의학박사 학위논문

Enhancement of the efficacy of genetically engineered antitumor T cell therapy using CTLA4-CD28 chimeric receptor

CTLA4-CD28 키메라 수용체를 이용한 항종양 유전자이입T세포요법의 개선효과

2015 년 2 월

서울대학교 대학원

의학과 면역학 전공

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지도 교수: 최 경 호

이 논문을 의학박사 학위논문으로 제출함
2014 년 10 월

서울대학교 대학원
의학과 면역학 전공
박 형 배

박 형 배의 의학박사 학위논문을 인준함
2015 년 1 월

위 원 장 _____ (인)

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Enhancement of the efficacy of genetically engineered antitumor T cell therapy using CTLA4-CD28 chimeric receptor

A thesis submitted by

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(Directed by Kyungho Choi, M.D., Ph.D.)

in partial fulfillment of the requirement

for the degree of Doctor of Philosophy

in Medical Science (Immunology)

Seoul National University, Seoul, Korea

January, 2015

Doctoral Committee:

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Part 1

Potentiation of autologous antitumor T cell therapy using CTLA4-CD28 chimeric receptor

Abstract

Potentiation of autologous antitumor T cell therapy using CTLA4-CD28 chimeric receptor

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T cell therapy has been touted as an ideal tumor therapy, based on the specificity of the immune response. However, due to tumor-induced tolerance, the therapeutic efficacy of T cell therapy has not been satisfactory. In theory, strategies to overcome this tolerance should improve the antitumor efficacy of T cell therapy. Although attempts have been made to modulate the immune response mediated by cytotoxic T lymphocyte-associated antigen 4 (CTLA4), a strong tolerance-inducing inhibitory receptor on T cells, unwanted side effects often resulted from systemic immune activation. Here, we developed a novel CTLA4-CD28 chimera, in which the intracellular signaling domain of CTLA4 was replaced with the CD28 signaling domain, and we confirmed the conversion of a negative signal into a positive signal. Selective tolerance breakage in antitumor specific T cells was achieved by retroviral transduction of CTLA4-CD28 into T cells. The CTLA4-CD28-transduced T cells exhibited increased *in vitro* reactivity and therapeutic potency in a tumor antigen-expressed animal model. Similar effects were also confirmed in a more physiologically relevant mouse melanoma model. Although direct tumor killing was

mainly induced by CD8 T cells, we confirmed that transduction of CTLA4-CD28 endowed CD4 T cells with a strong antitumor effect via excess IL-2 production.

Keyword: adoptive T cell therapy, CTLA4-CD28 chimera, antitumor effect, tolerance

Student number: 2005-30687

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Introduction

Immunotherapy was first introduced as an ideal antitumor therapeutic regimen that specifically activates the immune system to provide a therapeutic benefit without unwanted side effects. Theoretically, the specificity of the immune response allows it to discriminate tumor-specific antigens from surrounding normal tissues, which enables selective killing of the tumor and reduced side effects compared to those experienced with chemotherapy and radiation therapy. However, initial trials with tumor vaccines did not provide satisfactory therapeutic efficacy. Tumor-bearing host immune systems generally tolerate antitumor immune responses, and this prevents the initiation or amplification of the host response against the evolving tumor.[1-4] Antitumor immunotherapy focused on the modulation of immunological checkpoint molecules, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and programmed cell death protein 1 (PD1), which prevent excessive immune activation.[5-11] However, the therapeutic efficacy was limited in several tumors, and excessive systemic immune activation often occurred unwanted side effects. Therefore, active immunotherapy, utilizing host immune components did not lead to eradication of established tumors.[12-15]

Adoptive T cell transfer overcame many of the hurdles experienced in previous trials, which reinvigorated interest in antitumor immunotherapy.[16-18] Although antitumor reactive T cells exist in the peripheral circulation and tumor tissues, they generally do not eradicate established tumors. Both the inadequate number of antitumor reactive T cells and the reduced responsiveness of tumor-infiltrating T cells lead to insufficient tumor eradication.

Adoptive T cell transfer can overcome these problems. Isolation of tumor-infiltrating T cells from the peripheral blood or tumor tissues, and *ex vivo* expansion enables sufficient enrichment of antitumor reactive T cells. Since the *ex vivo* antitumor reactive T cell expansion strategy circumvents immune suppressive mechanisms in the host tumor environment, it is more efficient than the *in vivo* immunization-based expansion. Additionally, it is also possible to combine adoptive T cell therapy with chemotherapy and radiation therapy. In general, chemotherapy and radiation therapy are followed by systemic immunosuppression; therefore, the antitumor T cells are also affected. However, since *ex vivo* cultured T cells are administered after chemotherapy and radiation therapy, the T cells are not present during these immune compromising treatments; therefore, effective T cell combination therapy is possible. In fact, adoptive T cell therapy using autologous tumor-infiltrating lymphocytes mediated melanoma regression in approximately 50% of patients.[19-21)]

However, because most tumor-specific antigens are largely unidentified and tumor-infiltrating T cells are typically anergic, acquiring a sufficient number of antitumor specific T cells is a difficult task. In addition, adoptively transferred T cells encounter the immune suppressive environment of tumor-bearing host, which is another obstacle for successful antitumor T cell therapy. Until now, effective T cell therapy has been limited to some immunogenic tumors, such as melanoma and Epstein-Barr virus (EBV)-associated tumors.[22-24)]

After genetic engineering tools were introduced into the T cell therapy field, many of the existing obstacles were surmounted by genetic modifications.[25-34)] The low avidity and reduced activity of

antitumor reactive T cells were overcome by genetic manipulation of T cell receptor (TCR) and TCR-adhesion molecules.[30-32] *In vivo* tumor-targeting was improved by manipulation of chemokine-receptors, and a tolerogenic tumor environment was overcame by manipulation of co-stimulatory molecules and suppressive cytokines.[33-36] Introduction of an anti-apoptotic gene enhanced T cell survival *in vivo*, and introduction of a suicide gene enabled safety control of excessive immune response by selective killing of tumor-fighting and exhausted T cells.[37-41] Currently, gene-modified adoptive T cell therapy is regarded as one of the most promising therapeutic approaches in antitumor immune therapy, and additional improvements are expected in near future.

Blockade of immunological checkpoint molecules by treatment with antibodies or recombinant ligands was once a promising antitumor therapeutic approach.[11] The most promising approach was to attempt to overcome T cell exhaustion by using an anti-CTLA4 blocking antibody.[5-7] CTLA4 is a co-stimulatory molecule, that is mainly expressed on the surface of activated T cells. Through engagement with B7.1 (CD80) or B7.2 (CD86) ligand molecules on antigen presenting cells (APCs), CTLA4 attenuates excessive immune responses after the pathogen is cleared and the threat of disease is eliminated. CTLA4 competes for the same ligands with CD28, another T cell co-stimulatory molecule; however, the affinity of CTLA4 is 100 times higher than that of CD28, which enables CTLA4 to terminate excessive immune responses. Although the inhibitory role of CTLA4 endows it with a positive function under normal conditions by terminating excessive immune activation and preventing autoimmune responses, this may

also cause unexpected termination of antitumor reactions. Therefore, overcoming immune exhaustion by modulating CTLA4 should improve the therapeutic efficacy of antitumor T cell therapy.[42-47] In fact, systemic administration of a neutralizing anti-CTLA4 antibody led to tumor eradication in preclinical studies. Subsequently, a humanized anti-CTLA4 therapeutic antibody was developed, and enhanced survival was reported in a clinical study of metastatic melanoma patients. In 2011, FDA approved ipilimumab, an antagonistic antibody against CTLA4, therapeutic for melanoma. However, anti-CTLA4 antibody treatment also enhances systemic T cell reactivity, which leads to the adverse side effect of autoimmunity.[48-53]

Attempts to modulate negative regulatory receptors on T cells have yielded some positive results; however, unwanted side effects have prevented their clinical application. Therefore, in the development of antitumor T cell therapies that include the manipulation of immunological checkpoint molecules, we must aim to minimize side effects while enhancing therapeutic efficacy. To this end, we hypothesized that selective modulation of CTLA4 in antitumor-specific T cells will improve antitumor efficacy without systemic activation of T cells. To this end, we developed a novel chimeric receptor, consisting of the extracellular and transmembrane domain of CTLA4 and the intracellular signaling domain of CD28. In two preclinical animal studies, retroviral transduction of the gene encoding this chimeric receptor into antitumor-specific T cells resulted in potent, sustained T cell reactivity, which enhanced the antitumor effect without inducing systemic autoimmunity.

Materials and Methods

Mice

C57BL/6 (B6), B6.Thy1.1, OT-I and Pmel-1 mice were obtained from the Jackson Laboratory. OT-II mice were obtained from the Taconic Farms, and crossed with B6.Rag1$^{-/-}$ mouse to produce OT-II.Rag1$^{-/-}$ mouse. All of the mice were housed in specific pathogen-free (SPF) animal facility at the Research Institute National Cancer Center (Korea), and maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC).

Cells

E.G7, OVA-expressed T lymphoma EL4 cells and B16-F10 (B16) melanoma cells were derived from B6 mice. Phoenix GP and Phoenix Eco cell lines were provided by Garry Nolan (Stanford University). For some experiments, CD4^{+}CD25^{-} T cells were purified from B6 mice by negative selection using CD4^{+}CD25^{+} regulatory T cell isolation kit (Miltenyi Biotec).

DNA constructs

To generate CTLA4-CD28 chimeric gene, the extracellular and transmembrane domain of murine CTLA4 (nucleotides$_{1-567}$) and the cytoplasmic domain of murine CD28 (nucleotides$_{529-657}$) were amplified by polymerase chain reaction (PCR) from the plasmids containing murine CTLA4 and CD28 cDNA (ImaGenes). Each amplified fragments were joined by blunt end ligation and were cloned into a cloning vector. Subsequently, CTLA4-CD28 chimera cDNA was cloned into pMSCV-IRES-GFP-WRE (pMIGw)

retroviral expression vector (a gift from Yosef Refaeli, National Jewish Medical and Research Center). For CTLA4-decoy construct, the extracellular and transmembrane domain of CTLA4 (nucleotides$_{1-567}$) was amplified by PCR and was cloned into pMIGw. All the sequences were confirmed by automated DNA sequencing.

Luciferase Assay

Jurkat T cells (1×10^7), mixed with retroviral expression plasmid, RE/AP luciferase plasmid (a gift from Arthur Weiss, University of California), and pRL-TK *Renilla* luciferase control plasmid for normalization (Promega), were electroporated at 250V and 950uF in a 0.4 cm-gap cuvette using Gene Pulser (Bio-Rad Laboratories) and followed to recover for 24 h before stimulation. For stimulation, 96well plates coated with goat anti-mouse IgG (5μg/ml) and anti-hamster IgG (5μg/ml) overnight and washed and coated with anti-CD3 Ab (1μg/ml) along with normal hamster IgG or hamster anti-mouse CTLA4 (9H10; 2μg/ml) at RT for 2 h. Cells (1×10^5) were added to each well and incubated at 37°C for 6 h followed by cell lysis. For some experiments, soluble anti-CD28 Ab was added to the stimulation culture directly instead of plate-bound anti-CTLA4 Ab. Luciferase activity was measured with a luminometer using a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Western Blotting

The retroviral expression plasmids, without luciferase plasmids, were used in the above-mentioned transfection protocol for western blotting. For stimulation of the transfected cells, the cells

were treated either with mouse anti-human CD28 Ab or with normal hamster IgG or hamster anti-mouse CTLA4 Ab (2μg/ml) for 10 min on ice, followed by cross-linking with goat anti-mouse IgG or anti-hamster IgG (5μg/ml) for 10 min on ice. Then, cells were placed in a 37℃ water bath for 30 min, and the reaction was stopped by adding ice-cold PBS. The cell lysates were subjected to SDS-PAGE, transferred to a nitrocellulose membrane (Millipore), and probed with anti-phospho Akt or anti-Akt (Cell Signaling Technology) Abs. HRP-conjugated secondary Ab (Jackson Immuno Research Laboratories) was used to detect primary Abs. Blots were visualized by chemiluminescence reaction using SuperSignal West Pico (Pierce Chemical).

Production and transduction of retrovirus

The retroviral plasmids and a plasmid encoding VSV-G cDNA (pMD.G) were transiently transfected into Phoenix GP cell line using Lipofectamine 2000 (Invitrogen). After 48 h, the culture supernatant containing VSV-G pseudotyped retrovirus was harvested. Then, Phoenix Eco cell line was transduced with the retrovirus-containing supernatant overnight. After 3 to 5 days, green fluorescence protein (GFP)-positive Phoenix Eco cells were purified by FACS Aria (Becton Dickinson) to generate stable cell lines for producing ecotropic retroviruses. The culture supernatant containing ecotropic retrovirus was harvested and then concentrated 10-fold using a centricon (Amicon Ultra-15, 100kDa cut-off; Millipore) for murine T cell transduction.

For retroviral transduction of T cell, splenocytes from B6 mice or transgenic mice were stimulated with plate-bound anti-CD3 (10μg/ml; 145-2C11) and anti-CD28 (2μg/ml; 37.51) Abs or

antigenic peptides. After 24 h stimulation, T cells were transduced with the concentrated retroviruses by centrifuging the cells at 1,250g for 90 min (spin infection). This procedure was repeated once on the same day. During the spin infection, 6μg/ml Polybrene (Sigma-Aldrich) was added to the culture supernatant, or the cells were transduced in a RetroNectin-coated plate (15μg/ml; Takara) to enhance the transduction efficiency. After 48 h further stimulation, the transduced T cells were transferred to a fresh medium containing 30 U/ml mouse IL-2 (Invitrogen) and rested for 48 to 72 h without stimulation before functional analysis. After resting, more than 95% of the live cells were CD4 or CD8 T cells. Transduction efficiency was 50 to 90% as measured for GFP positivity for all cells tested.

Cytokine ELISA

GFP-positive T cells purified by FACS sorting were stimulated (2×10^4/well) with various concentrations of anti-CD3 Ab or antigenic peptides in the presence of irradiated splenocytes (2×10^5/well) for 48 h. The cytokines in the supernatant were measured using ELISA sets (BD Biosciences) according to the manufacturer's instructions.

Proliferation Assay

GFP-positive T cells purified by FACS sorting were stimulated (2×10^4/well) with various concentrations of anti-CD3 Ab or antigenic peptides in the presence of irradiated splenocytes (2×10^5/well) for 48 h, and pulsed with [^{3}H]-thymidine for an additional 24 h. Cells were harvested using a cell harvester, and radioactivity was counted in a Trilux 1450 scintillation counter (Wallac).

