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Optimization of lentiviral vector transduction into peripheral blood mononuclear cells in combination with the fibronectin fragment CH-296 stimulation

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Large scale T-cell expansion and efficient gene transduction are required for adoptive T-cell gene therapy. Based on our previous observations, human peripheral blood mononuclear cells (PBMCs) can be expanded efficiently while conserving a naïve phenotype by stimulating with both recombinant human fibronectin fragment (CH-296) and anti-CD3 monoclonal antibodies. In this article, we explored the possibility of using this co-stimulation method to generate engineered T cells using lentiviral vector. Human PBMCs were stimulated with anti-CD3 together with immobilized CH-296 or anti-CD28 antibody as well as anti-CD3/anti-CD28 conjugated beads and transduced with lentiviral vector simultaneously. Co-stimulation with CH-296 gave superior transduction efficiency than with anti-CD28. Next, PBMCs were stimulated and transduced with anti-CD3/CH-296 or with anti-CD3/CD28 beads. T-cell expansion, gene transfer efficiencies and immunophenotypes were analysed. Stimulation with anti-CD3/CH-296 resulted in more than 10-times higher cell expansion and higher gene transfer efficiency with conservation of the naïve phenotype compared with anti-CD3/CD28 stimulation method. Thus, lentiviral transduction with anti-CD3/CH-296 co-stimulation is an efficient way to generate large numbers of genetically modified T cells and may be suitable for many gene therapy protocols that use adoptive T-cell transfer therapy.

Keywords: adoptive T-cell transfer therapy/gene transfer/lentiviral vector/recombinant human fibronectin fragment (CH-296)/T-cell stimulation.

Abbreviations: ATC, adoptive T-cell; FN, Fibronectin; PBMCs, peripheral blood mononuclear cells; TCR, T-cell receptor; VSV-G, Vesicular stomatitis virus glycoprotein.

Fibronectin (FN), one of the major extracellular matrix proteins, is a disulphide-linked dimeric glycoprotein that has several functional domains with cell-binding properties (*1–3*). FN functions not only as an adhesion molecule but also as a signal inducer by binding to integrins expressed on T cells. FN acts with anti-CD3 to induce T-cell proliferation, which is thought to depend on an interaction between integrin very late activation antigen-4 (VLA-4) and the CS1 domain of FN (*4–6*). We previously showed that CH-296, a recombinant fragment of human FN (*7*), and anti-CD3 stimulates peripheral blood T-cell growth *in vitro* and anti-CD3/CH-296 stimulated T cells were more likely to maintain $CCR7^+CD45RA^+$ naïve phenotype and show longer persistence in an *in vivo* mouse model (*8*) than T cells stimulated by other methods. Cells with a naïve phenotype are more active in mediating tumour regression than cells with a more differentiated phenotype (*9*). The *ex vivo* expanded $CCR7^+CD45RA^+$ naïve phenotype cells showed the same pattern of cytokine production compared with un-stimulated naïve T cells and tumour antigen-specific CTLs could be induced from $CCR7^+CD45RA^+CD8^+$ cells but not from $CCR7^-CD45RA^-CD8^+$ cells (*10*). Thus, we hypothesize that $CCR7^+CD45RA^+$ naïve phenotype T cells obtained after expansion can differentiate into antigen-specific CTLs and naïve cells may be good agents for adoptive T-cell (ATC) transfer therapy. ATC transfer therapy, the infusion of tumour-reactive T cells into patients, has been demonstrated to be an effective treatment for metastatic cancers (*11*). One of the limitations of this technology is the difficulty in generating T cells that can recognize tumour targets. To render lymphocytes with antitumour reactivity, T cells can be genetically engineered with antitumour antigen receptors, such as naturally occurring T-cell receptor (TCR) genes (*12*, *13*) or recombinant chimeric TCR genes, which are hybrid proteins containing a single-chain antibody fragment (scFv) against tumour antigen linked to TCR signaling domains, achieving MHC-unrestricted recognition of target cells by T cells (*14*). Expression of these TCR in immunological effector cells should produce tumour-specific T cells that are capable of mounting an antitumour effect *in vivo*. Several mouse studies and clinical trials indicate that the differentiation state of the adoptively transferred T cells is critical to the success of T cell based approaches (*15*). In mice, naïve and early effector T cells mediate stronger *in vivo* antitumour responses than intermediate and late effector T cells (*16*). Moreover, less-differentiated T cells are ideal for ATC transfer therapy in humans (*17–19*). Based on these observations, obtaining naïve or less differentiated $CD8^+$ T cells that are specific for a tumour antigen may be an important approach for

improving current T-cell-based therapies. Anti-CD3/CH-296 stimulation is a promising method for ATC transfer therapy because it may reconstitute a long-lasting immune system in patients.

