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# Novel adoptive T-cell immunotherapy using a WT1-specific TCR vector encoding silencers for endogenous TCRs shows marked antileukemia reactivity and safety

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**Adoptive T-cell therapy for malignancies using redirected T cells genetically engineered by tumor antigen-specific *T-cell receptor* (*TCR*) gene transfer is associated with mispairing between introduced and endogenous TCR chains with unknown specificity. Therefore, deterioration of antitumor reactivity and serious autoimmune reactivity are major concerns. To address this problem, we have recently established a novel retroviral vector system encoding siRNAs for endogenous TCR genes (*siTCR* vector). In this study, to test the clinical application of *siTCR* gene therapy for human leukemia, we examined in detail the efficacy and safety of WT1-*siTCR*–transduced T cells. Compared with conventional WT1-*TCR* (WT1-*coTCR*) gene-transduced T cells, these cells showed significant enhancement of antileukemia reactivity resulting from stronger expression of the introduced WT1-specific TCR with inhibition of endogenous TCRs. Notably, WT1-*siTCR* gene-transduced T cells were remarkably expandable after repetitive stimulation with WT1 peptide in vitro, without any deterioration of antigen specificity. WT1-*siTCR* gene–transduced T cells from leukemia patients successfully lysed autologous leukemia cells, but not normal hematopoietic progenitor cells. In a mouse xenograft model, adoptively transferred WT1-*siTCR* gene-transduced T cells exerted distinct antileukemia efficacy but did not inhibit human hematopoiesis. Our results suggest that gene-immunotherapy for leukemia using this WT1-*siTCR* system holds considerable promise. (*Blood*. 2011;118(6):1495-1503)**

## Introduction

Recent identification of various tumor-associated antigens has encouraged the clinical development of cell-mediated immunotherapy for leukemia targeting leukemia-associated antigens.[1,2] Among various kinds of immunotherapy, adoptive tumor-specific T-cell therapy using ex vivo expansion of autologous tumor-responsive T cells seems to be an attractive option. Indeed, patients with advanced metastatic melanoma have been treated successfully with melanoma-specific T cells obtained from tumor-infiltrating T cells.[3-5] Although adoptive transfer of tumor-specific tumor-infiltrating T cells is a promising strategy, its general application for therapy would appear to be unlikely because of the complex procedures and difficulties involved in the timely preparation of sufficient numbers of tumor-specific cytotoxic T lymphocytes (CTLs) with adequate therapeutic quality.[6,7] To address these problems, an innovative approach involving substituting redirected T cells using predefined tumor antigen-specific *T-cell receptor* (*TCR*) gene transfer has been developed. In recent clinical trials, melanoma antigen-specific *TCR* gene-transferred T cells have been used for treatment of patients with advanced melanoma.[8,9] However, the clinical efficacy of *TCR* gene-engineered T cells is still not satisfactory, and serious autoimmune responses have been observed in some melanoma patients. In addition, adoptive immunotherapy using tumor antigen-specific *TCR* gene-transferred T cells targeting malignancies other than melanoma still remains in its infancy. Therefore, the development of *TCR* gene-immunotherapy targeting universal tumor-associated antigens is essential to popularize this strategy for cancer treatment.

Wilms tumor gene product 1 (WT1) is one of the zinc-finger transcriptional regulators that is abundantly expressed in the vast majority of acute leukemias, but not in normal cells.[10,11] In addition, the expression level of WT1 in tumor cells is clinically correlated with disease aggressiveness and prognosis.[12,13] Furthermore, a study using a model involving immunodeficient mice engrafted with human acute myelogenous leukemia has recently obtained important evidence that WT1 is expressed abundantly in chemotherapy-resistant acute myelogenous leukemia stem cells.[14] These data have prompted us and other groups to develop adoptive T-cell immunotherapy targeting leukemia stem cells using WT1-specific *TCR* gene transfer.[15-17]

To facilitate the clinical application of adoptive immunotherapy using genetically engineered WT1-specific CTLs, some important issues need to be addressed. First, there is the problem of mispairing between endogenous and introduced TCR chains that would reduce the expression of introduced TCR on the surface of gene-modified T cells, resulting in lower functionality.[18] Mispairing of TCR could also carry a risk of evoking severe autoimmunity.[19,20] Therefore, it is essential to clarify both the on- and off-target adverse effects mediated by WT1-*TCR* gene-engineered T cells using in vivo as well as in vitro systems. The other issue of concern is bone marrow suppression mediated by WT1-specific

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T cells because it has been reported that hematopoietic progenitor cells express *WT1* mRNA.[21,22] In previous trials of WT1 peptide vaccine, suppression of normal hematopoiesis has not been reported in most cases, even though WT1-specific CTLs were generated following vaccination.[23,24] However, long-term adverse effects on hematopoietic progenitors mediated by adoptively transferred WT1-*TCR* gene-engineered T cells should be considered because, in this therapy, a larger number of WT1-specific T cells are infused at one time into patients.

To overcome the aforementioned problems, we have recently developed a novel retroviral vector system for *TCR* gene transfer that can selectively express target antigen-specific TCR, whereas expression of intrinsic TCRs is suppressed by built-in siRNAs (*siTCR* vector).[25] MAGE-A4–specific *TCR* gene-engineered T cells prepared by this vector system successfully showed both up-regulated expression of the introduced TCR and enhanced anti-MAGE-A4 reactivity. We also constructed a novel WT1-*siTCR* retroviral vector encoding human leukocyte antigen (HLA)–A*24:02-restricted and WT1-specific *TCR* genes cloned from a CTL clone, TAK-1.[26] This WT1-*siTCR* vector similarly appeared capable of increasing the expression of the introduced WT1-specific TCR; however, the usefulness of WT1-*siTCR* for clinical application remains to be clarified before clinical trials can begin. In the present study, with the aim of clinically applying this WT1-*siTCR* gene transfer system for treatment of leukemia, we assessed in detail the efficacy and safety of adoptive immunotherapy using WT1-specific *TCR* gene-modified CTLs using both in vivo as well as in vitro experimental systems. On the basis of the data we have obtained, we discuss the feasibility of this novel gene immunotherapy for leukemia using a WT1-*siTCR* retroviral vector.