Adoptive T cell transfer

B6 mice were injected subcutaneously with E.G7 cells ($1 - 2 \times 10^6$) or B16 cells (1×10^5) on day 0. The retrovirus-transduced T cells were adoptively transferred into the mice on day 7. For the B16 melanoma models, the mice were lymphodepleted by non-myeloablative (4Gy) total body irradiation on the day of cell transfer. Tumor growth was measured using a caliper every 3 to 4 day, and their approximate sizes (square millimeters) were calculated using the following formula: length (millimeters) × width (millimeters) × π. The mice were euthanized when the tumor size exceeded $500mm^2$. For IL-2 neutralization experiments, rat anti-mouse IL-2 Ab (eBioscience; JES6-1A12) and normal rat IgG (Sigma-Aldrich) were purchased. For intracellular cytokine staining for the transduced T cells, the *ex vivo*-activated T cells were fixed and permeabilized (BD Cytofix/Cytoperm kit) and stained with PE-conjugated anti-mouse IL-2 or IFN-γ Ab. (BD Biosciences)

Results

Transduction of a chimeric CTLA4-CD28 gene enhances T cell reactivity through positive conversion of the negative CTLA4 signal

To develop a dominant-negative mutant of CTLA4 for expression in T cells, we constructed the pMIGw vector, which contains the pMSCV-IRES-GFP vector (pMIG) and a WRE element, which improves the expression of proteins. Initially, we designed a CTLA4 decoy receptor (CTdc), that blocked intracellular inhibitory signaling of CTLA4 (Figure 1), and expressed it in Jurkat T cells by transient transfection. We hypothesized that endogenous CTLA4, expressed on activated T cells, will compete with overexpressed CTdc on T cells for ligands on APCs. Then, the negative signaling of endogenous CTLA4 should be diminished and T cell stimulation should be elongated.

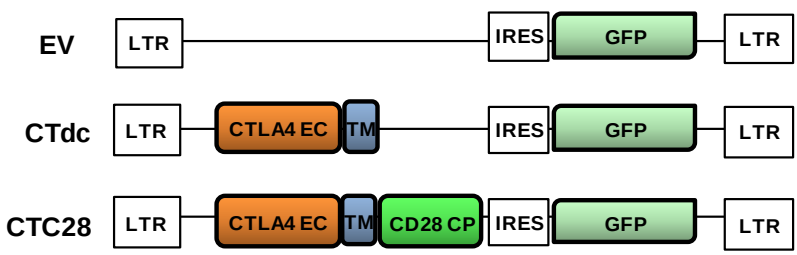

Figure 1. CTLA4-modified DNA constructs. The empty vector (EV) is pMIGw. CTdc or CTC28 cDNA was inserted in front of the internal ribosomal entry site (IRES)-GFP cassette of pMIGw. The IRES-GFP cassette was used to track virus-transduced cells. CTLA4 EC, extracellular domain of CTLA4; TM, transmembrane domain of CTLA4; CD28 CP, cytoplasmic domain of CD28; LTR, long term repeat.

When empty vector (EV)-transfected Jurkat T cells were stimulated with plate-bound anti-CD3 Ab plus a crosslinking anti-CTLA4 Ab, T cell stimulation was downregulated, as measured in a CD28 response element luciferase assay (Figure 2) and downstream Akt phosphorylation (Figure 3). CTdc-transfected Jurkat T cells were not affected by anti-CTLA4 Ab treatment (Figure 2). However, CTdc-transfected T cells exhibited an unexpected problem. When CTdc was overexpressed on T cells, CD28 on T cells also competed with CTdc, and the initial co-stimulation signal was insufficient to activate T cells. Therefore, we designed another DNA construct, consisting of the extracellular and transmembrane domains of CTLA4 and the intracellular signaling domain of CD28, and named it CTLA4-CD28 chimeric receptor (CTC28)(Figure 1). Because CTC28 provides additional CD28 stimulatory signals, CTC28-transfected T cells were initially stimulated more effective than CTdc-transfected T cells. Moreover, CTC28 competes with endogenous CTLA4 at later time points after stimulation, and T cell stimulation is elongated by conversion of the negative CTLA4 signal to a stimulatory CD28 signal. CTC28-transfected Jurkat T cells exhibited higher CD28 response element luciferase activity after plate-bound anti-CD3 Ab stimulation with anti-CTLA4 Ab than after anti-CD3 stimulation alone (Figure 2). This was also confirmed by the increased Akt phosphorylation after stimulation with an anti-CD3 Ab plus an anti-CTLA4 crosslinking Ab (Figure 3). Thus, CTC28 converts the negative signals of CTLA4 to positive signals, similar to that observed with CD28 co-stimulation.

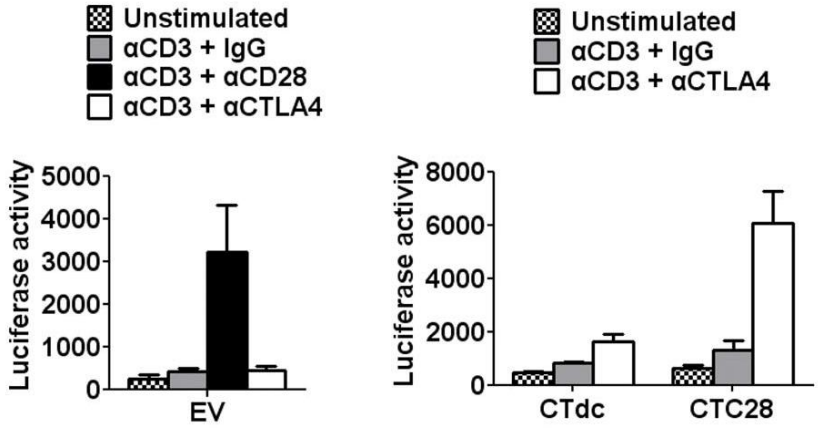

Figure 2. CD28 response element luciferase activity of CTLA4-modified Jurkat T cells. Jurkat T cells transiently transfected with the retroviral plasmid (10μg), RE/AP luciferase plasmid (10μg) and TK-*Renilla* luciferase plasmid (0.5μg) were stimulated with plate-bound anti-CD3 Ab plus soluble anti-CD28 Ab, plate-bound anti-CD3 Ab plus anti-CTLA4 Ab, or a control IgG for 6 h. Firefly luciferase activity in cell lysates was measured. Transfection efficiency was normalized to *Renilla* luciferase activity.

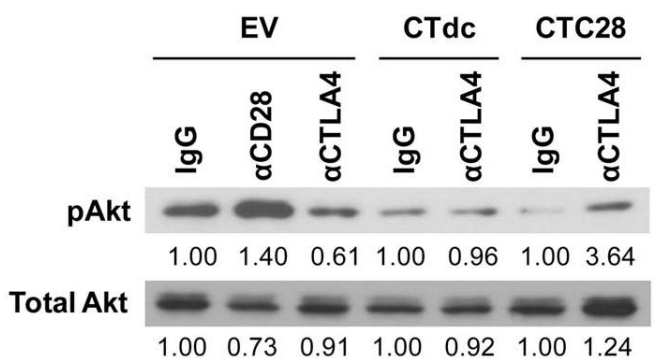

Figure 3. Western blotting of phosphorylated Akt in CTLA4-modified Jurkat T cells. Jurkat T cells transfected with the retroviral plasmids were stimulated with an anti-CD28 Ab or an anti-CTLA4 Ab cross-linked with secondary Abs for 30 min. Cell lysates were immunoblotted with anti-phospho-Akt or anti-Akt Abs.

To establish a method for stable and efficient transfection of the CTLA4-modified gene for subsequent experiments, we used a retroviral transduction method. Phoenix GP cells, a retrovirus packing cell line, was transiently transfected with the CTLA4-modified DNA constructs, and the culture supernatants were harvested. Then, Phoenix Eco cells were transduced with these supernatants. And high GFP expressing cells were FACS sorted and used as a stable cell line producing retrovirus containing the CTLA4-modified gene.

Next, we examined responses in CTC28-transduced mouse T cells. B6 mouse spleen cells were stimulated with plate-bound anti-CD3 Ab plus anti-CD28 Ab. After 24 h, stimulated cells were transduced with the CTLA4-modified gene-encoding retrovirus by spin infection. After stimulation for additional 2 days, cells were harvested and rested for 3 days. As the retrovirus construct was designed to express GFP along with the CTLA4-modified proteins, GFP-expressing T cells were sorted and used in subsequent experiments. Cells were stimulated with various concentrations of anti-CD3 Ab in the presence of irradiated splenocytes for 72 h. T cell proliferation was measured by [^{3}H]-thymidine uptake for the final 24 h of stimulation. T cell proliferation was downregulated by CTdc transduction, which was probably due to insufficient co-stimulation. CTC28-transduced T cells exhibited slightly increased proliferation than EV-transduced T cells (Figure 4).

An interferon gamma (IFN-γ) ELISA was performed using 48 h culture supernatants. IFN-γ is a T cell-producing effector cytokine that has a reported antitumor effect. CTdc-transduced T cells produced less IFN-γ than EV-transduced T cells. IFN-γ production was significantly increased in CTC28-transduced T

cells (Figure 5).

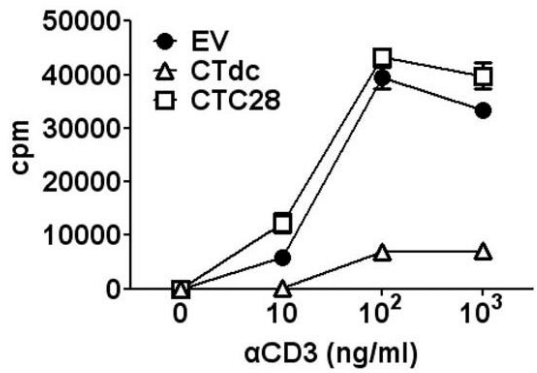

Figure 4. Proliferation of CTLA4-modified mouse T cells. Splenic T cells from normal B6 mice were activated and transduced with the retroviral CTLA4 constructs, and then rested for 3 days in the absence of stimulation. GFP-positive T cells were sorted, and then stimulated with various concentrations of anti-CD3 Ab in the presence of irradiated splenocytes for 48 h. [^{3}H]-thymidine (1μCi) was added to the culture, and the cells were incubated for additional 24 h. Cell proliferation was measured as [^{3}H]-thymidine uptake.

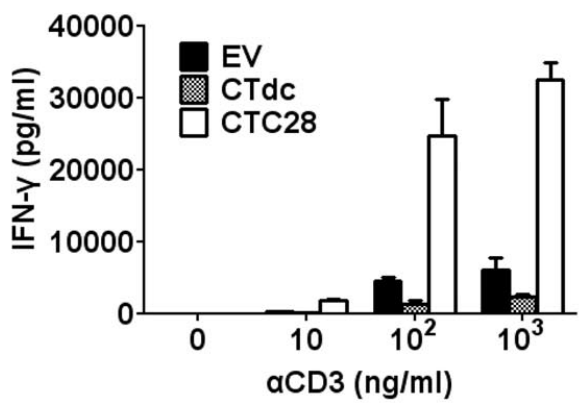

Figure 5. IFN-γ ELISA of CTLA4-modified mouse T cells. Splenic T cells from normal B6 mice were activated and transduced with the retroviral CTLA4 constructs, and then rested for 3 days in the absence of stimulation. GFP-positive

T cells were sorted and stimulated with various concentrations of anti-CD3 Ab in the presence of irradiated splenocytes for 48 h. The supernatant was harvested to measure IFN-γ production by ELISA.

CTLA4 expression was significantly enhanced in CTC28-transduced T cells compared to expression in EV-transduced cells, and this overexpressed CTLA4 competed with endogenous CTLA4 in activated T cells, and may have a dominant negative effect. Surface CD28 expression on CTC28-transduced T cell was maintained at levels similar to that on EV-transduced T cells (Figure 6).

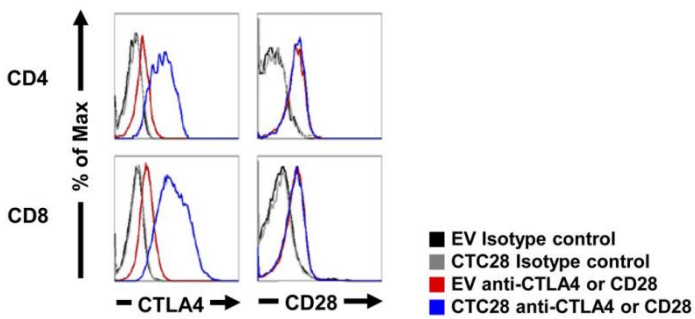

Figure 6. Expression of CTLA4 and CD28 on CTLA4-modified T cells. Transduced T cells were activated with anti-CD3 Ab (1μg/ml) in the presence of irradiated splenocytes for 48 h and then stained with APC-conjugated anti-CD4 Ab and PE-Cy5-conjugated anti-CD8 Ab along with PE-conjugated anti-CTLA4 Ab (intracellular staining), anti-CD28 Ab (surface staining), or relevant isotype control Ab. Then, GFP-positive populations were analyzed by flow cytometry.

Modified T cells were stimulated with anti-CD3 and anti-CD28 Abs in the presence or absence of crosslinking anti-CTLA4 Ab. While IFN-γ production was inhibited by CTLA4 crosslinking in EV-transduced T cells, this effect was not observed in CTC28-transduced T cells (Figure 7). Thus, the dominant negative effect of

CTLA4 molecules on CTC28-transduced T cells was confirmed.

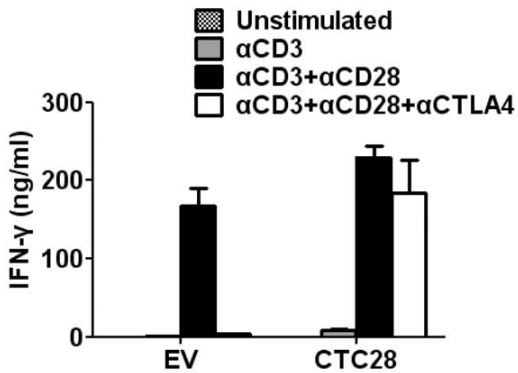

Figure 7. The dominant negative effect of CTLA4 molecules on CTC28-transduced T cells. Transduced T cells were stimulated with plate-bound anti-CD3 Ab (1μg/ml), anti-CD28 Ab (2μg/ml), or both in the presence or absence of anti-CTLA4 Ab (10μg/ml) cross-linked with plate-bound anti-hamster IgG (20μg/ml). After 72 h stimulation, the supernatant was harvested to measure IFN-γ production by ELISA.

Overall, CTC28-transduced T cells exhibit slightly enhanced proliferation and significantly increased IFN-γ production compared to those of control cells, presumably due to surrogate CD28 signaling and a dominant negative effect on endogenous CTLA4.

CTLA4-CD28 chimeric gene modification preferentially affects CD4 T cells over CD8 T cells

To test the antitumor activity of CTC28-transduced T cells, we first used a well-known Ag-expressing tumor model, E.G7, which is ovalbumin (OVA)-transfected murine EL4 lymphoma model. Ag-specific T cells were obtained from each OVA-specific CD4

transgenic mouse (OT-II) and CD8 transgenic mouse (OT-I). First, we confirmed the efficiency of cytokine production in CTC28-transduced OVA-specific T cells. T cells were isolated from OT-II and OT-I mice, and transduced with the modified retroviral construct. GFP-positive transduced T cells were sorted and stimulated with various concentrations of each cognate Ag OVA peptides in the presence of irradiated B6 splenocytes. After 48 h, culture supernatants were harvested, and IL-2 and IFN-γ were quantified by using a sandwich ELISA. IL-2 and IFN-γ were increased in both CD4 and CD8 T cells following CTC28 transduction, and the increase was higher in CD4 T cells than in CD8 T cells (Figure 8).