The heparin-binding domain of CH-296 has a high affinity to retroviral vectors and enhances gene transduction into a variety of cells, including haematopoietic stem cells (*20*). Lentiviral vectors have an advantage over gamma-retroviral vectors because they can transfer genes into non-dividing cells. This ability provides an attractive use of the lentiviral vector to generate genetically modified T cells for ATC transfer therapy. In the current study, we have investigated the use of lentiviral vector with simultaneous anti-CD3/CH-296 stimulation. Compared to other available T-cell stimulation procedures, our method of lentiviral transduction generated higher numbers of genetically modified T cells retaining naïve T-cell markers, including a homing receptor. In this report, we describe a novel *ex vivo* T-cell expansion and transduction method utilizing CH-296 stimulation, which is an efficient way to prepare large numbers of genetically modified T cells for ATC transfer therapy.

Materials and Methods

Lentiviral vectors and viral production

The lentiviral vector backbone used in this study, pLenti-TOPO, was purchased from Invitrogen (Carlsbad, CA, USA). The green fluorescent ZsGreen (Clontech, Mountain View, CA, USA) was inserted into the multiple cloning site of pLenti-TOPO. The resultant plasmid vector was designated pLenti-ZG (Fig. 1A). Recombinant lentiviral vector was generated by cotransfecting the lentiviral vector plasmid pLenti-ZG with the packaging plasmid mix (Invitrogen) into 293T cells following the manufacturer's instructions. Two days after the transfection, resultant VSV-G pseudotyped lentiviral supernatant was harvested and filtered with 0.45 μm filtre paper. For the titration of VSV-G pseudotyped Lenti-ZG vector, HT-1080 cells (ATCC CCL-121) were plated at 5×10^4 cells/well in 6-well plates 1 day before transduction and incubated for 3 days with serial dilutions of virus supernatants containing 8 μg of polybrene per ml. Transduced HT-1080 cells were run through a FACSCanto II flow cytometer (BD Biosciences) and ZsGreen positive HT1080 cells were detected. The infectious units (IFU) were determined by calculating numbers of infectious viral particles in the vector supernatant. The titre used in this experiment was 1×10^7 IFU/ml.

Stimulation and transduction of peripheral blood lymphocytes

Gene transfer study into primary healthy donors' blood samples for the purpose of research development of gene therapy was approved by the ethics committee of Takara Bio Inc. Peripheral blood samples were obtained from healthy volunteers who gave their written informed consent. Peripheral blood samples were collected by leukapheresis and washed using Cytomate (Baxter, Deerfield, IL, USA). Mononuclear cells were isolated by Ficoll-Paque PLUS (Amersham Biosciences) density gradient centrifugation and frozen until use.

To compare the lentiviral gene transfer efficiency with several stimulation methods, CH-296 or anti-CD28 antibody was used together with anti-CD3 antibody. For anti-CD3/CH-296 stimulation, a 24-well plate that was pre-coated with 25 μg/ml (5 μg/well) of CH-296 (RetroNectin, Takara Bio, Otsu, Japan) and 5 μg/ml (1 μg/well) of anti-CD3 (Orthoclone OKT3; Janssen pharmaceutical, Titusville, NJ, USA) was used. For anti-CD3/CD28 stimulation method, 5 μg/ml (1 μg/well) of anti-CD28 antibody (L293; BD Biosciences, Franklin Lakes, NJ, USA) together with 5 μg/ml (1 μg/well) of anti-CD3 (designated as anti-CD3/CD28 coat), or with anti-CD3/anti-CD28 paramagnetic beads (designated as anti-CD3/CD28 beads; Dynabeads® Human T-Activator CD3/CD28, Invitrogen) was used. The recovered peripheral blood mononuclear cells (PBMCs) were re-suspended at a concentration of 0.7×10^6 cells/ml in GT-T503 medium (Takara Bio), supplemented with 1% autologous plasma, 0.2% human serum albumin (HSA) (Baxter) and 600 IU/ml interleukin (IL)-2 (Chiron, Emeryville, CA, USA). The PBMCs were transduced with VSV-G pseudotyped Lenti-ZG vector at a multiplicity of infection of 10 at 1,000*g* for 30 min or 2 h at 32°C soon after the stimulation. All cells were