## Methods

### Cloning of WT1-specific *TCR* gene and construction of WT1-*TCR* retroviral vectors

The HLA-A*24:02-restricted and $WT1_{235-243}$-specific *TCR*-$\alpha$ and *TCR*-$\beta$ genes were cloned from our originally established CTL clone, TAK-1, using the 5′ RACE method (Clontech).[27] The *TCR*-$\alpha$ and *TCR*-$\beta$ genes of TAK-1 appeared to be V$\alpha$20/J33/C$\alpha$ and V$\beta$5.1/J2.1/C$\beta$2, respectively. Retrovirus vectors expressing TAK-1-derived *TCR* (WT1-*TCR*) genes were constructed as reported previously.[25] Briefly, the WT1-*TCR*-$\alpha$ and *TCR*-$\beta$ genes were bicistronically integrated into a conventional MS-bPa retroviral vector (WT1-*coTCR* vector).[28] Partially codon-optimized *TCR*-$\alpha$ and *TCR*-$\beta$ genes were similarly integrated into a novel MS-bPa–based retroviral vector encoding shRNAs that complementarily bind to the constant regions of the endogenous *TCR*-$\alpha$ and *TCR*-$\beta$ genes (WT1-*siTCR* vector) (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Ecotropic retroviral vectors were obtained by transient cotransfection of WT1-TCR-expression retroviral vector and other components (Takara Bio) to HEK293 cell line; subsequently, GaLV-pseudotyped retroviral vectors were obtained by sequential infection of ecotropic retroviral vectors to PG13 cell line. The Jurkat/MA cell line, lacking endogenous TCR and engineered with the hCD8$\alpha$ and NFAT-luciferase construct,[29] was transduced with the WT1-*TCR* vector to confirm that our retroviral vector was actually able to express functional WT1-specific and HLA-A*24:02-restricted TCR molecules on the cell surface (supplemental Figure 2).

### Cell lines, freshly isolated leukemia cells, and normal cells

Approval for this study was obtained from the Institutional Review Board of Ehime University Hospital. Written informed consent was given by all patients, healthy volunteers, and the parents of the cord blood donors in accordance with the Declaration of Helsinki. All cell lines and freshly isolated cells were cultured as described previously.[30] B-lymphoblastoid cell lines (B-LCLs) were established by transformation of peripheral blood B lymphocytes with Epstein-Barr virus. An $HBZ_{26-34}$ peptide-specific and HLA-A*02:01-restricted CTL clone, designated HBZ-1, was established as reported previously.[31] The *HLA-A*24:02* gene-transduced C1R cell line (C1R-A*24:02) was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 0.5 mg/mL hygromycin B (Invitrogen) and the *HLA-A*24:02* gene-transduced K562 cell line (K562-A*24:02) was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 1.0 $\mu$g/mL puromycin (Sigma-Aldrich). Peripheral blood mononuclear cells and bone marrow mononuclear cells from leukemia patients and healthy volunteers, and cord blood mononuclear cells from healthy donors were isolated and stored in liquid nitrogen until use. All leukemia samples contained more than 95% leukemia cells. In some experiments, $CD34^+$ cells from cord blood mononuclear cells were isolated using $CD34^+$ cell-isolating immunomagnetic beads (MACS beads; Miltenyi Biotec).

### Establishment of WT1-*TCR* gene-transduced CTL lines

$CD8^+$ T cells were isolated from peripheral blood mononuclear cells of healthy volunteers and leukemia patients in complete remission, and cord blood mononuclear cells using $CD8^+$ cell-isolating MACS beads and stimulated with 1 $\mu$g/mL anti-CD3 monoclonal antibody (MoAb, OKT-3; BioLegend). $CD8^+$ T cells were cultured in GT-T503 medium (Takara Bio) supplemented with 5% human serum, 0.2% human albumin, 50 U/mL recombinant human IL-2 (R&D Systems), 5 ng/mL IL-7 (R&D Systems), 10 ng/mL IL-15 (PeproTech), and 10 ng/mL IL-21 (Shenandoah Biotechnology). Then, $CD8^+$ T cells were transfected with the WT1-*TCR* retrovirus vector using RetroNectin (Takara Bio)-coated plates as described previously.[25] In some experiments, V$\beta$5.1-positive cells among WT1-*TCR* gene-transduced $CD8^+$ T cells were further isolated using fluorescein isothiocyanate (FITC)-conjugated V$\beta$5.1 MoAb (Beckman Coulter) and an anti-FITC-conjugated MACS beads system. To measure the expression levels of introduced WT1-specific TCR in gene-engineered $CD8^+$ T cells, the cells were labeled with anti-CD8, anti-CD4, anti-CD3 (BD Biosciences), and anti-V$\beta$5.1 MoAbs and phycoerythrin-conjugated HLA-A*24:02/$WT1_{235-243}$-tetramer or HLA-A*24:02/HIV-1 $Env_{584-592}$-tetramer (supplemental Figure 3).[32] The labeled cells were analyzed using a Gallios flow cytometer (Beckman Coulter) and FlowJo Version 7.2.2 software (TreeStar). To compare the expandability of WT1-*coTCR*–transduced and WT1-*siTCR*-transduced $CD8^+$ T cells by stimulation with WT1 peptide, WT1-*TCR* gene-transduced $CD8^+$ T cells were weekly stimulated with mitomycin-C (Kyowa Hakko)-treated and heteroclitic $WT1_{235-243}$ peptide (CYTWNQMNL)-pulsed HLA-A*24:02-positive LCLs.