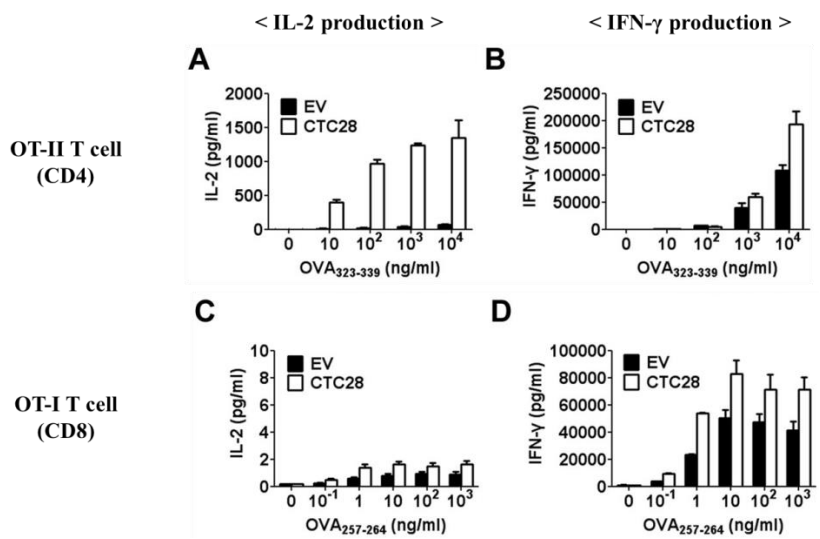

Figure 8. CTC28-modification preferentially affects CD4 T cells *in vitro*. Splenic T cells from OT-II or OT-I mice were stimulated with the antigenic peptides $OVA_{323-339}$ (ISQAVHAAHAEINEAGR; 10μg/ml) or $OVA_{257-264}$(SIINFEKL; 1μg/ml), respectively. Activated T cells were transduced with EV or CTC28 retrovirus by spin infection and stimulated for additional 2 days and then rested. After 2 days in the absence of Ag, GFP-positive T cells were FACS sorted. GFP-

positive OT-II or OT-I T cells were stimulated with various concentrations of these antigenic peptides in the presence of irradiated splenocytes for 48 h. IL-2 or IFN-γ levels in the culture supernatant were measured by ELISA.

CTLA4-CD28 chimeric gene modification enhances the therapeutic effect of both CD4 and CD8 T cells on antigen-expressing tumors

To assess the antitumor effect of CTC28-transduced T cells *in vivo*, we first used an Ag-expressing tumor model. E.G7, a murine OVA-expressing syngeneic lymphoma cell line, was subcutaneously injected into the back of B6 mice, and T cell therapy was administered 7 days later. Although no effect was observed in CD8 T cells following CTC28-modification, single therapy with OT-I CD8 T cells led to tumor regression. However, EV- and CTC28-transduced OT-II CD4 T cells did not exhibit any therapeutic effect in single therapy. Thus, we confirmed that in this model, direct tumor killing was mainly mediated by CD8 T cells (Figure 9).

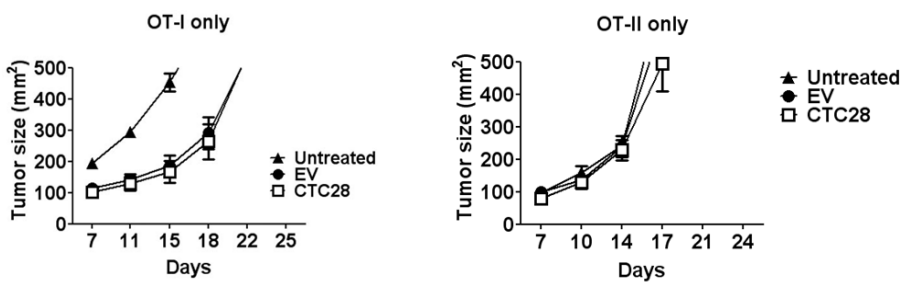

Figure 9. Antitumor effect of single therapy with modified OT-I or OT-II T cells. B6 mice were subcutaneously injected with E.G7 cells ($1 - 2 \times 10^6$). After 7 days, the mice were injected intravenously with (left) EV-transduced OT-I T cells (2×10^6), CTC28-transduced OT-I cells (2×10^6); (right) EV-transduced OT-II T cells (2×10^6), or CTC28-transduced OT-II cells (2×10^6) or untreated.

The mean tumor size of at least 5 mice per group was recorded.

Next, we assessed the antitumor effect of co-transfer therapy with various ratios of unmodified OT-I CD8 T cells and modified OT-II CD4 T cells. Increased tumor regression was observed in mice treated with a combination of OT-II CD4 T cells and unmodified OT-I CD8 T cells compared to that observed in mice administered single therapy with unmodified OT-I CD8 T cells. Tumor regression also increased as the ratio of OT-II CD4 T cells to CD8 T cells increased. Moreover, CTC28-transduced OT-II CD4 T cells enhanced the antitumor effect of unmodified OT-I CD8 T cells more than EV-transduced OT-II cells, and nearly 100% therapeutic efficacy was observed in co-transfer therapy at a 1:1 ratio of unmodified OT-I CD8 T cells to CTC28-transduced OT-II CD4 T cells (Figure 10).

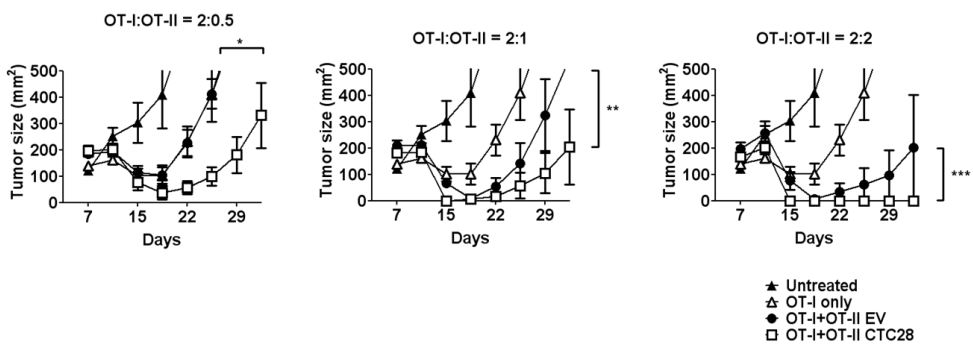

Figure 10. Antitumor effects of co-transfer therapy with unmodified OT-I and modified OT-II T cells. B6 mice were injected subcutaneously with E.G7 cells ($1 - 2 \times 10^6$). After 7 days, the mice were injected intravenously with un-transduced OT-I T cells (2×10^6) in combination with EV- or CTC28-transduced OT-II T cells at various ratios (2:0.5, 2:1 or 2:2). The mean tumor size of at least 5 mice per group was recorded (*P = 0.0391, **P = 0.0078, ***P = 0.0078; Wilcoxon matched pairs test).

Although striking effects were detected in CTC28-transduced CD4 T cells, CTC28-transduced CD8 T cells also exhibited a slight increase in reactivity (Figure 8). Therefore, we evaluated the antitumor effects of dual modified CD4 and CD8 T cells. Co-transfer therapy with CTC28-transduced CD8 and CD4 T cells further enhanced the antitumor effect compared to that observed in co-transfer therapy with unmodified OT-I CD8 T cells and CTC28-transduced OT-II CD4 T cells (Figure 11).

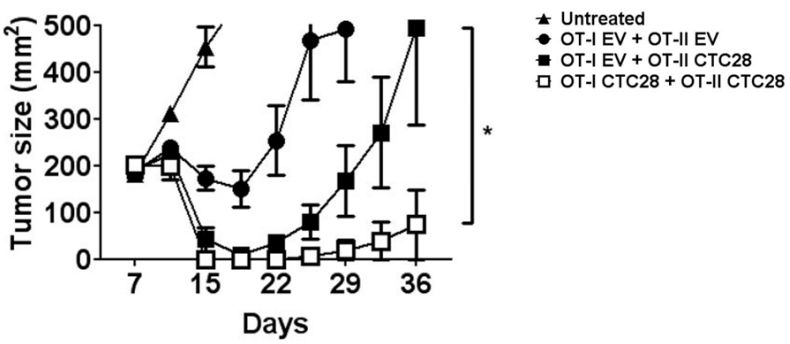

Figure 11. CTC28-modification of both OT-I and OT-II T cells increased the efficacy of T cell co-transfer therapy. B6 mice were injected subcutaneously with E.G7 cells ($1 - 2 \times 10^6$). After 7 days, the mice were injected intravenously with EV-transduced or CTC28-transduced OT-I T cells (2×10^6) in combination with EV-transduced or CTC28-transduced OT-II T cells (1×10^6) or left untreated. The mean tumor size of at least 5 mice per group was recorded ($*P = 0.0029$; Wilcoxon matched pairs test).

To evaluate the *ex vivo* reactivity of modified T cells in tumor-bearing mice, we sacrificed mice at 3 days after T cell therapy and isolated splenocytes for analysis. After *in vitro* re-stimulation with cognate OVA peptides in the presence of irradiated B6 splenocytes

for 6 h, cells were stained with anti-cytokine Abs conjugated with fluorescence dyes. IL-2 and IFN-γ production was higher in CTC28-transduced OT-II CD4 T cells than in EV-transduced CD4 T cells. CTC28-transduced CD4 T cell expansion was significantly increased compared to the expansion of EV-transduced CD4 T cells. IFN-γ production by *ex vivo* stimulated CD8 T cells from CTC28-transduced T cell-transferred mice was also increased (Figure 12).

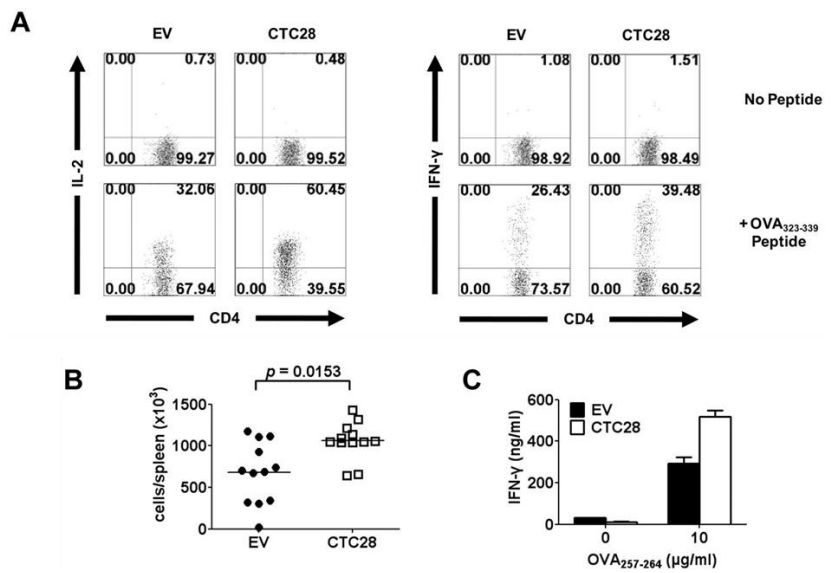

Figure 12. Adoptively transferred modified T cells retain enhanced reactivity to the antigens. Thy1.1 mice were subcutaneously injected with E.G7 cells (2×10^6). After 7 days, modified OT-I (1×10^7) and OT-II T cells (2×10^6) were adoptively transferred to tumor-bearing mice. After 3 days, splenocytes were isolated for analysis. (A) Splenocytes were stimulated with the $OVA_{323-339}$ peptides (10μg/ml) for 2 h and then further stimulated for an additional 4 h in the presence of GolgiStop solution. After fixation and permeabilization of the cells, intracellular IL-2 and IFN-γ were stained with fluorescence-conjugated Abs. (B) The absolute numbers of retrovirus-transduced OT-II T cells ($Thy1.2^+CD4^+$) in the spleen were calculated by flow cytometry and live cell counting. (C) CD8 T cells were purified using anti-CD8 microbeads and stimulated (2×10^4 cells/well)

with $OVA_{257-264}$ in the presence of irradiated splenocytes for 48 h. Supernatant was harvested after 48 h and IFN-γ production was measured by ELISA.

Transduction of the CTLA4-CD28 chimeric gene in both CD4 and CD8 T cells enhances the therapeutic effect of adoptive T cell therapy in a syngeneic melanoma model

The previous animal study was conducted to assess the immune response against OVA, an artificial tumor Ag in E.G7 tumors, which is not physiologically relevant. Therefore, we searched for a more physiological Ag-specific tumor model to test the effect of CTLA4-CD28 chimera-transduced T cell therapy. Because melanoma is one of the best studied tumors for T cell therapy and antigen-specific T cells are available, we used a mouse syngeneic melanoma (B16) model. B16 melanoma expresses glycoprotein 100 (gp100) as a tumor Ag, and Ag-specific T cells for T cell therapy are available in CD8 TCR transgenic mouse (Pmel-1).[54, 55]

B16 melanoma cells were subcutaneously injected into the back of B6 mice, and T cell therapy was administered 7 days later. Before the therapeutic T cells were transferred, tumor-bearing mice were exposed to 4Gy of total-body irradiation to induce lymphodepletion, and melanoma-specific CD8 T cells from a Pmel-1 mouse were adoptively transferred. Recently, lymphocyte depletion followed by adoptive T cell transfer was reported to significantly increase the therapeutic efficacy of T cell therapy.[56, 57] Because Ag-specific CD4 T cells were not available in this case, we used $CD4^+CD25^+$ regulatory T cell-depleted polyclonal CD4 T cells isolated from a B6 mouse, and assessed the effect of CD4 and CD8 T cell co-transfer therapy. Although CD4 T cells did not

acquire Ag specificity in this case, co-transfer of Pmel-1 CD8 T cells and polyclonal CD4 T cells was reported to enhance the antitumor effect in a melanoma model.[58)]

Transduction of the CTC28 chimera into both Pmel-1 CD8 T cells and polyclonal CD4 T cells, followed by adoptive transfer into 4Gy-irradiated B16 tumor-bearing mice provided a potent antitumor effect, as evidenced by reduced tumor size and enhanced survival (Figure 13).

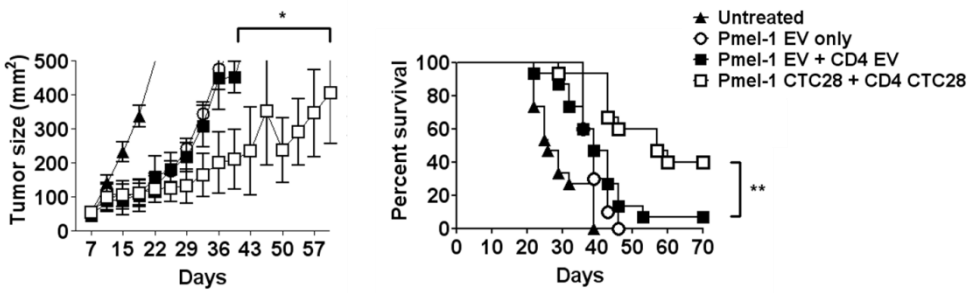

Figure 13. CTC28-transduction of CD4 and CD8 T cells potentiates the efficacy of adoptive T cell therapy in a melanoma model. B6 mice were injected subcutaneously with B16 melanoma cells. After 7 days, mice were subjected to total body irradiation (4Gy) to induce lymphodepletion, and then the mice were injected intravenously with retrovirus-transduced CD4 and CD8 T cells or left untreated. Regulatory T cell-depleted polyclonal CD4 T cells ($CD4^+CD25^-$) were purified from normal B6 mice, and Pmel-1 CD8 T cells were transduced with the retroviruses and then rested for 48 h before transfer. Equal numbers of transduced Pmel-1 CD8 T cells and CD4 T cells (2×10^6) were used for the adoptive transfer. The mean tumor size of 5 mice in each group was recorded (Left, $*P = 0.0105$; Wilcoxon matched pairs test). Survival data from the mice in all experiments are summarized (Right, n = 10 for Pmel-1 EV only; n = 15 for all other groups; $**P = 0.002$; log-rank test).