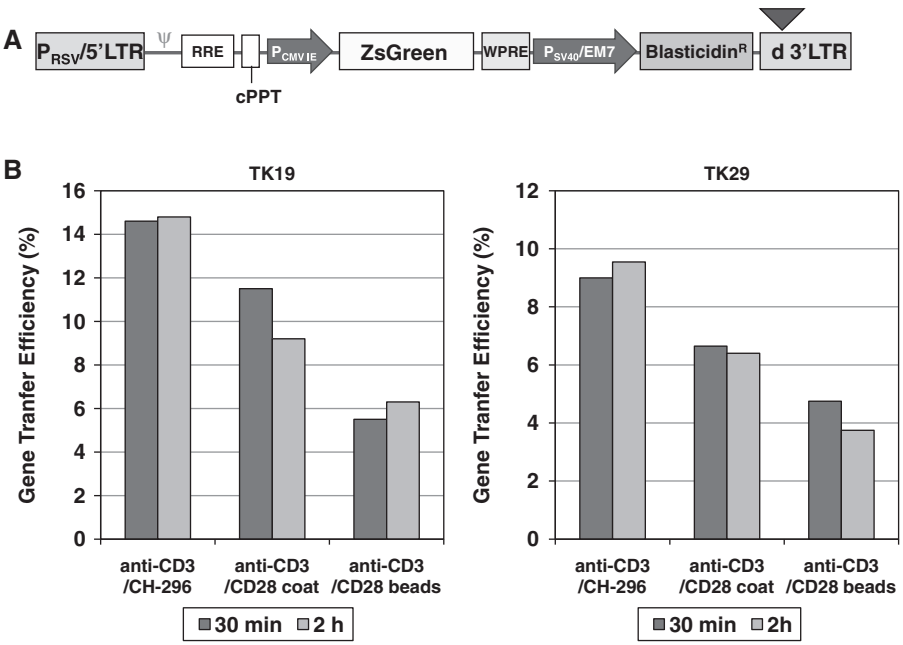

Fig. 1 Comparison of lentiviral gene transfer into PBMCs with different costimulation factors. (A) Schematic diagram of the lentiviral vector used in this study, (B) Lentiviral gene transfer efficiencies into PBMCs under minimally stimulated status using anti-CD3 together with anti-CD28 or CH-296. The recovered PBMCs were stimulated with anti-CD3/CH-296, anti-CD3/CD28 coat plate, or anti-CD3/CD28 beads (cell-to bead ratio of 1 : 1) and transduced with lentiviral vector simultaneously. To enhance the chance of lentiviral vector attachments to PBMCs, the plate was centrifuged for either 30 min or 2 h at 1,000*g*. The gene transfer efficiency was analysed by flow cytometry to detect the fluorescent protein ZsGreen expression on Day 7.

maintained until Day 7 when gene transfer efficiency of each condition was analysed by flow cytometry as described below.

To determine the optimal time for transduction in the presence of CH-296, anti-CD3/CH-296 was used for transduction on Day 0. For comparison, anti-CD3/CH-296 stimulated lymphocytes were transduced on Day 3 using CH-296 coated plate. Two kinds of transduction procedure, spinoculation and preloading, was also compared on both date as follows: (i) Day 0 transduction: the recovered PBMCs were resuspended at a concentration of 0.7×10^6 cells/ml in GT-T503 medium, supplemented with 1% autologous plasma, 0.2% HSA and 600 IU/ml IL-2 as previously described. About 0.4 ml of the cell suspension was mixed with 1 ml of lentiviral vector supplemented with 600 IU/ml IL-2 and added to an anti-CD3/CH-296 coated plate. The resultant multiplicity of infection was 35. The plate was subsequently centrifuged at 1,000*g* for 30 min or 2 h at 32°C (spinoculation). Centrifugation was followed by incubation at 37°C. For comparison, 1 ml of lentiviral vector was preloaded onto a well of anti-CD3/CH-296 plate and centrifuged at 2,000*g* for 2 h, then the supernatant was discarded and 2.8×10^5 of PBMCs were added onto the well (preloading). (ii) Day 3 transduction: PBMCs were activated with anti-CD3/CH-296 stimulation for 3 days and cells were adjusted at 0.7×10^6 cells/ml. 0.4 ml of the activated cell suspension was mixed with 1 ml of lentiviral vector supplemented with 600 IU/ml IL-2 and added to a well of CH-296 coated plate. The plate was subsequently centrifuged at 1,000*g* for 30 min or 2 h at 32°C (spinoculation). For comparison, 1 ml of lentiviral vector was preloaded onto a well of CH-296 coated plate and 2.8×10^5 of lymphocytes were transduced as previously described (preloading method). All cells were maintained until Day 10 when gene transfer efficiency of each condition was analysed by flow cytometry as described below.

To explore anti-CD3/CH-296 stimulation and transduction method for clinical scale, the expansion and immunophenotype pattern of the genetically modified cells was compared and evaluated with the previously reported method (*21*). The recovered PBMCs were resuspended at a concentration of 0.7×10^6 cells/ml, 0.4 ml of cell suspension was mixed with 1 ml of lentiviral vector supplemented with 600 IU/ml IL-2 and added to an anti-CD3/CH-296 coated plate. The plate was subsequently centrifuged at 1,000*g* for either 30 min or 2 h at 32°C, followed by incubation at 37°C for 4 days. At Day 4, cells were subcultured by diluting 16.7 times with GT-T503 medium, supplemented with 1% autologous plasma, 0.2% HSA and 600 IU/ml IL-2. At Day 7, cells were diluted twice with plasma free medium. At Day 10, cells were diluted twice with plasma free medium and maintained until Day 14. As a control for the stimulation method for clinical scale cell manufacturing, PBMCs were stimulated with anti-CD3/CD28 beads, and transduced with lentiviral vector in the presence of protamine and then cultured until Day 14 as previously described (*21*).

Flow cytometry analysis

Flow cytometry was used to analyse the surface phenotype and transduction efficiency. Monoclonal antibodies that detect surface proteins were used: PerCP-conjugated anti-CD3 antibody (BD Biosciences, Franklin Lakes, NJ, USA), APCcy7-conjugated anti-CD8 (BD Biosciences), PE-conjugated anti-CCR7 (R&D Systems, Franklin Lakes, NJ, USA), RD1-conjugated anti-CD45RA (Beckman Coulter, Fullerton, CA, USA), PEcy7-conjugated anti-CD62L (eBioscience). Samples were run through a FACSCanto II flow cytometer. The controls used were isotype-matched fluorochrome-conjugated irrelevant monoclonal antibodies. Data were analysed using FACSDiva software (BD Biosciences).