### $^{51}$Cr-release assays

To determine the cytotoxic activity of WT1-*TCR* gene-transduced $CD8^+$ T cells, standard $^{51}$Cr-release assays were performed as described previously.[30] Briefly, $5 \times 10^3$ $^{51}$Cr ($Na_2{}^{51}CrO_4$; New England Nuclear)–labeled target cells and various numbers of effector cells in 200 $\mu$L of RPMI 1640 medium supplemented with 10% fetal calf serum were seeded into 96-well round-bottomed plates. The target cells were incubated with or without WT1 peptide for 2 hours before adding the effector cells. To assess the HLA class I-restricted cytotoxicity, target cells were incubated with an anti-HLA class I framework MoAb (w6/32; ATCC) or an anti-HLA-DR MoAb (L243; ATCC) at an optimal concentration (10 $\mu$g/mL) for 1 hour before adding the effector cells. After incubation with the effector cells for 5 hours, 100 $\mu$L of supernatant was collected from each well. The percentage of specific lysis was calculated as: (experimental release cpm − spontaneous release cpm)/(maximal release cpm − spontaneous release cpm) × 100 (%).

### Detection of CD107a and intracellular IFN-$\gamma$ expression in WT1-*TCR* gene-transduced $CD8^+$ T cells

CD107a expression in WT1-*TCR* gene-transduced $CD8^+$ T cells in response to stimulation with WT1 peptide was assessed as described previously.[33] Briefly, $1 \times 10^5$ C1R-A*24:02 cells were seeded into a

96-well round-bottom plate and incubated with or without WT1 peptide for 2 hours. Then, $2 \times 10^5$ WT1-*TCR* gene-transduced $CD8^+$ T cells were seeded into each well along with FITC-conjugated CD107a MoAb (BioLegend) for 3 hours. Similarly, CD107a expression in the HBZ-1 cell line and WT1-*TCR* gene-transduced HBZ-1 cells in response to stimulation with $HBZ_{26-34}$ peptide-loaded HLA-A*02:01-positive T2 cells was analyzed. To investigate intracellular interferon-γ (IFN-γ) production, effector cells were incubated with target cells and 10 μg/mL brefeldin A for 4 hours. They were then collected, fixed, and permeabilized with fluorescence-activated cell sorter lysing solution and fluorescence-activated cell sorter permeabilizing solution (BD Biosciences).[34] After permeabilization, the washed cells were stained with FITC-conjugated IFN-γ MoAb (BD Biosciences). Finally, these effector cells were stained with anti-CD3, anti-CD8 MoAbs (BD Biosciences) and phycoerythrin-conjugated HLA-A*24:02/WT1-tetramer, and then analyzed using a Gallios flow cytometer and FlowJo Version 7.2.2 software.

### Quantitative analysis of *WT1* mRNA expression

Total RNA was extracted from each sample with an RNeasy Mini Kit (QIAGEN) in accordance with the manufacturer's instructions. Quantitative real-time polymerase chain reaction of *WT1* mRNA was performed using the QuantiTect SYBR Green PCR Kit (QIAGEN) and primers as follows: forward: 5′-AGCACAGGGTACGAGAGCGATAAC-3′, reverse; 5′-TATTGCAGCCTGGGTAAGCACA-3′ (Takara Bio). *GAPDH* mRNA as an internal control was prepared as described previously.[30] These samples were analyzed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The expression level of *WT1* mRNA was corrected by reference to that of *GAPDH* mRNA, and the amount of *WT1* mRNA in each sample relative to that in the K562 leukemia cell line, which strongly expresses *WT1* mRNA (shown as 1.0), was calculated by the comparative $\Delta C_t$ method.

### In vivo antileukemia effect of WT1-*siTCR* gene-transduced CTLs

Six-week-old NOD/scid/$\gamma c^{null}$ (NOG) female mice[35] were purchased from the Central Institute for Experimental Animals and maintained in the institutional animal facility at Ehime University. All in vivo experiments were approved by the Ehime University animal care committee. For xenografting of human leukemia cells, NOG mice were inoculated subcutaneously in the left flank with $5 \times 10^6$ K562-A*24:02 cells, which had been preincubated with $2.5 \times 10^7$ effector cells for 5 hours. Then, $1 \times 10^7$ effector cells were additionally administered intravenously via the tail vein every week for a total of 5 times. The mice were monitored for tumor growth and survival after inoculation; the tumors were measured at 5-day intervals, and the tumor area was determined.