Blood analysis, which was performed at 4 weeks after adoptive

transfer, showed greater survival of CTC28-transduced CD4 and CD8 T cells compared to that of EV-transduced T cells. CD4 T cell expansion was dramatically higher than CD8 T cell expansion (Figure 14). Consequently, CTC28 modification enhances the *in vivo* survival of T cells and promotes T cell reactivity.

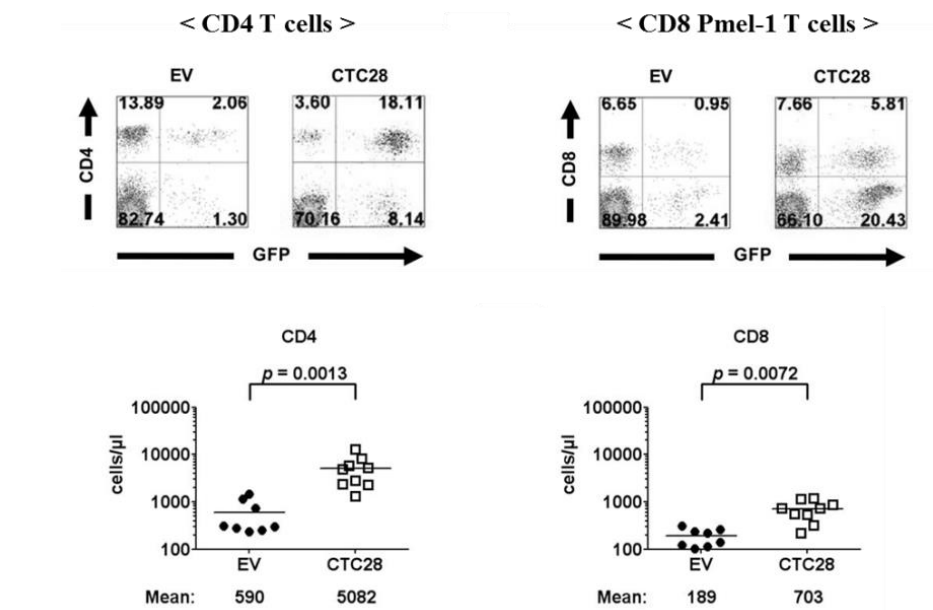

Figure 14. CTC28 modification enhances the *in vivo* survival of therapeutic T cells. Four weeks after the adoptive transfer, peripheral blood leukocytes were counted and analyzed by flow cytometry. Retrovirus-transduced CD4 and Pmel-1 T cells were identified by GFP expression. Representative flow cytometry profiles and the absolute numbers of the transduced CD4 and Pmel-1 T cells are shown (*P* value; Student's *t*-test).

Finally, we evaluated the effect of CTC28 modification in individual T cells on therapeutic potential in a melanoma model. As previously shown in the OVA-expressing E.G7 tumor model, CTC28-transduction of CD4 T cells provided a significant antitumor effect in the melanoma model. A more potent antitumor

effect was obtained by CTC28 modification of both CD4 and CD8 T cells, and nearly complete tumor regression was achieved. However, this antitumor effect was nearly abrogated by treatment with IL-2 neutralizing Ab. Therefore, increased production of IL-2 through CTC28 modification might play an important role in this antitumor effect (Figure 15).

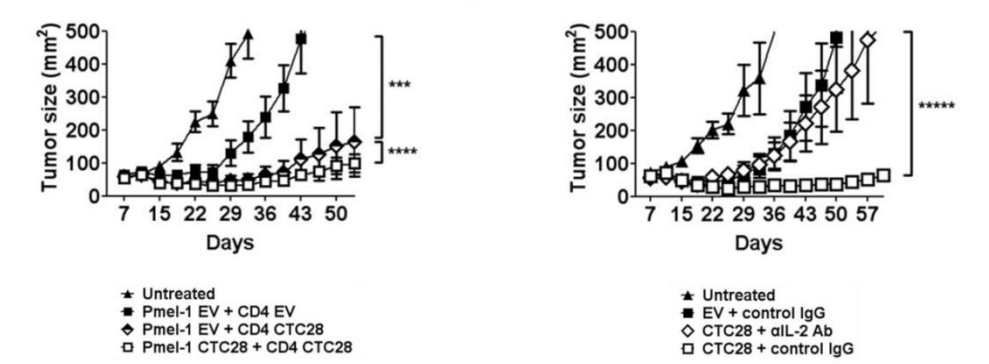

Figure 15. Modification of CTC28 provides a potent antitumor effect, which is dependent on IL-2 production. B16 tumor-bearing mice generated as described in Figure 13 were lymphodepleted and injected with various combinations of retrovirus-transduced polyclonal CD4 and Pmel-1 T cells at 1:1 ratio (2×10^6). The mean tumor size of 5 mice in each group was recorded (left, ***P = 0.0001; ****P = 0.0006). Anti-IL-2 neutralizing Ab or a control rat IgG (100μg/EA) was injected intravenously every 3 days beginning the day of cell transfer. The mean tumor size of 5 mice in each group was recorded (right, *****P = 0.0021; Wilcoxon matched pairs test).

Discussion

CTLA4 is a major receptor on T cells that plays a negative regulatory role and is critical for termination of excessive T cell responses after pathogen clearance and prevention of autoimmune response. However in antitumor therapy, this precise off switch could lead to premature termination of the immune response, which often resulted in reduced therapeutic efficacy. Previous attempts have been made to modulate immune responses through regulation of immunological check point molecules such as CTLA4 and PD1, and an anti-CTLA4 blocking antibody has been approved by the US FDA as therapeutic for melanoma.[5-11] Systemic blockade of CTLA4 by antibody treatment resulted in a sustained T cell response and an enhanced antitumor effect; however, it was often followed by harmful side effects as a result of uncontrolled activation. Therefore, we designed this study to modify the T cell response by modulating the T cell itself instead of a systemic blockade. We paid attention to the competition between CTLA4 and CD28 on T cells for B7 molecules on APCs determines the T cell response. We first hypothesized that overexpression of CTLA4 decoy receptor (CTdc), which contained a disrupted intracellular inhibitory signaling domain, in T cells would compete with endogenous CTLA4 and prevent inhibitory T cell signaling. However, this concept had a flaw. High affinity binding of CTdc to B7 molecules also competed with the CD28 molecules on T cells and prevented T cell priming. Next, we postulated that insertion of the cytoplasmic domain of CD28 into the deletion site of CTdc could provide a surrogate CD28 co-stimulatory signal to the T cell priming process. This was shown in Jurkat T cell transfection experiments by the results of a CD28

response element luciferase assay and Akt phosphorylation (Figure 2, 3). Therefore, expression of a chimeric CTLA4-CD28 receptor (CTC28) on T cells can induce a potent and sustained T cell response by providing a surrogate CD28 co-stimulatory signal and competing with endogenous CTLA4 (Figure 16).

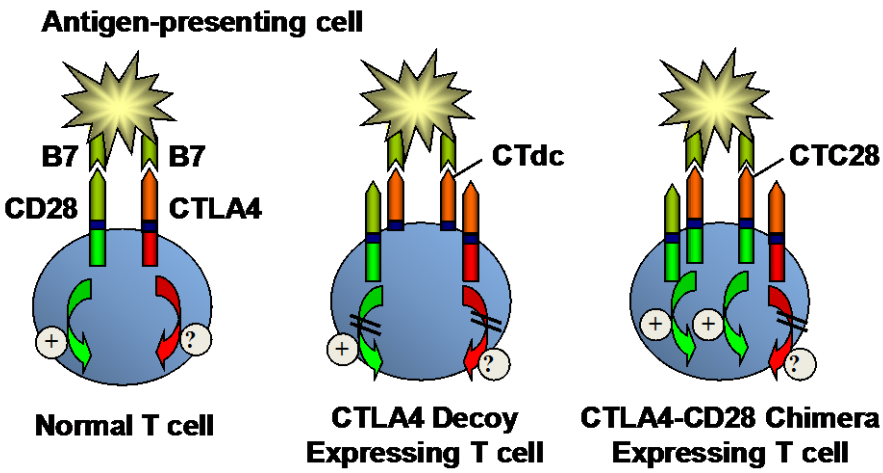

Figure 16. Conceptual design and mode of action of CTLA4-decoy (CTdc) and CTLA4-CD28 chimera (CTC28)

CTC28-modified T cells exhibited enhanced IL-2 and IFN-γ production, and this effect was especially remarkable in CD4 T cells (Figure 8). Predominant affection of CTLA4 on CD4 T cells has been reported in various studies using CTLA4$^{-/-}$ mice.[47, 48] Generally, homozygous CTLA4$^{-/-}$ mice died at 3 - 4 weeks of age due to massive polyclonal expansion of T cells and infiltration into non-lymphoid organs, such as the heart, liver, lung and pancreas. However, depletion of CD4 T cells rescued mice from this lymphoproliferative disease, which demonstrated the contribution of CTLA4 in CD4 T cells.[59] Although CD4 T cells were not the

primary killer of tumor cells, they reportedly augmented the antitumor effect of CD8 T cells.[60, 61] In this study, addition of CTC28-modified OT-II CD4 T cells significantly enhanced the antitumor effect of OT-I CD8 T cells in a dose-dependent manner (Figure 10). The CD4 T cell contribution in antitumor effect was also demonstrated by the retarded tumor development and enhanced survival in the melanoma model (Figure 15). Although melanoma antigen-specific CD4 T cells were not available, regulatory T cell-depleted polyclonal CD4 T cells have been reported to be able to assist CD8 T cells.[58] *In vivo* IL-2 depletion study also demonstrated the contribution of robust IL-2 production in CTC28-modified T cells to the antitumor effect. Although systemic depletion of IL-2 induced by treatment with a neutralizing antibody completely abrogates the antitumor effect of CD8 T cells, the antitumor effect of unmodified CD8 T cells was not inhibited in our experimental setting (Figure 15). Therefore, the enhanced antitumor effect of CTC28-modified T cells mainly resulted from excessive production of IL-2.

Although the influence of CTC28 modulation on cytokine production in CD8 T cells was not as dramatic as that in CD4 T cells (Figure 8), CTC28-transduction of both CD4 and CD8 T cells maximized the antitumor therapeutic efficacy and nearly eradicated established tumors (Figure 11, 15). Therefore, inhibition of CTLA4 in both CD4 and CD8 T cells might be the most effective approach in antitumor T cell therapy.

To propagate in an immune competent host, tumor cells generally establish a special environment to escape the antitumor immune response.[62, 63] Down-regulation of MHC molecules and ligands of co-stimulatory molecules on APCs or the tumor itself provided

insufficient stimulatory signals to T cells, and often resulted in unresponsive T cells.[64] Moreover, the suppressive cytokine milieu in the tumor microenvironment hampered efficient T cell responses against the tumor.[65, 66] In this environment, adoptively transferred T cells often lost their immune competency and failed to kill the tumor. However, CTC28-transduced T cells expanded well in tumor-bearing mice and survived for a long time (Figure 14). In addition, the expanded T cells were not tolerated in the tumor-bearing mice and their enhanced reactivity against antigens was conserved (Figure 12). Therefore, tolerance in tumor antigen-specific T cells was partly suppressed by CTC28-modification of the T cells.

In vitro CTC28-modification of CD4 T cells enhanced IL-2 production, which increased the antitumor effect of CD8 T cells, and CTC28-modification of CD8 T cells further increased the antitumor therapeutic efficacy. Adoptively transferred modified T cells expanded well without additional IL-2 injection and retained reactivity against the tumor antigen in tumor-bearing mice. The potential of CTC28 modification was partly provided by the surrogate CD28 co-stimulatory signals and resulted from sustained activation through competition with endogenous CTLA4. This individual cell-based modulation of CTLA4 via transduction of a CTLA4-CD28 chimeric gene could eliminate the possible harmful side effects that result from systemic blocking of CTLA4, followed by polyclonal activation of T cells. Therefore, we conclude that positive conversion of negative CTLA4 signaling by transduction of the CTLA4-CD28 chimera is conceptually reasonable and was verified in preclinical tumor Ag-specific models.

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국문초록

CTLA4-CD28 키메라수용체를 이용한 자가 항종양 T세포 치료의 강화

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면역반응의 특이성에 기초를 한 T세포 치료법은 이상적인 항종양 치료법으로 소개되었지만, 종양 유래의 관용현상으로 인해 항종양 치료 효율은 현재까지 기대만큼 만족스럽지는 않다. 이러한 관용현상을 극복하는 전략은 항종양 T세포 치료법의 치료효율을 개선시켜 줄 것이다. 비록 T세포 상의 강력한 관용 유도 수용체인 cytotoxic T lymphocyte-associated antigen 4 (CTLA4)의 조절을 통한 면역관용의 극복은 시도되어왔지만, 전신의 T세포 활성화로 인한 원치 않는 부작용의 발생이 수반되었다. 본 연구에서는 이러한 문제점들을 해결하기 위한 새로운 방법으로 CTLA4-CD28 키메라 유전자를 개발하였다. CTLA4-CD28 키메라는 CTLA4의 세포 내 신호전달 부위를 CD28의 세포 내 신호전달 부위로 치환함으로써, CTLA4 수용체가 T세포에 제공하는 면역반응의 억제 신호를 CD28 수용체가 제공하는 면역반응 촉진 신호로 전환시켜 준다. CTLA4-CD28 키메라 유전자를 레트로바이러스 운반체를 통해 항종양 특이적 T세포에 형질도입 시킴으로써 항종양 특이적 T세포에만 선별적으로 면역관용을 회피할 수 있을 것으로 기대한다. CTLA4-CD28 키메라 형질도입 결과, T세포의 반응성이 증가됨이 확인되었고, 모델 항원을 표지 하는 종양동물모델 실험에서 치료효과의 개선을 보였다. 항종양 치료효과는 쥐의 흑색종 (melanoma) 동물모델 실험에서도 탁월하게 확인되었다. 비록 종양세포의 직접적인 살상은 CD8 T세포에

의해 진행되지만, CTLA4-CD28 키메라 형질도입의 효과는 CD8 T세포보다 CD4 T세포에서 더 극명하게 나타남을 확인하였으며 항종양 치료효과에는 인터루킨-2의 과 생산이 중요하게 작용함을 확인하였다.

주요어: T세포 입양전달, CTLA4-CD28 키메라 수용체, 항종양 효과, 면역관용

학 번: 2005-30687

Part 2

Development of allogeneic antitumor T cell therapy using CTLA4-CD28 chimeric receptor

Abstract

Development of allogeneic antitumor T cell therapy using CTLA4-CD28 chimeric receptor

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Reduced-intensity conditioning allogeneic hematopoietic cell transplantation (HCT) has widened therapeutic opportunity to weak and old patients with hematologic malignancy. However, it is associated with increased relapse rates, which require an additional treatment regimen. Delayed donor lymphocyte infusion (DLI) was introduced as a promising T cell therapeutic regimen against recurring hematologic malignancy, and it exhibited satisfactory results against a many leukemias, except acute lymphoid leukemia (ALL). We hypothesized that potentiation of T cells would improve the therapeutic efficacy of the current DLI regimen. To potentiate DLI T cells, we transduced them with the CTLA4-CD28 chimeric gene and evaluated the graft-versus-tumor (GVT) effect in an EL4 tumor-bearing mixed chimera model, which was slightly modified to mimic ALL. A strong GVT effect was only observed with dual CTLA4-CD28-modified CD4 and CD8 T cells, and *in vivo* expansion of the infused CD8 T cells was significantly correlated with this GVT effect. An additional GVT effect was provided by the enhanced effector function of the CTLA4-CD28-modified CD4 T cells. Modified T cell-derived IL-2 was required for the GVT

effect, but was not required for the expansion of the DLI T cells. The alloreactive responses that provided the GVT effect also developed moderate, but non-lethal graft-versus-host-disease (GVHD).