Results

Comparison of lentiviral gene transfer efficiency with different costimulation factors

To evaluate effect of several costimulation factors on lentiviral transduction efficiencies, CH-296 or anti-CD28 was used together with anti-CD3. Frozen PBMCs, obtained from two healthy donors, were recovered, stimulated with anti-CD3/CH-296, anti-CD3/CD28 coat plate or anti-CD3/CD28 beads (cell-to bead ratio of 1 : 1) and transduced with lentiviral vector simultaneously. To enhance the chance of lentiviral vector attachments to PBMCs, the plate was centrifuged for either 30 min or 2 h at 1,000*g*. The gene transfer efficiency was determined by analysing ZsGreen expression on Day 7. Among these transduction conditions, stimulation with anti-CD3/CH-296 exhibited the highest gene transfer efficiencies (Fig. 1B).

Comparison of gene transfer efficiency with CH-296 at different times

Because the lentiviral vector has been shown to have the potential to transduce genes into non-dividing cells, we compared the gene transfer efficiencies of minimally activated PBMCs (Day 0) with activated PBMCs (Day 3). To determine whether anti-CD3/CH-296 costimulation followed by lentiviral gene transfer is useful for preparing T cells for ATC transfer therapy, we stimulated PBMCs with anti-CD3/CH-296 and transduced simultaneously on Day 0. For comparison, anti-CD3/CH-296 activated PBMCs were transduced with the lentiviral vector on Day 3 with a CH-296 coated plate using either the preloading method or spinoculation. Frozen PBMCs, obtained from healthy donor, were recovered, stimulated and transduced with lentiviral vector and then expanded until Day 10 (Fig. 2A). Lentiviral transduction was performed once at Day 0 or Day 3. The gene transfer efficiency was determined by analyzing ZsGreen expression on Day 10. Among these transduction conditions, PBMCs transduced on Day 0 with anti-CD3/CH-296 exhibited the highest gene transfer efficiencies (Fig. 2B). This result suggests that transduction on Day 0, when the cells are minimally activated, provides the most efficient gene transfer into PBMCs.

Comparison of the effect of different stimulation methods on cell growth and gene transfer efficiency

To investigate whether the stimulation of T cells with anti-CD3/CH-296 followed by lentiviral gene transfer is useful for preparing T cells for ATC transfer therapy, we compared the anti-CD3/CH-296 stimulation method with the anti-CD3/CD28 beads stimulation method. Frozen PBMCs obtained from healthy donors were recovered and stimulated by each method until Day 4 (Fig. 3). Lentiviral transduction was also performed on Day 0 (Fig. 3). Transduced populations were expanded and cultured in maintenance media for 14 days after the initiation of the culture (Fig. 3). The experiments were performed using PBMCs from two donors (TK18 and TK29). Comparing the two culture conditions, PBMCs stimulated with anti-CD3/CH-296 exhibited the highest cell growth (Fig. 4A). The anti-CD3/CH-296 stimulated cell population that was transduced for 30 min expanded 258-fold (TK18) and 198-fold (TK29) in the 14-day culture period, compared to 18-fold (TK18) and 16-fold (TK29) when cells were stimulated with anti-CD3/CD28 beads. The growth of anti-CD3/CH-296-stimulated cells decreased when the cells were transduced for 2 h. Gene transfer efficiency was measured on Day 10 by analysing ZsGreen expression. As shown in Fig. 4B, gene transfer mediated by lentiviral transduction in combination with