### In vivo differentiation of human hematopoietic stem cells in humanized mice

$CD34^+$ cells were isolated from cord blood mononuclear cells (hCB-$CD34^+$ cells), and then $5 \times 10^4$ of the cells were coincubated with $2.5 \times 10^5$ autologous WT1-*siTCR*–transduced or non–gene-modified CTLs generated from cord blood $CD8^+$ T cells for 5 hours. hCB-$CD34^+$ cells were then reisolated from the cell mixture and injected intravenously into 7-week-old NOG mice that had been irradiated with 1.5 Gy. Three months later, these mice were killed to study the engraftment and differentiation of human hematopoietic cells in both the bone marrow and spleen. Human leukocytes were discriminated from murine cells using anti-hCD45 MoAb (BD Biosciences). Human cells were further stained with MoAbs against cell lineage-related surface molecules, including CD3, CD8, CD4, CD19, CD33, CD34, CD38, CD41a, and GPA (BD Biosciences). The expression of HLA-A*24:02 in the engrafted human cells was also measured using FITC-conjugated anti-HLA-A24 MoAb (One Lambda), and the immunostained cells were analyzed using a Gallios flow cytometer and FlowJo Version 7.2.2 software.

## Results

### Comparison of WT1-TCR expression and WT1-specific cytotoxic reactivity of WT1-*siTCR*- and WT1-*coTCR*–transduced $CD8^+$ T cells

First, we confirmed the augmented and inhibitory efficacies of the WT1-*siTCR* vector for expression of the respectively introduced and endogenous TCRs. To do so, we transduced WT1-*siTCR* into HBZ-1, which is an HLA-A*02:01-restricted and $HBZ_{26-34}$-specific $CD8^+$ T-cell clone. As shown in Figure 1A, positivity for HLA-A*24:02/WT1-tetramer staining in nontreated, WT1-*coTCR*- and WT1-*siTCR*–transduced HBZ-1 was $< 1\%$, 29%, and 65%, respectively, whereas the corresponding values for HLA-A*02:01/HBZ-tetramer staining were 98%, 20%, and 4%, respectively. For functional assessment of the efficacy of the *siTCR* vector for suppression of endogenous HBZ-TCR, CD107a assays were performed. We evaluated the extent of the decreased responsiveness to the cognate HBZ peptide mediated by WT1-*coTCR*– and WT1-*siTCR*–transduced HBZ-1 compared with that mediated by the parent HBZ-1. As shown in Figure 1B, WT1-*siTCR*–transduced HBZ-1 exhibited an apparent loss of responsiveness to the HBZ peptide-loaded T2 cells, indicating that sufficient functional suppression of endogenous TCR is achievable using the WT1-*siTCR* vector. The reactivity of WT1-*coTCR*–transduced HBZ-1 to stimulation with HBZ peptide also appeared to decrease compared with that of the parental HBZ-1 clone; however, the inhibitory effect of the *siTCR* vector appeared to be higher than that of the *coTCR* vector at high concentrations of the HBZ peptide. On the basis of these data, we were able to confirm the efficacy of the WT1-*siTCR* vector for augmentation of introduced TCR expression and inhibition of endogenous TCR expression.

Next, we further investigated whether enhanced expression of the introduced TCR by the WT1-*siTCR* vector actually up-regulated the function of engineered $CD8^+$ T cells. To do this, we compared the amounts of intracellular IFN-γ production and degrees of granular exocytosis by WT1-*coTCR*– and WT1-*siTCR*–transduced $CD8^+$ T cells in response to stimulation with WT1 peptide. As shown in the upper column of Figure 1C, the proportion of tetramer-positive cells in WT1-*siTCR*–transduced $CD8^+$ T cells was higher than that in WT1-*coTCR*–transduced $CD8^+$ T cells. The data for intracellular IFN-γ production and CD107a expression in WT1 tetramer-positive cells are shown in the lower columns. The degrees of both IFN-γ production and CD107a expression in WT1-*siTCR*–transduced $CD8^+$ T cells in response to stimulation with WT1 peptide appeared to be higher than those in WT1-*coTCR*-transduced $CD8^+$ T cells.

Similarly, as shown in Figure 1D, up-regulation of cytotoxicity mediated by WT1-*siTCR*–transduced $CD8^+$ T cells against both WT1 peptide-loaded target cells and HLA-A*24:02-positive leukemia cells was detected compared with that mediated by WT1-*coTCR*–transduced $CD8^+$ T cells.

### Enhanced expandability without deterioration of antigen-specific responsiveness of WT1-*siTCR*–transduced $CD8^+$ T cells

Efficient expansion of transferred T cells in vivo as well as in vitro is an important issue affecting the efficacy of adoptive T-cell