Keyword: donor lymphocyte infusion (DLI), graft-versus tumor (GVT), acute lymphoid leukemia (ALL), graft-versus-host disease (GVHD)

Student number: 2005-30687

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Introduction

Selective expansion of autologous antitumor-specific T cells from sick patients is a particularly laborious and time-consuming process. Although using T cells from a healthy volunteer is an alternative, the specificity of the immune response, discriminating self from non-self, limits the compatibility of allogeneic donor and recipient T cells, and host immune cells reject adoptively-transferred T cells. However, this hurdle was overcome in the hematology field by the first successful allogeneic hematopoietic stem cell transplantation (HSCT) in 1959.[1-2] Allogeneic HSCT was initially developed to treat individuals with inherited anemia or immune deficiencies by replacing the abnormal hematopoietic system with a healthy one. It has also allowed the delivery of myeloablative doses of radiation and chemotherapy in patients with cancer.[3-4] In hematologic malignancies, neoplastic blood cells spread throughout the body; therefore, systemic chemotherapy and radiotherapy are the main medical treatments. Treatment of patients with myeloablative conditioning to maximize the killing of leukemia cells must be followed by an infusion of hematopoietic cells to rescue the hematopoietic destruction induced by these excessive therapeutic operations.[5] Additional antitumor therapeutic effects of HSCT were confirmed to be due to the allogeneic responses of small contaminating populations of mature T cells in donor marrow. Expanded T cells from grafts mainly recognize minor histocompatibility antigens on recipient tumor cells and eradicate residual malignancy. This process is referred to as the graft-versus-tumor (GVT) effect. However, these T cell responses also occur against normal tissues, and can develop into

the detrimental and often life-threatening side effect, graft-versus-host disease (GVHD). GVT and GVHD phenomena observed in preclinical mouse models were later identified as T cell-mediated symptoms.[6-8] In humans, GVHD is associated with a decreased rate of malignant relapse.[9, 10] Like this, adoptive T cell therapeutic response against hematologic malignancy was inadvertently developed in HSCT, and it was regarded the first cancer immune therapy and the earliest example of individualized cancer therapy.

Although the myeloablative conditioning regimen has an efficient therapeutic antitumor effect, it is considered to be too toxic for sick and old patients and is often accompanied with unwanted even lethal side effects, such as an increased probability of infection and bursts of severe pro-inflammatory cytokines.[5, 7, 11, 12] Therefore, non-myeloablative conditioning, which has been used along with reduced intensity chemotherapy and radiotherapy, is commonly used at present. In this case, mixed chimerism is initially established by the compatibility of the donor and recipient hematopoietic systems. Then, a small population of alloreactive T cells that contaminated the donor marrow initiates a potent allogeneic response against the recipient hematopoietic cells. Finally full donor chimerism is established. Host hematopoietic malignant cells are also killed in this process, mainly by the allogeneic immune response.[13-21]

However, non-myeloablative conditioning is relatively inefficient at eradicating residual tumor cells and full donor chimerism is not established in some patients, which results in a higher relapse rate. Such relapses require another therapeutic method; therefore, donor lymphocyte infusion (DLI) was introduced to treat relapsed patients after allogeneic hematopoietic cell transplantation (HCT).[5, 17, 22]

With DLI, the infused donor lymphocytes are not rejected by the recipient T cells due to tolerance induced by the allogeneic HCT process; thus, they can eliminate recurring tumor cells, primarily via allogeneic immune responses. In the late 1990s, DLI was developed to treat Epstein-Barr virus (EBV)-related lymphoma and post-transplant lymphoproliferative disease (PTLD), which occur in approximately 1% of individuals undergoing allogeneic HSC transplants who receive immunosuppressive drugs to prevent GVHD.[23, 24] It is currently applied to treat various types of leukemia.[25, 26] Therefore, DLI is one example of an effective adoptive T cell therapy that is widely applied for many hematologic malignancies.

Allogeneic T cell responses that occur after DLI sometimes destroy normal tissues. Although both GVHD and GVT originate with the same allogeneic responses between the infused T cells and recipient antigen presenting cells (APC), GVHD is preferentially induced in highly inflammatory cytokine milieus. Extravasation of activated T cells into target tissues is facilitated in highly inflammatory environments, which can develop into severe GVHD.[27, 28] Potent GVT induction without GVHD is the ultimate goal in the field of hematology, and delayed administration of DLI has been reported to be a good method to achieve this.[29, 30] Delayed DLI administration provides enough time to achieve mixed chimerism in non-myeloablative-conditioned HSCT recipients, and to subside inflammation caused by the conditioning process. Using this regimen, diminished the extravasation of infused donor-derived allogeneic T cells into normal tissue and significantly reduced GVHD. Therefore, delayed DLI could be a good starting point for

the development of an allogeneic T cell therapy without harmful side effects.

Currently, DLI following allogeneic HSCT is an established general therapeutic method for various hematologic malignancies. DLI shows 70 – 80% therapeutic efficacy in chronic myeloid leukemia (CML) patients.[8] However, the therapeutic efficacy of DLI in acute lymphoid leukemia (ALL) patients is not satisfactory, and only 10 – 20% therapeutic efficacy was reported.[31-33] Therefore, the therapeutic efficacy needs to be improved. In addition, the only currently available preclinical animal model for delayed DLI is a tumor preventive model, in which DLI is performed in the absence of tumors, and then animals are inoculated with tumor cells. This does not reflect the real clinical situation of ALL patients.

In this study, we hypothesized that potentiation of DLI T cells will provide an enhanced GVT effect in a delayed DLI model. To potentiate T cell reactivity, we introduced a CTLA4-CD28 chimeric receptor gene into allogeneic T cells, and these cells were used for DLI. Previously, we confirmed that transduction of a CTLA4-CD28 chimeric receptor results in the potentiation of T cells and enhancement of syngeneic tumor regression.[34] To test an ALL-like clinical situation, EL4 cells were inoculated into a mixed chimera mouse prior to DLI. Retroviral transduction of the CTLA4-CD28 chimeric receptor gene into DLI T cells resulted in a potent and sustained allogeneic T cell response in the recipients. Therefore, an enhanced GVT effect was induced in this preclinical ALL-like animal model.

Materials and Methods

Mice

Female C57BL/6 (B6; H-2^b) and B10.A (H-2^a) were purchased from Orient Bio (Korea) and SLC (Japan), respectively. Male BALB/c nude mice were purchased from SLC. B6.IL-$2^{-/-}$ mice were purchased from Jackson Lab (USA) and cross mated with B10.A to exchange genetic background. B10.A.IL-$2^{-/-}$ mice were obtained from mating between B10.A.IL-$2^{+/-}$ mice. Although complete genetic background switch to B10.A is necessary to backcross with B10.A for 8 generations or more, substitution of major histocompatibility complex (MHC) from B6 to B10.A is possible by only this cross mating, and confirmed by FACS staining with FITC-conjugated anti-H-2D^d Ab. Donor mice were aged 5 – 6 weeks, and recipient B6 mice were aged 9 – 12 weeks at the time of bone marrow transplantation (BMT). All of the mice were housed in specific pathogen-free (SPF) animal facility at the Research Institute National Cancer Center (Korea), and maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC).

Cells

EL4 is a B6-derived lymphoid leukemia cell line expressing H-2^b and was originally acquired from American Type Culture Collection (ATCC). Hybridoma producing cytotoxic anti-CD8 monoclonal antibody 2.43 (ATCC TIB-210) was also purchased from ATCC for *in vivo* CD8 depletion study. 2.43 ascites were generated in BALB/c nude, which were pre-treated with incomplete Freund's adjuvant, and used in CD8 depletion study. Phoenix GP and Phoenix Eco cell

lines were provided by Garry Nolan (Stanford University).

DNA constructs

To generate CTLA4-CD28 chimeric gene, the extracellular and transmembrane domain of murine CTLA4 (nucleotides$_{1-567}$) and the cytoplasmic domain of murine CD28 (nucleotides$_{529-657}$) were amplified by polymerase chain reaction (PCR) from the plasmids containing murine CTLA4 and CD28 cDNA (ImaGenes). Each amplified fragments were joined by blunt end ligation and were cloned into a cloning vector. Subsequently, CTLA4-CD28 chimera cDNA was cloned in to pMSCV-IRES-GFP-WRE (pMIGw) retroviral expression vector (a gift from Yosef Refaeli, National Jewish Medical and Research Center).

Production and transduction of retrovirus

The retroviral plasmids and a plasmid encoding VSV-G cDNA (pMD.G) were transiently transfected into Phoenix GP cell line using Lipofectamine 2000 (Invitrogen). After 48 h, the culture supernatant containing VSV-G pseudotyped retrovirus was harvested. Then, Phoenix Eco cell line was transduced with the retrovirus-containing supernatant overnight. After 3 to 5 days, green fluorescence protein (GFP)-positive Phoenix Eco cells were purified by FACS Aria (Becton Dickinson) to generate stable cell lines for producing ecotropic retroviruses. The culture supernatant containing ecotropic retrovirus was harvested and then concentrated 10-fold using a centricon (Amicon Ultra-15, 100kDa cut-off; Millipore) for murine T cell transduction.

For retroviral transduction of T cell, lymphocytes from lymph nodes and spleen from B10.A mice or B10.A.IL-2$^{-/-}$ mice were

stimulated with plate-bound anti-CD3 (10μg/ml; 145-2C11) and anti-CD28 (2μg/ml; 37.51) Abs. After 24 h stimulation, T cells were transduced with the concentrated retroviruses by centrifuging the cells at 2,500 rpm for 90 min (spin infection). This procedure was repeated once on the same day. During the spin infection, 6μg/ml Polybrene (Sigma-Aldrich) was added to the culture supernatant to enhance the transduction efficiency. After 48 h further stimulation, the transduced T cells were transferred to a fresh medium containing 20 U/ml recombinant mouse IL-2 (Invitrogen) and rested for 72 h without stimulation. After resting, more than 95% of the live cells were CD4 or CD8 T cells. Transduction efficiency was checked by GFP expression and surface CTLA4 expression level was also confirmed by multi-color flow cytometry using PE-conjugated anti-CTLA4 Ab (UC10-4F10-11).

Bone Marrow Transplantation (BMT)

Recipient B6 mice were lethally (split 9Gy) irradiated, followed the next day by intravenous injection of a mixture of T cell-depleted donor (B10.A; 1.5×10^7cells) and recipient (B6; 0.5×10^7 cells) bone marrow cells. Recipients were provided antibiotics by drinking water for 5 weeks starting from 1 week before BMT. BM cells were flushed out from tibia and femur of donor mice and T cell depletion was performed by complement lysis method using Ab cocktail with anti-Thy1.2 (30-H12), anti-CD4 (GK1.5), and anti-CD8 (53-6.7) Abs, and guinea pig complement (Cedarlane). Mixed chimerism in peripheral blood was assessed at 7 weeks after transplantation by multi-color flow cytometry using FACS Calibur cytometer (Becton Dickinson). Fluorescein isothiocyanate (FITC)-

conjugated anti-H-2D^{d} Ab (34-2-12) was used to distinguish between donor (B10.A) and recipient (B6) hematopoietic cells.

Donor Lymphocytes Infusion (DLI)

To establish lymphoid leukemia in mixed chimera recipients, EL4 (1×10^{4}) cells were administered intravenously through the tail vein on at least 8 weeks after BMT. In this case, nearly 100% of lethality was confirmed before 40 days. DLI was operated in tumor-bearing recipients at 4 – 6 h later on the same day.

For naïve DLI, B10.A splenocytes were harvested and lysis red blood cells (RBC) by ACK-lysis buffer. Single cell suspensions were filtered through nylon mesh and administered (2×10^{7} cells) by intravenous injection. For pre-activated DLI, B10.A splenocytes were stimulated with anti-CD3 (10μg/ml; 145-2C11) and anti-CD28 (2μg/ml; 37.51) Abs. After 72 h stimulation, T cells were transferred to a fresh medium containing 20 U/ml mouse IL-2 (Invitrogen) and rested for 72 h without stimulation. Rested T cells were harvested and administered (5×10^{6} cells) by intravenous injection. For Unfractionated CTLA4-CD28 chimera (CTC28)-transduced DLI, T cell transduction was performed using method described above and administered (5×10^{6} cells) by intravenous injection. Finally, for fractionated CTC28-transduced DLI, CD4 and CD8 T cells were purified from B10.A mice by positive selection using CD4 and CD8 microbeads (Miltenyi Biotec) and T cell transduction was performed using method described above. Transduced CD4 and CD8 T cells were mixed by 1:1 ratio and administered (each $3 – 5 \times 10^{6}$ cells) by intravenous injection.

For *in vivo* CD8 T cell depletion study, 2.43 AF (100μl, measured by 10 mg/ml concentration) were injected every other day for the

first week after tumor and DLI co-inoculation, and then injected once a week. Above 98% depletion of CD8 cells in blood was confirmed by flow cytometry. Control group was injected with rat IgG (1mg) by the same schedule.

GVT effect of DLI was evaluated by survival percentage of recipients receiving EL4 and DLI. Survival was monitored until 70 – 80 days after tumor injection. Autopsy was performed in all dead mice to determine whether the death was leukemia-related or caused by GVHD.

Analysis of peripheral bloods

After DLI in mixed chimera, tracing of DLI cells and mixed chimerism shift was evaluated at various times. Peripheral blood was collected and lysis RBC by ACK-lysis buffer. *In vivo* tracing of modified donor T cells was determined by GFP-expressing CD4 and CD8 T cells. Mixed chimerism shift, resulted from allogeneic responses of DLI cells was also evaluated by multi-color flow cytometry. The following antibodies were used for DLI cell tracing and chimerism analysis: APC-conjugated anti-CD4 (RM4-5), PE-Cy5-conjugated anti-CD8 (53-6.7), and PE-conjugated H-2K^{b} (AF6-88.5.5.3) Abs. Flow cytometry was performed using a FACS Calibur flow cytometer (Becton Dickinson) and analysis was performed FlowJo software (TreeStar Inc.).

Assessment of graft-versus-host-disease (GVHD)

Body weights were measured once or twice weekly during the whole experiments. Clinical evidence of GVHD was also scored during the whole experiments by assessment of changes in skin (alopecia, inflamed or scaly skin), generalized signs (fur texture,

posture, activity), inflammation of the eyes, and diarrhea. Each parameter was quantified by scoring as follows: 0, normal; 1, mild; 2, moderate; 3, severe (for scoring of eye inflammation, 4 indicate eyes severely inflamed and closed). Total clinical GVHD scores were defined as the sum of the scores from each parameter.[35)]

For histopathological analysis of GVHD target tissues, samples were collected from liver, lung, colon and skin (from the neck) and fixed in 10% formalin. Formalin-fixed tissue samples were embedded in paraffin, cut into 5μm thick sections, and stained with Hematoxylin and Eosin (H&E) for histological examination.