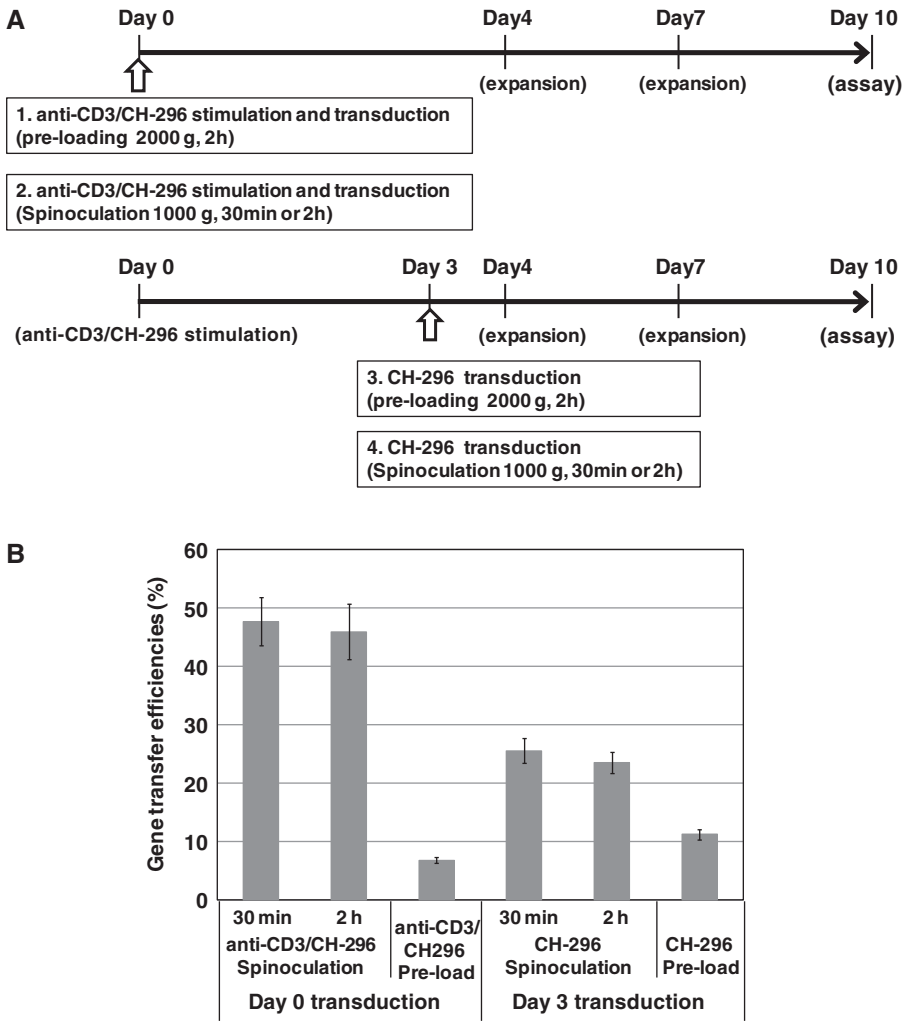

Fig. 2 Comparison of gene transfer timings and transduction methods. (A) Outline of experimental procedure to determine the optimal transduction timing of the lentiviral vector; to determine the optimal time for transduction in the presence of CH-296, anti-CD3/CH-296 was used for transduction on Day 0. For comparison, anti-CD3/CH-296 stimulated lymphocytes were transduced on Day 3 using CH-296 coated plate. Two kinds of transduction procedure, spinoculation and preloading, was also compared on both date as described in 'Materials and Methods' section. All cells were maintained until Day 10 when gene transfer efficiency of each condition was analysed by flow cytometry. (B) Results of gene transfer efficiencies when different transduction conditions were used.

anti-CD3/CH-296 stimulation was more efficient than with anti-CD3/CD28 beads stimulation in the presence of protamine. These results suggest that the anti-CD3/CH-296 stimulation method is beneficial to gene therapy of T cells because it enhances cell growth and promotes efficient gene transfer.

Immunophenotype of expanded populations

We analysed immunophenotype markers of stimulated, transduced and expanded T cells. We obtained similar results in PBMCs from both TK29 and TK18. Thus, representative results from PBMCs from TK29 are shown (Fig. 5). Prior to stimulation, ~51% of the recovered PBMCs were $CD4^+$ and 38% were $CD8^+$. However, on Day 14, the $CD8^+$ population increased to 88.6% with the anti-CD3/CH-296 stimulation method and 74.6% with anti-CD3/CD28 beads stimulation method (Fig. 5A).

To assess the status of T-cell differentiation in transduced T cells, PBMCs that had been expanded for 14 days were analysed by flow cytometry for CD45RA and CCR7 expression. Before stimulation, 22.0% of PBMCs had a $CD45RA^+/CCR7^+$ naïve phenotype (Fig. 5B). After 14 days of expansion, 54.2% of cells stimulated with anti-CD3/CD28 beads and 75.6% of cells stimulated with anti-CD3/CH-296 had a naïve phenotype (Fig. 5B). We obtained 20-times (TK18) and 17-times (TK29) more cells with a naïve phenotype when cells were expanded after anti-CD3/CH-296 simulation, compared with anti-CD3/CD28 beads stimulation. When cells were gated and analysed for ZsGreen, immunophenotype results were similar to those obtained for un-gated cells (Fig. 5B), suggesting that lentiviral vector transduction on Day 0 results in uniform transduction into PBMCs with no deflections. Estimated numbers of GMCs were calculated from the result of fold expansion, gene transfer efficiencies and immunophenotype analysis (Fig. 5D). The anti-CD3/CH-296 stimulation method can yield more than 10^9 GMCs from 10^7 PBMCs. The majority of these GMCs exhibited a naïve phenotype. As we have previously demonstrated, T lymphocytes with a naïve phenotype