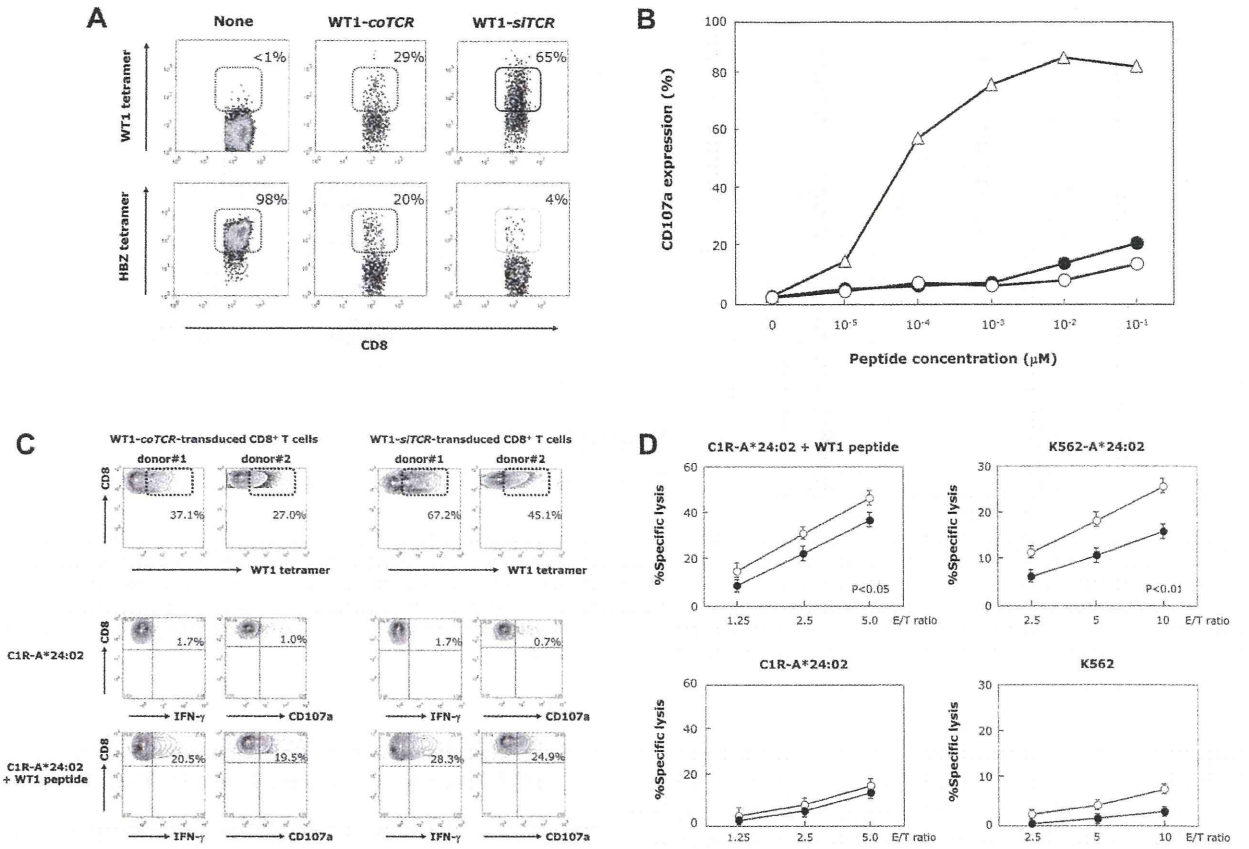


**Figure 1. Enhanced expression of introduced WT1-specific TCR and augmented functionality in WT1-*siTCR*–transduced CD8$^+$ T cells.** (A) An HLA-A*02:01-restricted $HBZ_{26-34}$-specific CTL clone (HBZ-1) was transduced with the WT1-*siTCR* or WT1-*coTCR* vector. Expression of the introduced WT1-specific and intrinsic HBZ-specific TCRs in *TCR* gene-modified HBZ-1 cells was examined using either HLA-A*24:02/WT1 tetramer or HLA-A*02:01/HBZ tetramer. A non–gene-modified HBZ-1 clone was used as a negative control. (B) HBZ-1 cells (△), WT1-*coTCR*–transduced HBZ-1 cells (●), and WT1-*siTCR*–transduced HBZ-1 cells (○) were cocultured with HLA-A*02:01-positive T2 cells loaded with various concentrations of HBZ peptide for 3 hours. Thereafter, surface CD107a expression was analyzed as detailed in "Detection of CD107a and intracellular IFN-γ expression in WT1-*TCR* gene–transduced CD8$^+$ T cells." (C) IFN-γ production and degranulation of WT1-*siTCR*–transduced and WT1-*coTCR*–transduced CD8$^+$ T cells in response to stimulation with WT1 peptide. Populations of WT1 tetramer-positive cells in WT1-*coTCR*– and WT1-*siTCR*–transduced CD8$^+$ T cells before stimulation are shown in the upper column. The CD8$^-$/WT1-tetramer$^+$ cells shown with a broken line in each sample were analyzed for intracellular IFN-γ production and surface CD107a expression. One set of data obtained from experiments performed using CD8$^+$ T cells from 2 different donors are representatively shown. (D) Cytotoxic activities of WT1-*siTCR*–transduced CD8$^+$ T cells (○) and WT1-*coTCR*–transduced CD8$^+$ T cells (●) against C1R-A*24:02 cells loaded with or without WT1 peptide and K562 cells transduced with or without *HLA-A*24:02* gene were examined by standard 5-hour $^{51}$Cr-release assays at various effector/target (E/T) ratios.

therapy. Therefore, we compared the expandability of WT1-*siTCR*–transduced and WT1-*coTCR*–transduced CD8$^+$ T cells after repeated stimulation with WT1 peptide. Representative data are shown in Figure 2A. WT1-*siTCR*–transduced CD8$^+$ T cells showed good expansion after stimulation with WT1 peptide and maintained their antigen specificity. In contrast, WT1-*coTCR*–transduced CD8$^+$ T cells showed rapid growth, but their WT1 specificity declined rapidly. A summary of this experiment using WT1-*siTCR*–transduced CD8$^+$ T cells generated from 5 donors and WT1-*coTCR*–transduced CD8$^+$ T cells generated from 3 donors is shown in Figure 2B.

We further confirmed the WT1 specificity and HLA-A*24:02 restriction of cytotoxicity mediated by WT1-*siTCR*–transduced CD8$^+$ T cells that had been cultured and expanded for more than 2 months with repeated WT1 peptide stimulation. Representative data for 5 experiments are shown in Figure 3A. WT1-*siTCR*–transduced CD8$^+$ T cells after $>$ 2 months of culture appeared to be totally positive for TCR-Vβ5.1 expression, and $>$ 70% of the cells were positive for HLA-A*24:02/WT1-tetramer staining. These WT1-*siTCR*–transduced CTLs exerted strong cytotoxicity against WT1 peptide-loaded but not peptide-unloaded C1R-A*24:02 cells. Similarly, they exerted strong cytotoxicity against HLA-A*24:02-positive but not HLA-A*24:02-negative leukemia cell lines, as shown in Figure 3B. Their cytotoxicity against HLA-A*24:02-positive leukemia cell lines was significantly abrogated by anti-HLA class I MoAb. These data indicate that stimulation of WT1-*siTCR*–transduced CD8$^-$ T cells with WT1 peptide might be effective for their expansion while maintaining their antigen specificity.