Statistical analysis

Survival differences between groups were analyzed by the log-rank (Mantel-Cox) test using Prism software (GraphPad Software Inc.). Other statistical differences between groups were analyzed by Student's paired *t*-test using SigmaPlot software (Systat Software Inc.).

Results

CTLA4-CD28 chimeric gene-modified DLI shows a GVT effect in a tumor co-injection model

Although delayed DLI is often administered to patients without signs of recurrence to prevent tumor relapse in real clinical situation, the only current preclinical model of delayed DLI does not reflect the physiological situation of tumor relapse followed by therapy. Therefore, we modified the injection schedule for the tumor cells and DLI cells to test whether delayed DLI will work in a relapsed ALL-like model. EL4, a T lymphoma cell line derived from B6 mice, grew aggressively in a syngeneic mouse, and inoculation of only 10^4 cells is sufficient for lethal tumor development. A bone marrow (BM) mixed chimera was established in B6 mice by injection of a BM mixture from B10.A and B6 mice, and then the mice were left untreated for approximately 2 months to allow the inflammation generated by bone marrow transplantation (BMT) to subside. After mixed chimerism was confirmed by blood analysis, EL4 cells were inoculated, followed by DLI on the same day. In this model, the DLI therapy is administered when the T lymphoma cells are present in the blood stream to mimic ALL. We called this the "tumor co-injection model." [36, 37)]

First, we evaluated the antitumor effect of naïve allogeneic DLI in the tumor co-injection model. Splenocytes derived from donor B10.A mice were used as the naive DLI cells. Most mixed chimeric mice, which were inoculated with 10^4 EL4 cells, were killed by the aggressive developing tumor 40 days after tumor injection. Preventive naïve allogeneic DLI, in which naïve DLI was administered 7 days before tumor injection, which is the current

widely used delayed DLI preclinical model, resulted in 100% survival of EL4-inoculated mice. In contrast, naïve allogeneic DLI did not exhibit any GVT effect in the tumor co-injection model (Figure 1).

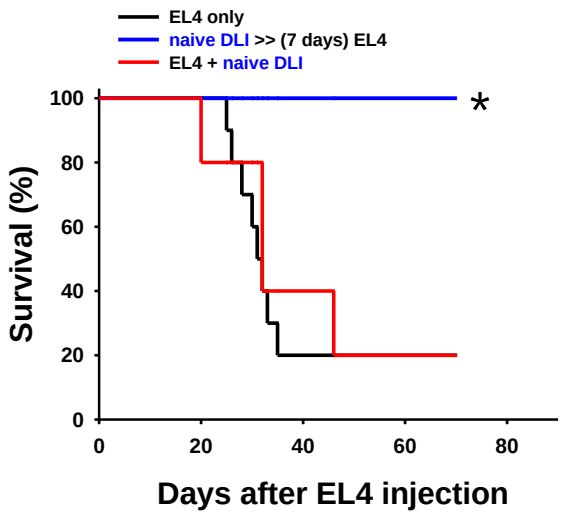

Figure 1. Naïve allogeneic DLI does not exhibit a GVT effect in the tumor co-injection model. Lethally irradiated B6 mice were reconstituted with a mixture of T cell-depleted (TCD) bone marrow (BM) cells from B10.A and B6 mice. EL4 (1×10^4) cells were injected intravenously at least 8 weeks after BM transfer. Naïve DLI cells were prepared from B10.A splenocytes and administered (2×10^7) 7days before EL4 injection (blue; prevention model) or on the same day in tumor-bearing mice (red; co-injection model). Data were pooled from 2 independent experiments. ($*P = 0.0087$; log-rank [Mantel-Cox] test)

When naïve DLI T cells were injected into mixed chimeric mice, allogeneic T cells started to react against host allogeneic antigens, and then differentiated into effector T cells. It takes approximately 7 – 10 days to acquire antitumor effector functions *in vivo*. However, the aggressively proliferating EL4 cells were overburdening the mice during that time, and GVT was not

observed in the tumor co-injection model. Conversely, in the preventive DLI model, a 7 day priming period before tumor inoculation could impart the naïve DLI with antitumor effector functions. Therefore, we tested the GVT effect of pre-activated T cells in the tumor co-injection model, because the recall response of pre-activated T cells requires shorter priming time than naïve T cells. Splenocytes derived from donor B10.A mice were stimulated with anti-CD3 and anti-CD28 Abs and these rested T cells were used as pre-activated DLI cells in the co-injection model. However, enhanced survival was not detected (Figure 2).

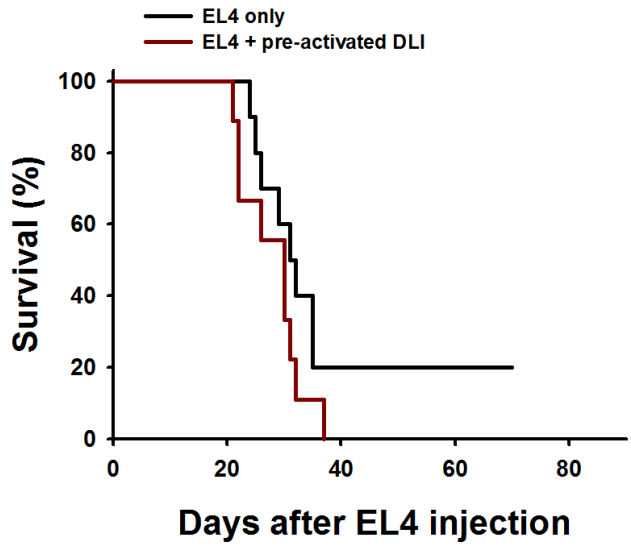

Figure 2. Pre-activated DLI does not show a GVT effect in the tumor co-injection model. B10.A splenocytes were stimulated with anti-CD3 (10μg/ml) and anti-CD28 (2μg/ml) Abs. After 72 h of stimulation, T cells were transferred to fresh medium containing 20 U/ml mouse IL-2 and rested for 72 h without stimulation. Rested T cells were harvested and administered intravenously (5×10^6) to EL4-inoculated (1×10^4) mixed chimeric mice. Data were pooled from 2 independent experiments.

Next, to induce T cell reactivity, we introduced the CTLA4-CD28 chimeric gene (CTC28) into DLI cells and tested them for a GVT effect in the tumor co-injection model. CTC28 modification of DLI T cells was accomplished by retroviral transduction of CTC28, which was previously described in our Ag-specific solid tumor model study.[34] CTC28 transduction efficiency, as measured by GFP expression, was approximately 90% in CD4 T cells and 75% in CD8 T cells, and stable surface expression of the CTLA4 molecule was confirmed by flow cytometry (Figure 3).

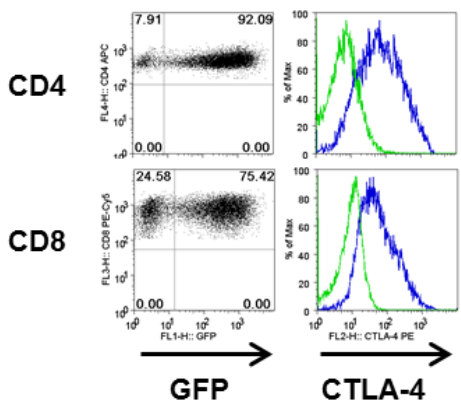

Figure 3. A CTLA4-CD28 chimeric gene was transduced and expressed in DLI T cells. Lymphocytes from B10.A mice were stimulated with a plate-bound anti-CD3 (10μg/ml) and an anti-CD28 (2μg/ml) Abs. After 24 h of stimulation, T cells were transduced with the concentrated retroviruses by centrifuging the cells at 2,500 rpm for 90 min, and this procedure was repeated once on the same day. After 48 h of additional stimulation, the transduced T cells were transferred to fresh medium containing 20 U/ml mouse IL-2, and then rested for 72 h without stimulation. After resting, transduction efficiency was assessed by measuring GFP expression. Surface CTLA4 expression was also measured by multi-color flow cytometry. Representative flow cytometry profiles from separate experiments are shown.

We transduced CTC28 into splenocytes from donor B10.A mice, and then tested these cells in the tumor co-injection model. However, no GVT effect was observed (Figure 4).

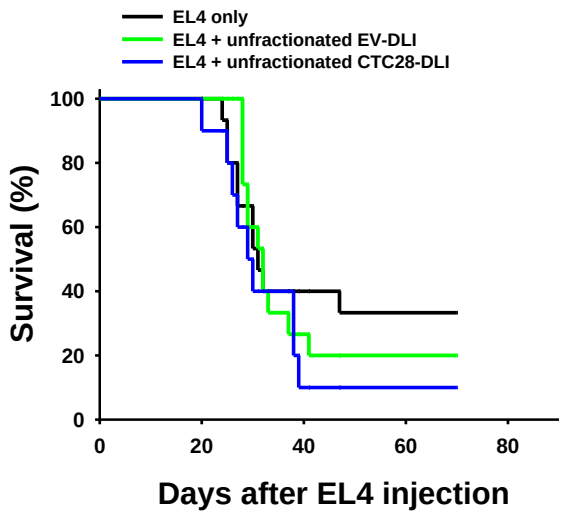

Figure 4. Unfractionated CTC28-DLI does not exhibit a GVT effect in the tumor co-injection model. B10.A splenocytes were stimulated with an anti-CD3 (10μg/ml) and an anti-CD28 (2μg/ml) Abs. After 24 h of stimulation, T cells were transduced with concentrated retroviruses by spin infection. Then after an additional 48 h of stimulation, the transduced T cells were transferred to fresh medium containing 20 U/ml mouse IL-2, and then rested for 72 h without stimulation. Rested transduced T cells were harvested and injected intravenously (5×10^6) into EL4-inoculated (1×10^4) mixed chimeric mice. Data were pooled from 2 independent experiments.

Although the numbers of CD4 and CD8 T cells were nearly identical in naïve mouse splenocytes, *in vitro* stimulation for preparation of activated or gene-modified T cells resulted in a 3 - 4 fold greater expansion of CD8 T cells than that of CD4 T cells. This dominant CD8 T cell expansion might be the result of their differential kinetics. Although CD8 T cells directly killed the tumor

cells, CD4 T cells were reported to enhance therapeutic efficacy in adoptive T cell therapy.[38-40] In addition, donor-derived CD4 T cells recognize tumor antigens as foreign and generate an antigen-specific immune response that contributes to the GVT effect.[41, 42] Moreover, in our previous solid tumor model study, the CTC28 modification effect was predominantly occurred in CD4 T cells.[34] Therefore, we first fractionated CD4 and CD8 T cells from donor splenocytes, and then transduced these cells with CTC28 chimera. Equal numbers of CTC28-modified CD4 and CD8 T cells were used as DLI cells and tested in the tumor co-injection model. Unlike unfractionated CTC28-modified T cells, fractionated CTC28-modified T cells exerted a significant GVT effect, and the survival rate was significantly increased (Figure 5).

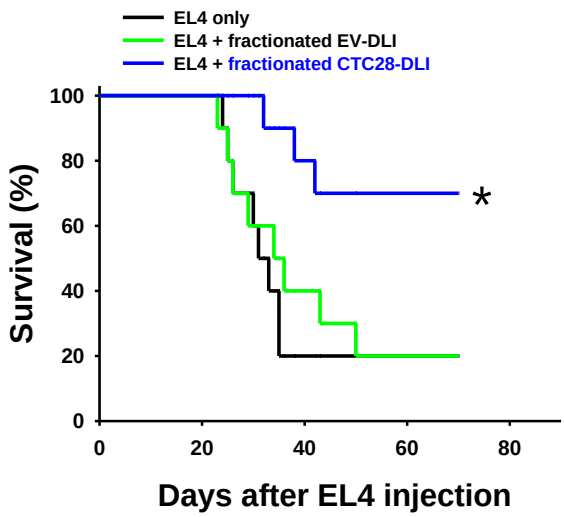

Figure 5. Fractionated CTC28-DLI exhibits a GVT effect in the tumor co-injection model. CD4 and CD8 T cells were purified from B10.A mice by positive selection using microbeads and stimulated with anti-CD3 (10μg/ml) and anti-CD28 (2μg/ml) Abs. T cells were transduced using the method described above during the polyclonal T cell stimulation process. Transduced CD4 and CD8 T cells

were mixed at a 1:1 ratio and injected intravenously ($3 - 5 \times 10^6$ cells of each type) into EL4-inoculated (1×10^4) mixed chimeric mice. Data were pooled from 2 independent experiments. ($*P = 0.0077$; log-rank [Mantel-Cox] test)

Dual modification of CD4 and CD8 T cells is required for expansion of CD8 T cells and the GVT effect in the tumor co-injection model

Although CTC28 modification of both CD4 and CD8 T cells almost completely eradicated established tumors in the Ag-specific solid tumor model, the effect was predominantly due to CD4 T cells.[34] To evaluate the effect of CTC28 modification in the two T cell types in the DLI model, we prepared EV-transduced and CTC28-transduced CD4 and CD8 T cells and tested them for a GVT effect in the tumor co-injection model. Dual CTC28-modified CD4 and CD8 T cells exhibited a significant GVT effect. However, injection of either CTC28-transduced CD4 or CD8 T cells did not confer enhanced survival (Figure 6).

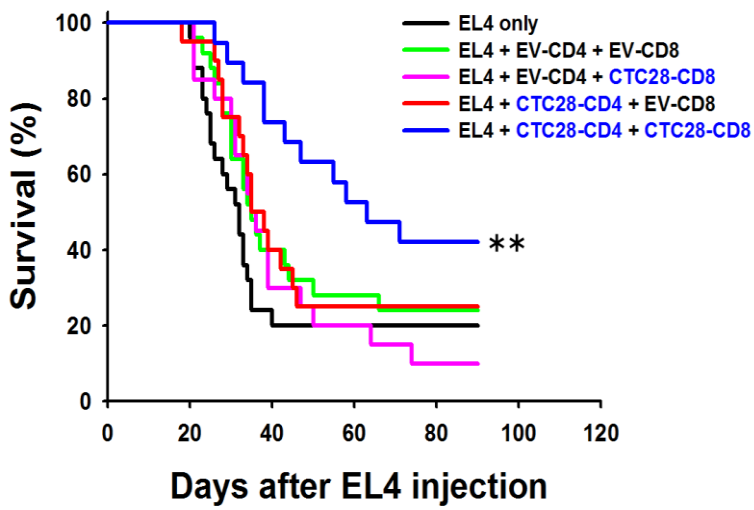

Figure 6. Injection of CTC28-modified CD4 and CD8 T cells confers a GVT

effect in the tumor co-injection model. CD4 and CD8 T cells were purified from B10.A mice by positive selection using microbeads and stimulated with an anti-CD3 (10μg/ml) and anti-CD28 (2μg/ml) Abs. T cells were transduced using the above mentioned method during the polyclonal T cell stimulation process. Various combinations of EV- or CTC28-transduced CD4 and CD8 T cells were mixed at a 1:1 ratio and injected intravenously ($3 - 5 \times 10^6$ cells of each type) into EL4-inoculated (1×10^4) mixed chimeric mice. Data were pooled from 4 independent experiments. (**$P = 0.0067$; log-rank [Mantel-Cox] test).

We conducted a mechanistic study of the CTC28-modified DLI-mediated GVT effect in the tumor co-injection model. First, a blood analysis was conducted after DLI. The *in vivo* fates of the injected DLI cells were evaluated by tracing the GFP-expressing T cells. Although the CTC28-transduced CD4 T cells expanded sufficiently, the CTC28-transduced CD8 T cells did not. *In vivo* CD8 T cell expansion was only observed when dual CTC28-modified CD4 and CD8 T cells were used (Figure 7). Therefore, the enhanced GVT effect observed in mice, that were injected with CTC28-transduced CD4 and CD8 T cells, might be partly due to the enhanced expansion of CD8 T cells.