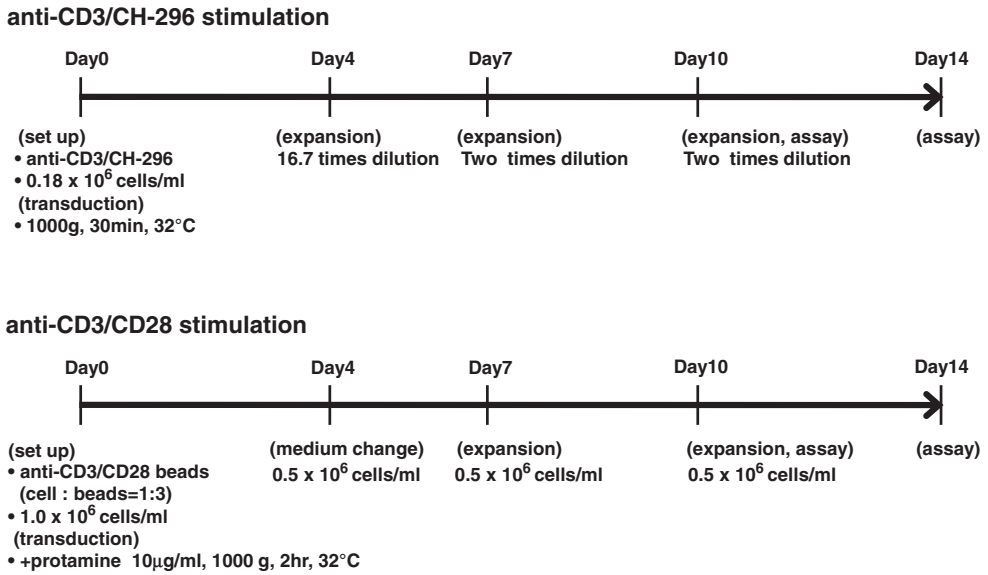

Fig. 3 Outline of experimental procedure to determine the optimal lentiviral transduction and cell expansion method. To evaluate the expansion and immunophenotype pattern of the genetically modified cells, the recovered PBMCs were directly mixed with lentiviral vector and added to an anti-CD3/CH-296 coated plate. The plate was subsequently centrifuged at 1,000*g* for either 30 min or 2 h at 32°C, followed by incubation at 37°C for 4 days. At Day 4, cells were subcultured by diluting 16.7 times with the growth medium. At Day 7, cells were diluted twice with plasma free medium. At Day 10, cells were diluted twice with plasma free medium and maintained until Day 14. For comparison, PBMCs were stimulated with anti-CD3/CD28 T-cell expander superparamagnetic beads and transduced with lentiviral vector in the presence of protamine and then cultured until Day 14 as previously described.

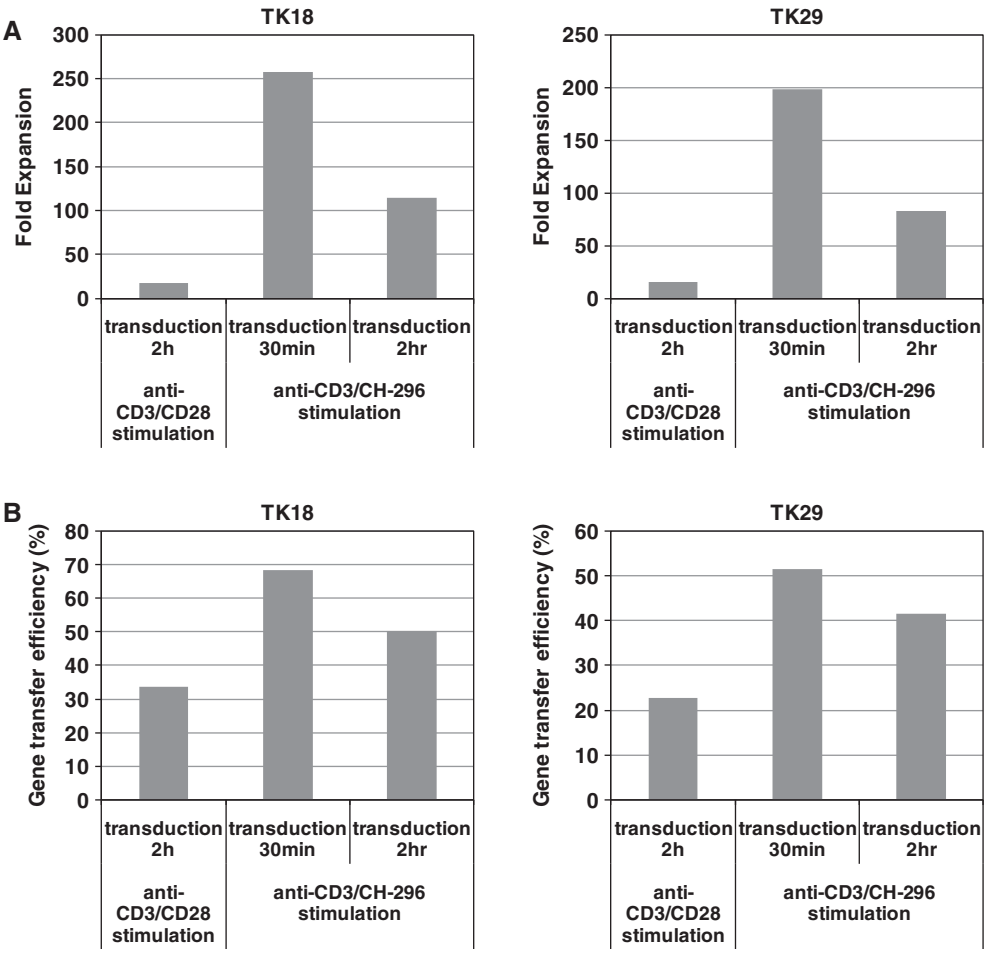

Fig. 4 Lymphocytes fold expansion and gene transfer efficiencies for different stimulation conditions. PBMCs from two donors (TK18 and TK29) were stimulated with either anti-CD3/CH-296 or anti-CD3/CD28 beads and expanded until Day 14. Fold expansion (A) and gene transfer efficiencies (B) were compared. Anti-CD3/CH-296 stimulation method was superior in both cell expansion and gene transduction.