### Lysis of autologous leukemia cells and lack of damage to autologous hematopoietic progenitor cells by WT1-*siTCR*–transduced CTLs

For clinical application of adoptive T-cell therapy using WT1-*TCR* gene-engineered CTLs, it is essential to obtain evidence that autologous leukemia cells are indeed lysed and that autologous hematopoietic progenitor cells are not damaged by WT1-*siTCR*–transduced CTLs. We therefore performed cytotoxicity assays using WT1-*siTCR*–transduced CTLs as effector cells, and autologous leukemia cells from leukemia patients and hematopoietic

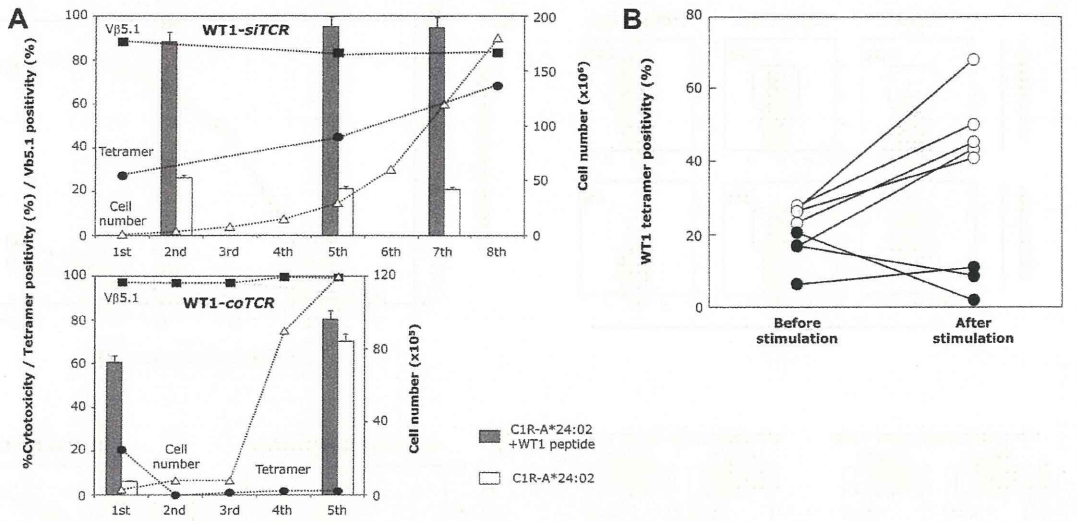


**Figure 2. Enhanced expandability of WT1-*siTCR*–transduced CD8⁺ T cells by repetitive stimulation with WT1 peptide in vitro.** (A) WT1-*siTCR*–transduced CD8⁺ T cells (top) and WT1-*coTCR*–transduced CD8⁺ T cells (bottom) were repetitively stimulated with HLA-A*24:02-positive LCLs loaded with WT1 peptide in vitro. Total cell number (△), percentage of Vβ5.1-positive cells (■), and percentage of HLA-A*24:02/WT1 tetramer-positive cells (●) were monitored after stimulation with WT1 peptide. Cytotoxic activities of WT1-*siTCR*–transduced CD8⁺ T cells and WT1-*coTCR*–transduced CD8⁺ T cells against WT1 peptide-loaded (gray bars) or -unloaded C1R-A*24:02 cells (white bars) are also shown. (B) Percentages of HLA-A*24:02/WT1 tetramer-positive cells among WT1-*siTCR*–transduced CD8⁺ T cells from 5 donors (○) and those in WT1-*coTCR*–transduced CD8⁺ T cells from 3 donors (●) before and after stimulation with WT1 peptide.

progenitors from cord blood as target cells. As shown in Figure 4A, WT1-*siTCR*–transduced CTLs generated from peripheral blood CD8⁺ T cells of patients with leukemia exerted cytotoxicity against autologous leukemia cells. On the other hand, those generated from cord blood CD8⁺ T cells exerted no cytotoxicity against autologous hematopoietic progenitor cells. As shown in Figure 4B, all WT1-*siTCR*–transduced CTL lines made from cord blood CD8⁺ T cells used in this experiment appeared to efficiently lyse WT1 peptide-loaded HLA-A*24:02-positive LCLs and HLA-A*24:02-positive leukemia cell lines without the addition of exogenous WT1 peptide.

### In vivo antileukemia efficacy of adoptively transferred WT1-*siTCR*–transduced CTLs in a xenograft mouse model

We further examined the in vivo antileukemia efficacy of adoptive transfer with WT1-*siTCR*–transduced CTLs using 3 cohorts of a therapeutic xenograft mouse model. In the first group, NOG mice were inoculated with K562-A*24:02 cells that had been preincubated with non–gene-modified CD8⁺ T cells (control CTLs), and additionally treated by intravenous infusion of control CTLs weekly for a total of 5 times. Control CTLs were prepared by stimulation of peripheral blood CD8⁺ T cells with anti-CD3 MoAb