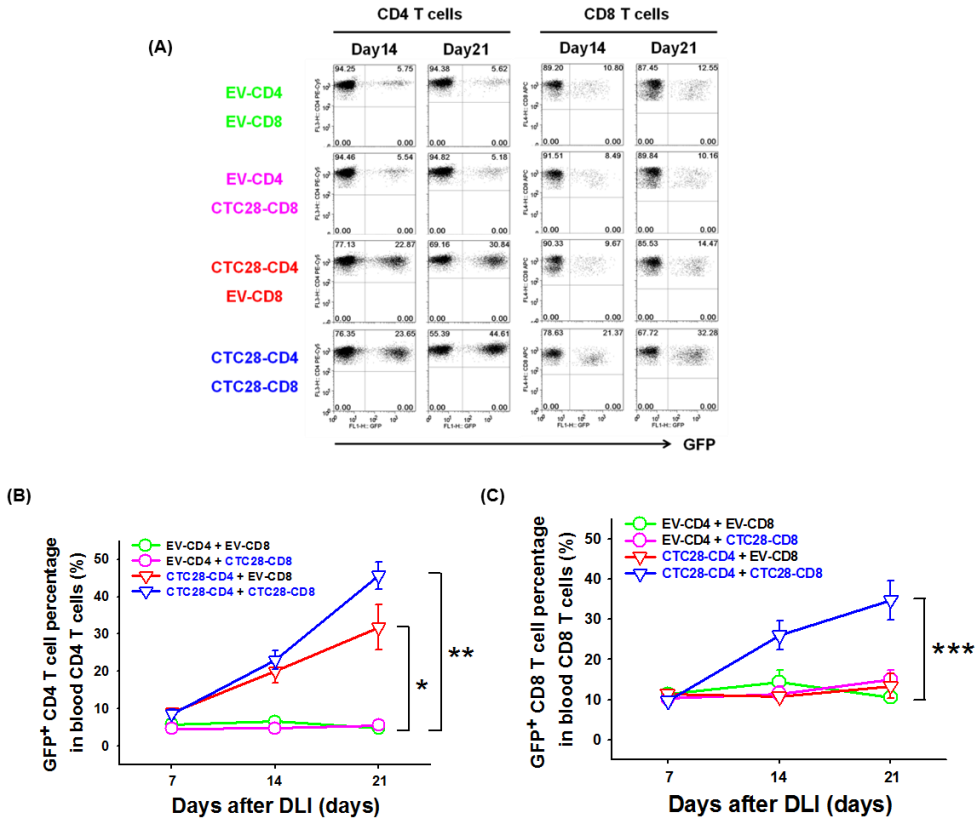

Figure 7. CTC28 modification of both CD4 and CD8 T cells enhanced the expansion of CD8 T cells *in vivo*. Fractionated, CTC28-modified DLI T cells were injected into tumor-bearing mice, as described in Figure 6. Peripheral blood was collected at various times, and the *in vivo* expansion of transduced-DLI cells (GFP^+) was analyzed by flow cytometry. (A) Representative flow cytometry profiles from separate experiments are shown. (B, C) Percentage of GFP^+ CD4 and CD8 T cells in blood. Data were pooled from 2 independent experiments. ($*P < 0.0053$, $**P < 0.0002$, $***P < 0.05$; Student's paired t-test).

The contribution of CD8 T cell expansion to the GVT effect was further confirmed in an *in vivo* CD8 depletion study. To deplete CD8 T cells *in vivo*, mice were injected with 2.43 ascites or control rat IgG every other day for the first week after tumor and CTC28-modified DLI co-inoculation, and then injected once a week. Greater than 98% of the CD8 T cells in blood were depleted

beginning 4 days after antibody injection, and this was maintained for 28 days. Although a slight enhancement of survival was detected in the CTC28-modified DLI plus anti-CD8 depletion Ab-injected group, compared to the tumor only group, these results show that a major portion of the GVT effect induced by CTC28-modified DLI was abrogated by CD8 T cell depletion (Figure 8).

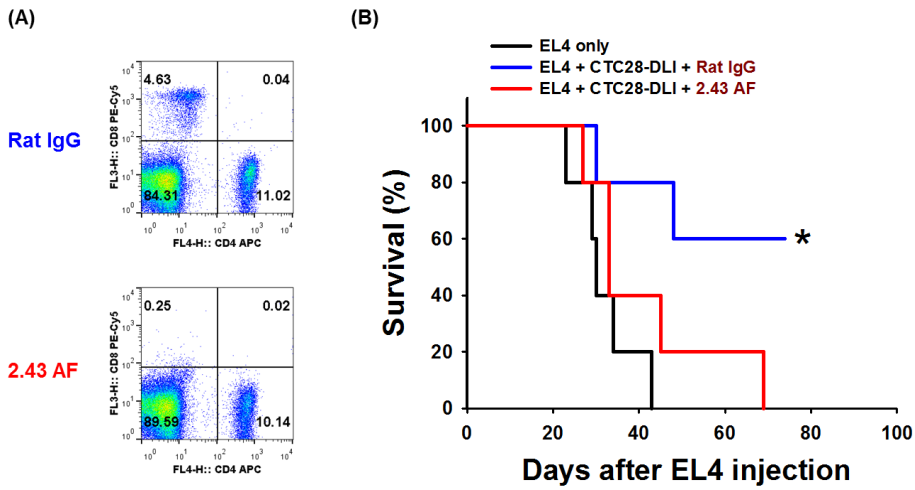

Figure 8. CD8 T cells are the main mediator of the GVT effect in the tumor co-injection model. Fractionated CTC28-modified DLI T cells were injected into tumor-bearing mice, as described in Figure 6. To deplete CD8 T cells, 2.43 ascites or control rat IgG were injected in mice every other day for the first week and then once a week for 28 days. (A) Representative flow cytometry profiles of CD8 depletion in blood samples. (B) Survival curve of the CD8 depletion experiment. (*P = 0.0143; log-rank [Mantel-Cox] test).

The functional activity of CTC28-modified T cells is maintained after *in vivo* expansion

In a functional assay of *in vivo* transferred DLI cells, we analyzed spleen cells at 10 days after DLI injection. Robust expansion of CTC28-modified T cells was detected in splenocytes (Figure 9A,

9B), as previously observed in blood sample analysis (Figure 7). The percentage of IFN-γ-producing CD4 T cells in the CTC28-modified cells was higher than that in the EV-transduced cells. Although the percentage of IFN-γ-producing CD8 T cells among the EV- and CTC28-transduced T cells were similar, the absolute number of IFN-γ-producing CD8 T cells was significantly higher in the CTC28-modified cells, due to the extensive expansion (Figure 9C, 9D).

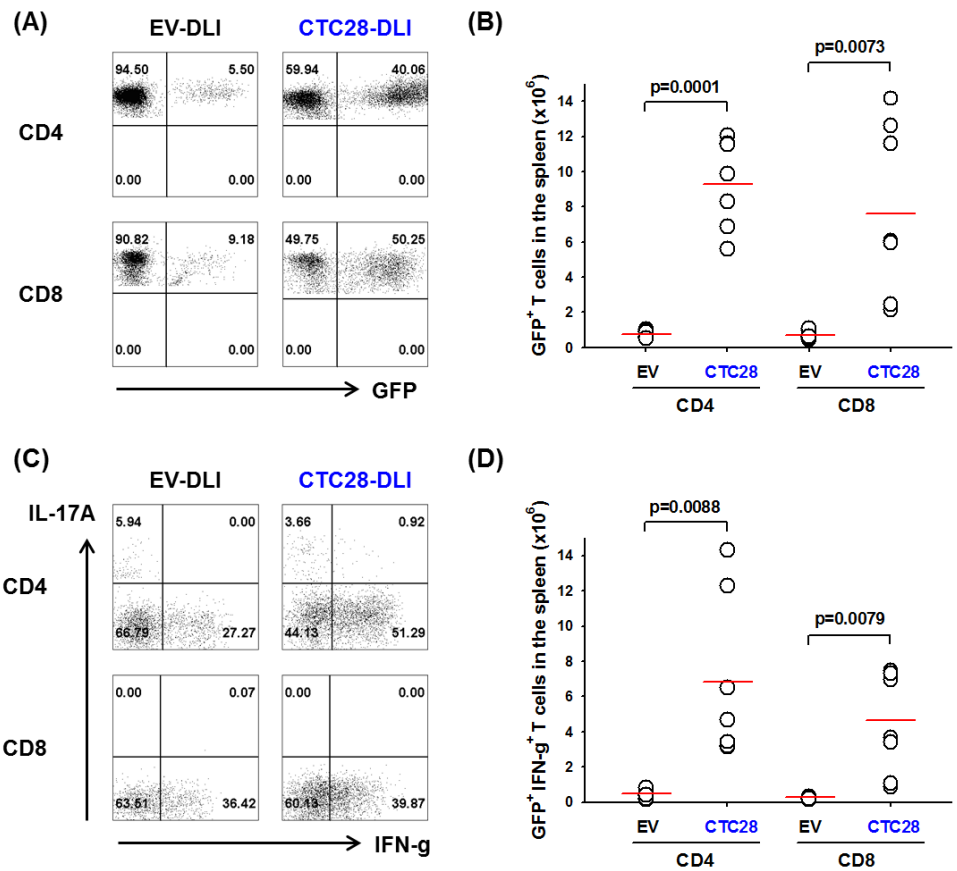

Figure 9. CTC28 modification induces robust expansion of therapeutic T cells, which maintain their functionality *in vivo*. Ten days after the adoptive transfer into tumor-bearing mixed chimeric mice, spleens were extracted, and the cells were analyzed by flow cytometry. The retrovirus-transduced cells were

identified by GFP expression. (A) Representative flow cytometry profiles of splenocytes. (B) Absolute numbers of transduced CD4 and CD8 T cells are shown (*P* value; Student's paired *t*-test). (C, D) Splenocytes were re-stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of Golgi-plug for 4 h. Then the cells were fixed and permeabilized with 4% paraformaldehyde and Triton buffer, respectively, and intracellular cytokine production was confirmed by multi-color flow cytometry. (C) Representative flow cytometry profiles of intracellular cytokine staining (GFP^+ cell gating). (D) Absolute numbers of IFN-γ-producing CD4 and CD8 T cells are shown (*P* value; Student's paired *t*-test).

IL-2 production by DLI T cells is necessary for the therapeutic effect of CTC28-modified T cells

In our previous Ag-specific solid tumor model study, the antitumor effect of CTC28-modified T cells was abrogated by treatment with an IL-2 neutralizing antibody, which demonstrated the important role of IL-2 in the antitumor effect.[34] Although CTC28 modification enhanced IL-2 production in CD4 T cells more than that in CD8 T cells and the *in vivo* antitumor effect of CTC28-modified T cells was abrogated by treatment with an IL-2 neutralizing antibody in our previous study, we could not conclude that the production of IL-2 by the CTC28-modified CD4 T cells was critical for the antitumor effect in CTC28-modified T cell therapy. To assess the contribution of IL-2 produced by CD4 and CD8 T cells in the tumor co-injection model, we prepared CTC28-modified DLI T cells from wild type (WT) and IL-2 knockout (KO) mice, and evaluated the GVT effect of various combinations of these CD4 and CD8 T cells in the tumor co-injection model. DLI T cells derived from WT and IL-2 KO mice were comparably expanded *in vivo* (data not shown). DLI groups containing CD4 T cells, CD8 T cells, or both CD4 and CD8 T cells prepared from WT mice,

exhibited a GVT effect in the tumor co-injection model. However, mice injected with DLI prepared with CD4 and CD8 T cells from IL-2 KO mice did not exhibit enhanced survival (Figure 10).

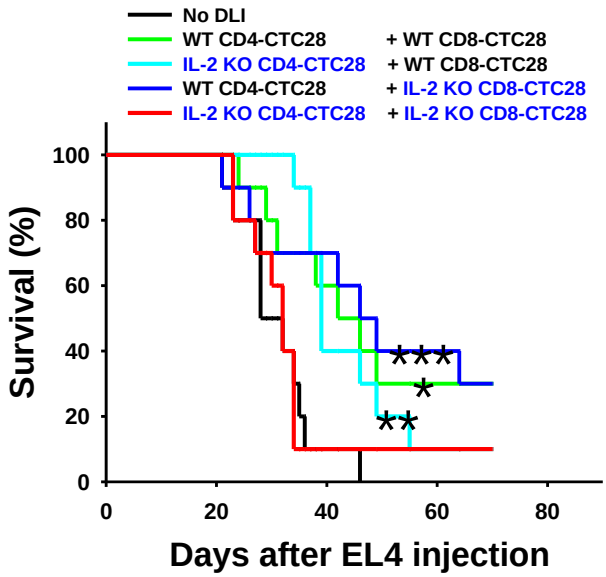

Figure 10. IL-2 production by either CD4 or CD8 T cells is necessary for the GVT effect. CD4 and CD8 T cells were purified from B10.A WT and B10.A.IL-2 KO mice by positive selection using microbeads, and stimulated with an anti-CD3 (10μg/ml) and an anti-CD28 (2μg/ml) Abs. CTC28-transduction was performed as described above during the polyclonal T cell stimulation process. CTC28-transduced CD4 and CD8 T cells from WT and IL-2 KO mice were mixed in various combinations at a 1:1 ratio and injected intravenously ($3 - 5 \times 10^6$ cells of each type) into EL4-inoculated (1×10^4) mixed chimeric mice. Data were pooled from 2 independent experiments. ($*P = 0.0111$, $**P = 0.0011$, $***P = 0.0155$; log-rank [Mantel-Cox] test).

CTC28-modified DLI provides a GVT effect in the tumor co-injection model; however, it is followed by a harmful side effect, GVHD

Although the potent, sustained immune responses of the CTC28-modified DLI cells against host allogeneic antigens provided therapeutic efficacy in the tumor co-injection model, the probability of GVHD was also increased. To evaluate the effect of CTC28-modified T cells in GVHD development, we transferred these modified T cells into mixed chimeric mice, without tumor injection. The transferred T cells encountered host allogeneic antigens, proliferated, and differentiated into effector T cells. A sustained T cell response often provokes an inflammatory milieu *in vivo*, and this in turn facilitates extravasation of activated T cells into normal tissue, followed by the development of GVHD.[28)]

GVHD was assessed by measuring weight loss, the clinical GVHD scores described in the Material and Methods, and histological evaluation.[35)] The CTC28-modified DLI group exhibited significant GVHD symptoms (Figure 11), whereas in the EV-modified DLI group, body weight loss and clinical GVHD symptoms were not observed during the experimental period. Conversely, body weight loss in the CTC28-transduced DLI treated group started at 7 days after DLI injection and progressed until the mice lost approximately 25% of their initial body weight (Figure 11A). Clinical GVHD scores also increased with elapsed time after DLI (Figure 11B). Histological H&E staining, which was performed at 28 days after DLI injection, showed severe infiltration of mononuclear cells in GVHD target tissues in the CTC28-transduced DLI group (Figure 11C). However, even though GVHD developed early after DLI and rapidly progressed, it was relatively non-lethal, and long-term survival was different from that with lethal acute GVHD (Figure 11D).