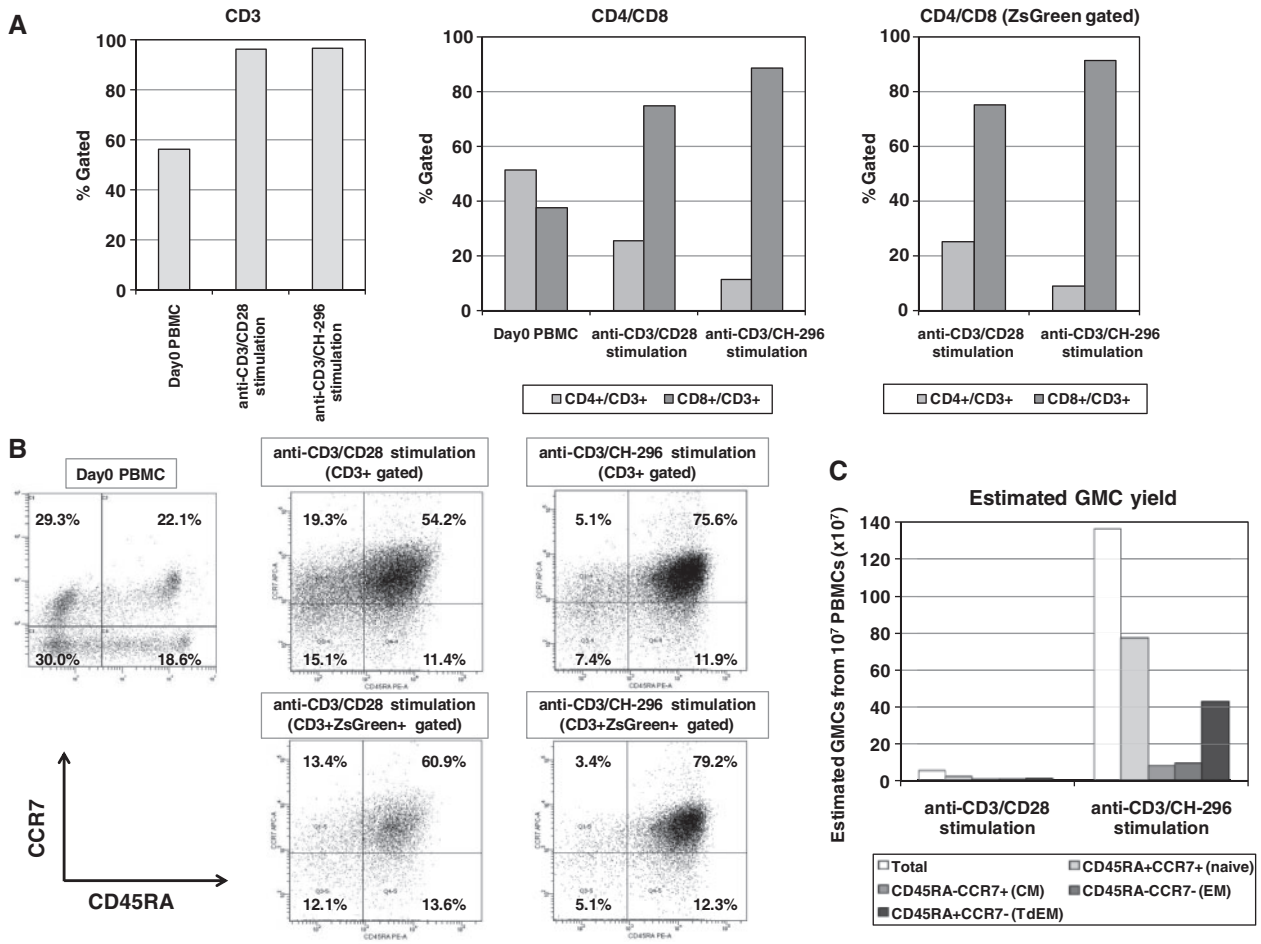

Fig. 5 Analysis of PBMCs and expanded lymphocytes for different stimulation conditions. On Day 14, the immunophenotypes of expanded populations and un-stimulated peripheral blood lymphocytes were compared. (A) CD3 positive percent was measured in the recovered PBMC and expanded cells by gating the live cells based on the FSC/SSC parameters during the flow cytometry analysis (left panel). After gating for CD3, cells were analysed for $CD4^+$ or $CD8^+$ expression (middle panel). $CD4^+$ or $CD8^+$ expression in genetically modified cells was also analysed by gating $CD3^+ZsGreen^+$ cells (right panel). (B) $CD3^+$ cells and genetically modified $CD3^+ZsGreen^+$ cells were also analysed by flow cytometry for CD45RA and CCR7 expression. (C) Estimated numbers of genetically modified cells (GMCs) were calculated from the result of fold expansion, gene transfer efficiencies and immunophenotype analysis. CM, central memory; EM, effector memory; TdEM, terminally differentiated effector memory.

exhibit a longer *in vivo* persistence of transduced T cells because of their highly proliferative potential (*8*). These results suggest an intriguing potential for the use of the anti-CD3/CH-296 stimulation method in combination with lentiviral vector transduction for adoptive T cell gene therapy.