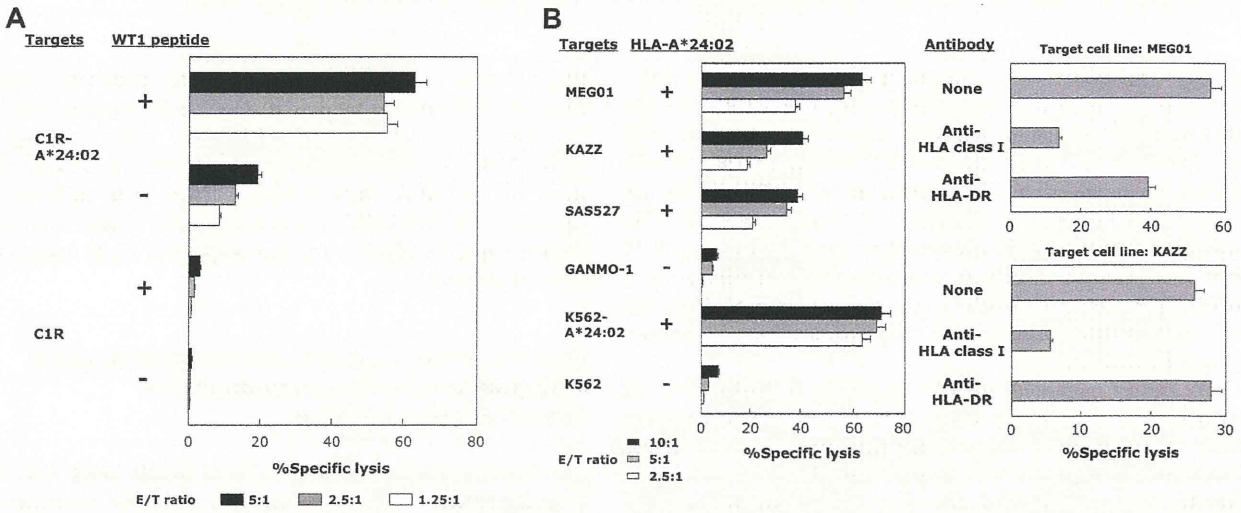


**Figure 3. HLA-A*24:02-restricted and WT1 peptide-specific cytotoxicity mediated by WT1-*siTCR*–transduced CTLs after repeated stimulation with WT1 peptide.** (A) Cytotoxicity of WT1-*siTCR*-transduced CD8⁺ T cells that had been cultured continuously for > 2 months against WT1 peptide-loaded or -unloaded C1R-A*24:02 cells and C1R cells was examined by 5-hour $^{51}$Cr-release assays. (B) Cytotoxicity of WT1-*siTCR*–transduced CD8⁺ T cells that had been cultured continuously for > 2 months against HLA-A*24:02-positive and HLA-A*24:02-negative leukemia cell lines was examined by 5-hour $^{51}$Cr-release assays. HLA class I-restriction of cytotoxicity mediated by WT1-*siTCR*–transduced CTLs against MEG01 and KAZZ cell lines was examined by 5-hour $^{51}$Cr-release assays at an E/T ratio of 5:1 in the presence or absence of anti-HLA class I MoAb or anti-HLA-DR MoAb.

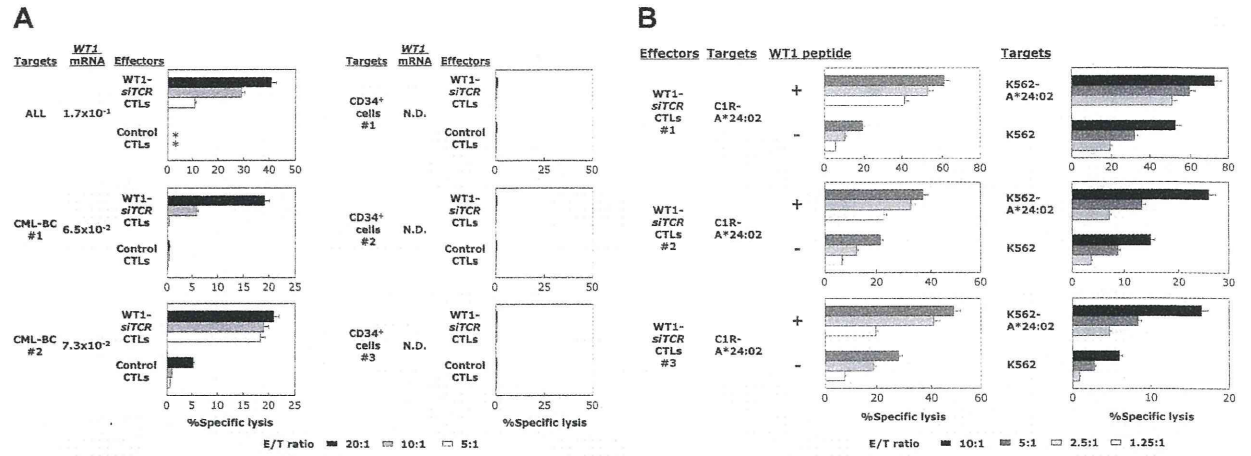


**Figure 4. Cytotoxicity mediated by WT1-*siTCR*–transduced CTLs against autologous leukemia cells and hematopoietic progenitor cells.** (A) Cytotoxic activities of WT1-*siTCR*–transduced CTLs against autologous leukemia cells and autologous normal hematopoietic progenitor cells were examined by 5-hour $^{51}$Cr-release assays. WT1-*siTCR*–transduced CTLs were generated from peripheral blood CD8$^+$ T cells from a patient with acute lymphoblastic leukemia (ALL) in complete remission, 2 patients with blastic crisis of chronic myelogenous leukemia (CML-BC) in chronic phase after chemotherapy, and cord blood CD8$^+$ T cells from 3 donors. Their cytotoxicity against autologous freshly isolated leukemia cells or autologous hCB-CD34$^+$ cells was examined by standard $^{51}$Cr-release assays at various E/T ratios. The relative expression levels of *WT1* mRNA in target cells are shown. N.D. indicates not detectable. (B) Cytotoxicity mediated by WT1-*siTCR*–transduced CTLs generated from cord blood CD8$^+$ T cells against C1R-A*24:02 cells with or without loaded WT1 peptide, K562-A*24:02 cells, and K562 cells was examined by 5-hour $^{51}$Cr-release assays at various E/T ratios. Each number of effector cells (#1, #2, and #3) corresponds to that of the hCB-CD34$^+$ cell sample shown in Figure 4A, respectively.

