s40425-019-0629-6
40. Wagner DL, Fritsche E, Pulsipher MA, Ahmed N, Hamieh M, Hegde M, Ruella M, Savoldo B, Shah NN, Turtle CJ, et al. Immunogenicity of CAR T cells in cancer therapy. *Nat Rev Clin Oncol.* 2021;18:379–393. doi:10.1038/s41571-021-00476-2
41. Wu L, Brzostek J, Sankaran S, Wei Q, Yap J, Tan TYY, Lai J, MacAry PA, Gascoigne NRJ. Targeting CAR to the peptide-MHC complex reveals distinct signaling compared to that of TCR in a jurkat T cell model. *Cancers (Basel).* 2021;13. doi:10.3390/cancers13040867
42. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, Royal RE, Topalian SL, Kammula US, Restifo NP, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science.* 2006;314:126–129. doi:10.1126/science.1129003
43. Bajwa G, Lanz I, Cardenas M, Brenner MK, Arber C. Transgenic CD8alpha beta co-receptor rescues endogenous TCR function in TCR-transgenic virus-specific T cells. *J Immunother Cancer.* 2020;8:e001487. doi:10.1136/jitc-2020-001487