Discussion

We previously demonstrated that T-cell stimulation with CH-296 and anti-CD3 enhanced cell proliferation and resulted in a high level of retroviral transgene expression (*8*). This method of stimulation also preserved the naïve phenotype of the T-cell population, resulting in increased persistence of genetically modified T cells *in vivo* (*8*). In this study, we assessed the use of lentiviral vector instead of gamma-retroviral vectors. Lentiviral vectors have a number of potential advantages over γ-retroviral vectors, including a safer integration site profile and the ability to transduce genes into non-dividing cells or minimally stimulated PBMCs (*22*, *23*). There has been one report on the clinical use of lentiviral vector-transduced T cells in humans (*24*). This study reported high levels of *ex vivo* transduction with no adverse events attributed to the lentiviral vector gene transfer system. Based on this initial report of the safety of this vector platform and the potential biological advantages of lentiviral vectors to engineer human T cells, lentiviral vectors may be useful for human gene therapy, including adoptive immunotherapy for cancer.

We previously reported that the culture fluid of retroviral producer cells contained molecules that inhibit transduction and that using this culture fluid hampered gene transfer (*20*). We found that preloading the culture fluid onto CH-296 removes the inhibitory molecules, leaving purified retrovirus bound to CH-296, which dramatically enhanced gene transfer efficiencies (*20*). However, gene transfer was not hampered for VSV-G enveloped vector, even if we used the viral vector supernatant directly without purification. Based on these observations, we used non-purified and

non-concentrated VSV-G pseudotyped lentiviral vector for transduction. To increase the chance of lentiviral vector contacting target cells, the PBMCs were mixed with lentiviral vector and centrifuged at 1,000*g* in the anti-CD3/CH-296 coated plate. We tested the lentiviral transduction of PBMCs on Day 0, when the cells were minimally activated and simultaneously stimulated by either anti-CD3/CH-296 or anti-CD3/anti-CD28. It was reported that the majority of HIV isolates are not able to infect and replicate in unstimulated PBMCs (*25*), most of which are in the G_0 stage of the cell cycle (*26*, *27*). Accordingly, freshly isolated PBMCs in the G_0 phase are poorly transduced with lentiviral vectors (*28*). In this regard, despite the ability of lentiviral vector to infect non-proliferating cells, PBMCs are needed to be stimulated for lentiviral transduction. The cell growth from Day 0 to Day 4 in anti-CD3/CH-296 stimulation was 3- to 4-folds higher than that of anti-CD3/CD28 (data not shown). We speculate that efficient stimulation leaded the result of efficient transduction. In addition, our results indicate that anti-CD3/CH-296 enhanced gene transfer efficiency and expansion of cells that retained a naïve phenotype. This finding suggests that CH-296 promotes lentiviral gene transfer by colocating lentiviral vectors and PBMCs on the CH-296 molecule. The transgene ratios gated in the immunophenotypes of naïve, central memory and effector memory were similar. This similarity suggests that lentiviral vector transduction on Day 0 results in uniform transduction into PBMCs with no deflections. Anti-CD3/CH-296 stimulated PBMCs contained a considerably larger numbers of naïve phenotype cells expressing the transgene than PBMCs stimulated with anti-CD3/CD28 beads. Because effector cells derived from naïve $CD8^+$ T cells have been shown to mediate antitumour immunity (*29*), expansion of genetically transduced $CD8^+$ T cells in combination with anti-CD3/CH-296 will be a powerful approach for adoptive T-cell gene therapy.

Leukapheresis is commonly used to collect PBMCs for adoptive T cell gene therapy because more than 10^9 cells are necessary to provide a therapeutic effect *in vivo* (*30*). In this report, PBMCs were expanded between 83- and 258-fold with the anti-CD3/CH-296 stimulation method. This expansion was significantly higher than the expansion observed with the anti-CD3/CD28 stimulation method. This level of expansion would provide more than 10^9 cells or nearly 10^{10} cells from a small amount of blood, such as 50 ml, and could simplify the cell processing method because leukapheresis would not be required. To simplify the cell processing method, PBMCs from a small amount of blood will be used with this protocol although monocytes and are still included, because non-stimulated monocytes are not able to proliferate in the presence of anti-CD3 and expected to be less transduced by lentiviral vector. After the stimulation, the majority of cells came to be CD3 positive T lymphocytes. We compared cell growth and immunophenotype of PBMCs with purified T cells and no significant difference was observed (data not shown). Thus, the simultaneous anti-CD3/CH-296 stimulation and lentiviral transduction method could provide *ex vivo* gene therapy for use in any kind of gene therapy protocols using adoptive T-cell immunotherapy. We expect that anti-CD3/CH-296 costimulation of T cells with lentiviral gene transduction will lead to the next generation of adoptive T-cell immunotherapy that promises more effective therapy, due to longer persistence of the cells in patients.

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Conflict of interest
None declared.

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