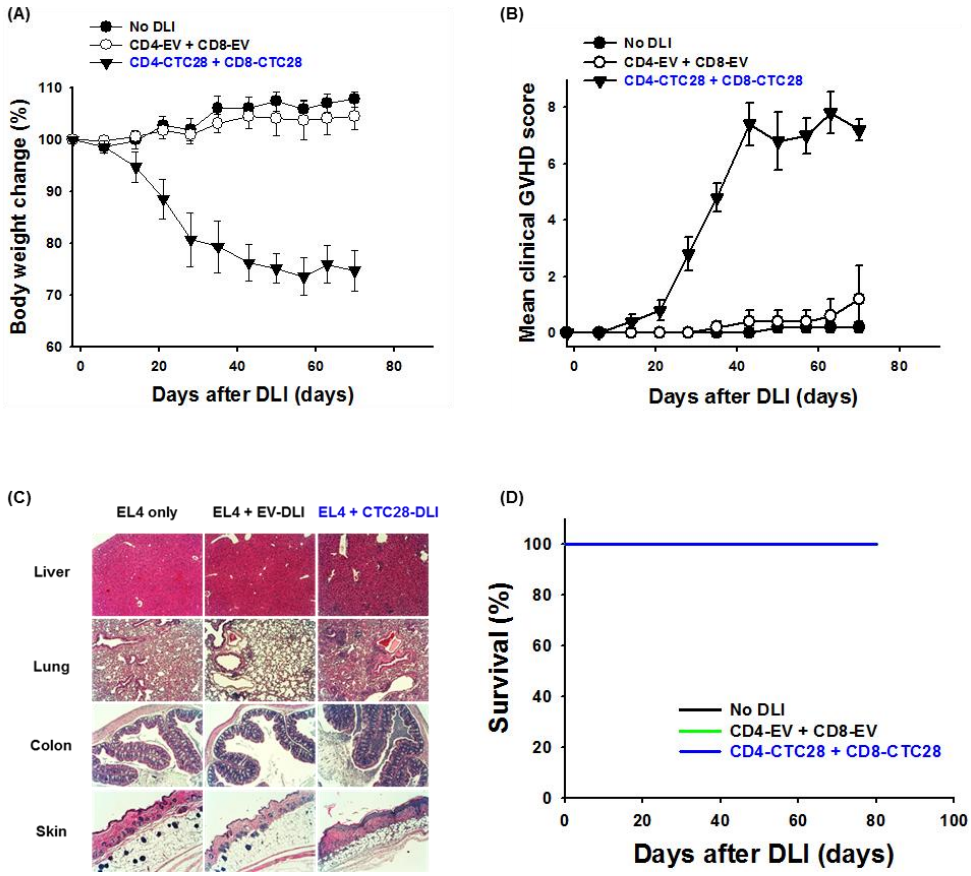

Figure 11. CTC28-transduced DLI-treated mice exhibited symptoms of non-lethal GVHD. CD4 and CD8 T cells were purified from B10.A mice by positive selection using microbeads and stimulated with an anti-CD3 (10μg/ml) and anti-CD28 (2μg/ml) Abs. T cells were transduced using the above mentioned method during polyclonal T cell stimulation. Transduced CD4 and CD8 T cells were mixed at a 1:1 ratio and injected intravenously ($3 - 5 \times 10^6$) into tumor-free mixed chimeric mice. Recipients were followed for body weight changes (A) and clinical GVHD scoring (B). (C) GVHD target tissues (liver, lung, colon, and skin) were fixed in 10% formalin and stained with H&E at 28 days after DLI. (D) Long-term survival of CTC28-transduced DLI group mice.

Discussion

The prospect of effective antitumor T cell therapy against hematologic cancers was demonstrated by the observation of a robust immune response due to a small population of mature T cells in bone marrow transplantation. This effect was later confirmed by the T cell response against allogeneic antigens.[5, 8-10] Antitumor T cell therapy using allogeneic T cells has considerable merits over Ag-specific T cells. Generally, alloreactive T cells comprise 10 - 20% of the T cell pool, and this abundance can reduce the laborious efforts required to isolate and expand the relatively small population of Ag-specific T cells. In addition, healthy volunteers are easily recruited without HLA matching for allogeneic transplantation. However, many hurdles also exist in allogeneic T cell therapy. In immune competent hosts, grafted T cells are rejected due to host alloreactive responses. Preconditioning prior to administration of T cells might permit the coexistence of allogeneic grafts in immunocompromised patients for a limited time period. However, uncontrolled responses against ubiquitously expressed allogeneic antigens inevitably results in aggressive disruption of normal tissues, and this will thoroughly offset the positive antitumor effect.[43]

DLI, which is currently widely used to treat hematologic malignancies, is a type of allogeneic T cell therapy. The mixed chimerism established as a result of reduced intensity conditioning imparts tolerance against donor T cells and enables DLI without alloreactive rejection. After the excessive inflammatory cytokine levels that are induced by BMT subside, DLI could provide an antitumor effect without GVHD.[22-25] Although delayed DLI is a

promising antitumor T cell therapeutic method in the hematology field, the current therapeutic efficacy in some leukemia, such as ALL is not satisfactory.[31, 32] Therefore, we attempted to potentiate the therapeutic efficacy of DLI by introducing a CTLA4-CD28 chimeric gene, and evaluated CTLA4-CD28 DLI in a tumor co-injection model. To reflect clinical ALL, we inoculated EL4 T cell lymphoma cells into mixed chimera, followed by DLI on the same day. This schedule is different from that of the current, widely used delayed DLI preclinical model, in which DLI was administered 7 days before tumor injection.[38]

Both naïve and pre-activated DLI did not work in the tumor co-injection model (Figure 1, 2). Theoretically, we expected that pre-activated T cells would rapidly respond to host allogeneic APCs and exert their effector functions to eradicate residual tumors.[44] However, no GVT effect was observed with pre-activated DLI in the tumor co-injection model, and additional potentiation of the DLI T cells was required. In our previous study, transduction of a CTLA4-CD28 chimeric gene into T cells enhanced cytokine production and the antitumor effect in an Ag-specific solid tumor model.[34] In this study, we confirmed that the CTLA4-CD28 chimera worked well in a delayed DLI model; therefore, this novel chimeric receptor could be applied in allogeneic T cell therapy (Figure 5). However, the contribution of CTLA4-CD28 modification in each T cell subset was somewhat different from that observed in the previous study. In the Ag-specific solid tumor model, CTLA4-CD28 modification of CD4 T cells was critical for antitumor therapeutic efficacy. Although dual modification of both CD4 and CD8 T cells resulted in the best therapeutic efficacy in the Ag-

specific solid tumor models, transduction of the CTLA4-CD28 chimera into CD4 T cells was also sufficient for the antitumor effect.[34] However, in the tumor co-injection model, modification of either CD4 or CD8 T cells with the CTLA4-CD28 chimera did not provide an antitumor effect for delayed DLI (Figure 6). This might be due to the EL4 cells that were used in the ALL-like preclinical model, which aggressively propagated in the syngeneic host and exhibited higher lethality than the E.G7 or B16 melanoma cells that were subcutaneously injected in the Ag-specific solid tumor model. Blood analysis after DLI injection revealed a critical difference between CD4 and CD8 T cell expansion in the tumor co-injection model. While CD4 T cell expansion in allogeneic hosts was dependent on CTLA4-CD28 modification of CD4 T cells, CD8 T cell expansion required modification of both CD4 T and CD8 T cells (Figure 7). Expansion of CD8 T cells accurately correlated with the GVT effect in the tumor co-injection model, and this could be a critical factor for the antitumor effect. This was also confirmed by a CD8 T cell depletion study (Figure 8). The antitumor effect of CTC28-modified DLI in the tumor co-injection model was abrogated by CD8 depletion. Because the contribution of CTC28-modified CD4 T cells to the antitumor effect was insignificant in the absence of CD8 T cells, we concluded the CD8 T cells were largely responsible for the tumor killing in this tumor co-injection model, and additional CTC28-modification of CD8 T cells was critical for the antitumor effect.

In addition to the robust expansion of the CTC28-modified DLI T cells, the enhanced functional activity of the CTC28-modified T cells also contributed to the antitumor effect in the tumor co-

injection model. Generally, numerous tumor-associated factors play a significant role in immune system subversion and contribute to tumor growth and metastasis.[45-47] The tumor itself or tumor accessory cells can deplete tryptophan from the environment by expressing indoleamine 2,3-dioxygenase (IDO), which induces effector T cell anergy.[48, 49] Alternatively, the tumor can recruit regulatory T cells (Treg) by secreting suppressive cytokines, such as TGF-β and IL-10, and induce a tolerogenic tumor microenvironment by suppressing effector T cells.[50, 51] The CTC28-modification effect might be partly due to disruption of the tolerance of effector T cells in the tumor environment. *Ex vivo* analysis of the functional activity of DLI T cells at 10 days after inoculation in tumor-bearing mice, revealed significantly higher expansion of the IFN-γ-producing CTC28-modified CD4 T cells than the IFN-γ-producing EV-modified cells (Figure 9). Although the percentage of IFN-γ-producing CD8 T cells was not increased by CTC28 modification, the absolute number of IFN-γ-producing CD8 T cells was significantly increased by CTC28 modification as a result of robust expansion (Figure 9). Therefore, the enhanced GVT effect provided by CTC28 modification was the a result of the induction or maintenance of IFN-γ-producing effector functions in addition to the increased *in vivo* expansion of DLI T cells.

The impact of DLI T cell-derived IL-2 on the GVT effect was also evaluated in the tumor co-injection model. IL-2 has been reported to increase antitumor effects and has been used in many clinical settings.[52, 53] IL-2 has been reported to induce IFN-γ production by stimulating T cell proliferation or via a STAT5-dependent mechanism.[54-56] IL-2 also drives the development of naïve CD8 T cells into effector/memory cytolytic T lymphocytes

through induction of IFN-γ, perforin and granzymes.[57-59] In our previous study with an Ag-specific solid tumor model, systemic blocking of IL-2 completely abrogated the antitumor effect that was imparted by modification of T cells with a CTLA4-CD28 chimera.[34] However in that study, the contribution of each T cell subset was not confirmed. Here, we used WT and IL-2 KO mice as DLI T cell source, and we could evaluate the impact of DLI T cell-produced IL-2 on the GVT effect. As predicted, DLI with CD4 and CD8 T cells prepared from IL-2 KO mice did not exhibit a GVT effect in the tumor co-injection model. However, when the CD4 or CD8 T cells or both were prepared from WT mice, the survival of tumor-bearing mice was extended (Figure 10). Interestingly, *in vivo* expansion of the DLI T cells was similar for all combinations of CD4 and CD8 T cells derived from WT and IL-2 KO mice (data not shown). Therefore, DLI T cell-produced IL-2 was necessary for the GVT effect in this tumor co-injection model, and the antitumor effect was not merely the result of T cell expansion. Studies aimed at determining the mechanism underlying the contribution of DLI T cell-derived IL-2 to the antitumor effect of CTC28-modified DLI are currently in progress.

The allogeneic T cell response induces a GVT effect; however, it also increases the probability of promoting GVHD. Selective induction of GVT without GVHD is the objective of hematologic malignancy therapy, and to a certain extent, it is possible through delayed DLI.[27, 60-62] In this experiment, although the CTC28-modified DLI enhanced the survival of tumor-bearing mice, the surviving mice showed signs of GVHD. Therefore, we checked whether CTC28-modified DLI induced GVHD in tumor-free mixed

chimeric mice. Indeed, mice administered with the CTC28-modified DLI developed GVHD. However, it was not lethal, acute GVHD. The development of GVHD was slightly delayed compared to the tumor-induced lethality of untreated mice, and it was nearly non-lethal (Figure 11). Therefore, we hypothesized that GVHD can be chronologically separated from tumor-induced lethality. Since DLI-induced acute GVHD has been successfully blocked by insertion of a suicide gene in clinical trials [63-67] and GVHD development in our model may be separable from tumor eradication, we designed another retrovirus that co-expresses CTLA4-CD28 chimera and herpes simplex virus-derived thymidine kinase (HSV-TK) gene in the same construct and assessed in tumor co-injection model.[63-65] However, unwanted elimination of HSV-TK-transduced DLI T cells as a consequence of immune responses toward the HSV-TK gene product was observed in mixed chimeric mice (data not shown). Now, we move to another safety switch, inducible caspase 9 (iCasp9$_M$), and will test if the iCasp9$_M$ is compatible with our tumor co-injection model.[66, 67] In future studies, we will determine if the GVT effect of the CTLA4-CD28 chimera is conserved in a CTLA4-CD28-iCasp9$_M$ chimera, and evaluate the control of GVHD.

Overall, the CTLA4-CD28 chimera potentiates the GVT effect of DLI in tumor-bearing mice. The GVT effect was correlated with the expansion of CD8 T cells and was accomplished by dual modification of CD4 and CD8 T cells with the CTLA4-CD28 chimera. The GVT effect was increased by the enhanced effector function of CTC28-modified CD4 T cells. CTC28-modified T cell-derived IL-2 was required for the GVT effect, but was redundant for the expansion of DLI T cells. Alloreactive responses that

provided a GVT effect also induced moderate GVHD. However, it was not-lethal, acute GVHD and might be possible to separate from tumor-induced lethality.

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국문초록

CTLA4-CD28 키메라수용체를 이용한 동종 항종양 T세포치료법의 개발

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혈액종양 환자의 치료에서 감소된 세기 조건화 (reduced intensity conditioning) 방법과 동종 조혈모세포 이식 방법의 사용으로 심신이 허약하고 고령인 환자에게까지 치료범위가 넓어진 반면, 동시에 완치 후 재발률의 상승도 가져오게 되어 재발 환자에 대한 새로운 치료방법의 필요성이 대두되고 있다. 지연 공여자림프구주입술 (delayed donor lymphocyte infusion)은 재발된 혈액종양에 대한 유망한 T 세포치료법으로 소개되었으며, 현재까지 급성 림프구성 백혈병을 제외한 여러 종류의 백혈병에 대해서는 만족할만한 치료효과를 보였다. 본 연구에서는 T 세포를 강화시킴으로써 현재의 공여자림프구주입술의 치료효과를 개선시킬 수 있을 것으로 가정하여, 공여자림프구주입술에 사용되는 T 세포에 CTLA4-CD28 키메라 유전자를 형질도입하고, 이 T 세포를 이용하여 급성 림프구성 백혈병을 구현한 동물모델에서 항종양 효과를 평가해 보았다. 강력한 항종양 효과는 CD4 T 세포와 CD8 T 세포 모두에 CTLA4-CD28 키메라 유전자를 형질도입 한 경우에만 관찰되었으며, 이 때에만 체내에 이입된 CD8 T 세포가 증식됨을 확인할 수 있었다. CTLA4-CD28 키메라 유전자 형질도입 CD4 T 세포의 인터페론감마 생성 증가는 부가적인 항종양 효과를 제공할 것으로 사료된다. CTLA4-CD28 키메라 유전자 형질도입 T 세포 유래의 인터루킨-2는 체내에 이입된 세포의 증식에는 영향을

미치지 않았지만, 항종양 효과에는 필수적임이 확인되었다. 항종양 효과를 제공하는 동종 면역반응은 중등도의 이식편대숙주병 (graft-versus-host disease)을 동반하지만 치명적이지는 않았다.

주요어: 공여자림프구주입술, 항종양 효과, 급성 림프구성 백혈병, 이식편대숙주병

학 번: 2005-30687