and cultured in IL-2–containing medium. In the second group, NOG mice were inoculated with K562-A*24:02 cells that had been preincubated with WT1-*siTCR*–transduced CTLs, without additional cell transfer. In the third group, NOG mice were inoculated with K562-A*24:02 cells that had been preincubated with WT1-*siTCR*–transduced CTLs and received additional cell transfer with WT1-*siTCR*–transduced CTLs weekly 5 times. The growth curves of the inoculated leukemia cells are shown in Figure 5. K562-A*24:02 cells grew rapidly in all mice treated with control CTLs and died within 40 days. Compared with mice treated with control CTLs, the survival of mice inoculated with K562-A*24:02 cells that had been preincubated with WT1-*siTCR*–transduced CTLs was significantly prolonged. Furthermore, additional transfer of WT1-*siTCR*–transduced CTLs further significantly prolonged the survival period of K562-A*24:02-inoculated mice. Notably, no tumor formation was detected in mice during adoptive transfer of WT1-*siTCR*–transduced CTLs. These results clearly show the efficacy of adoptive T-cell therapy using WT1-*siTCR*–transduced CTLs for treatment of human leukemia.

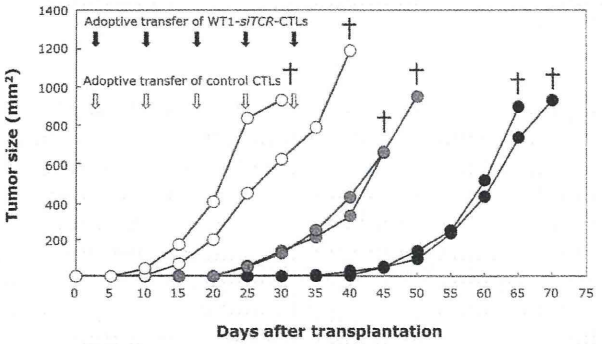


**Figure 5. Antileukemia effect of adoptively transferred WT1-*siTCR*–transduced CTLs in a xenograft mouse model.** WT1-*siTCR*–transduced CTLs and non–gene-modified human CD8$^+$ T cells (control CTLs) were prepared from peripheral blood CD8$^+$ T cells. NOG mice were inoculated with K562-A*24:02 cells preincubated with WT1-*siTCR*–transduced CTLs or control CTLs with or without additional cell therapy. (○) represents the growth of leukemia cells in 2 control mice inoculated with K562-A*24:02 cells preincubated with control CTLs and into which control CTLs were transferred weekly; gray circles, leukemia cell growth in 2 mice inoculated with K562-A*24:02 cells preincubated with WT1-*siTCR*–transduced CTLs without additional transfer of CTLs; and (●), the growth of leukemia cells in 2 mice inoculated with K562-A*24:02 cells preincubated with WT1-*siTCR*–transduced CTLs, and into which WT1-*siTCR*–transduced CTLs were additionally transferred weekly. The time points (in days) after transplantation of K562-A*24:02 cells when the mice died are indicated.

## No deteriorative effect of WT1-*siTCR*–transduced CTLs on engraftment and differentiation of autologous hematopoietic progenitor cells in humanized mice

Finally, we addressed the issue of whether WT1-*siTCR*–transduced CTLs exert an inhibitory effect on the proliferation and differentiation of normal hematopoietic progenitor cells, as it has been reported that WT1 expression is detectable in normal hematopoietic progenitor cells.[21,22,36] The hCB-CD34$^+$ cells that had been preincubated with WT1-*siTCR*–transduced CTLs or control CTLs were transplanted into NOG mice. Three months later, these mice were killed and analyzed for engraftment and differentiation of human hematopoietic cells. HLA-A*24:02 appeared to be efficiently expressed in human blood cells that had proliferated in humanized mice (data not shown). Representative data for 3 experiments are shown in Figure 6A. It is clearly evident that human CD34$^+$ cells preincubated with WT1-*siTCR*–transduced CTLs were successfully engrafted and differentiated into human peripheral blood cell components, including hCD45$^+$/CD33$^+$ myeloid cells, hCD45$^+$/CD19$^+$ B cells, and hCD45$^+$/CD3$^+$ T cells in the spleen, as was the case for NOG mice transplanted with human CD34$^+$ cells that had been preincubated with control CTLs. In bone marrow, not only hCD45$^+$/CD33$^+$ myeloid cells, but also hCD45$^+$/CD34$^+$ hematopoietic progenitor cells, hCD45$^+$/hGPA$^-$ erythroid immature cells, and hCD45$^+$/CD41a$^+$ megakaryocytic immature cells were efficiently engrafted and differentiated from human CD34$^+$ cells preincubated with WT1-*siTCR*–transduced CTLs as well as control CTLs. Although interindividual differences in engraftment efficacy were detected among NOG mice transplanted with human CD34$^+$ cells, it was concluded that

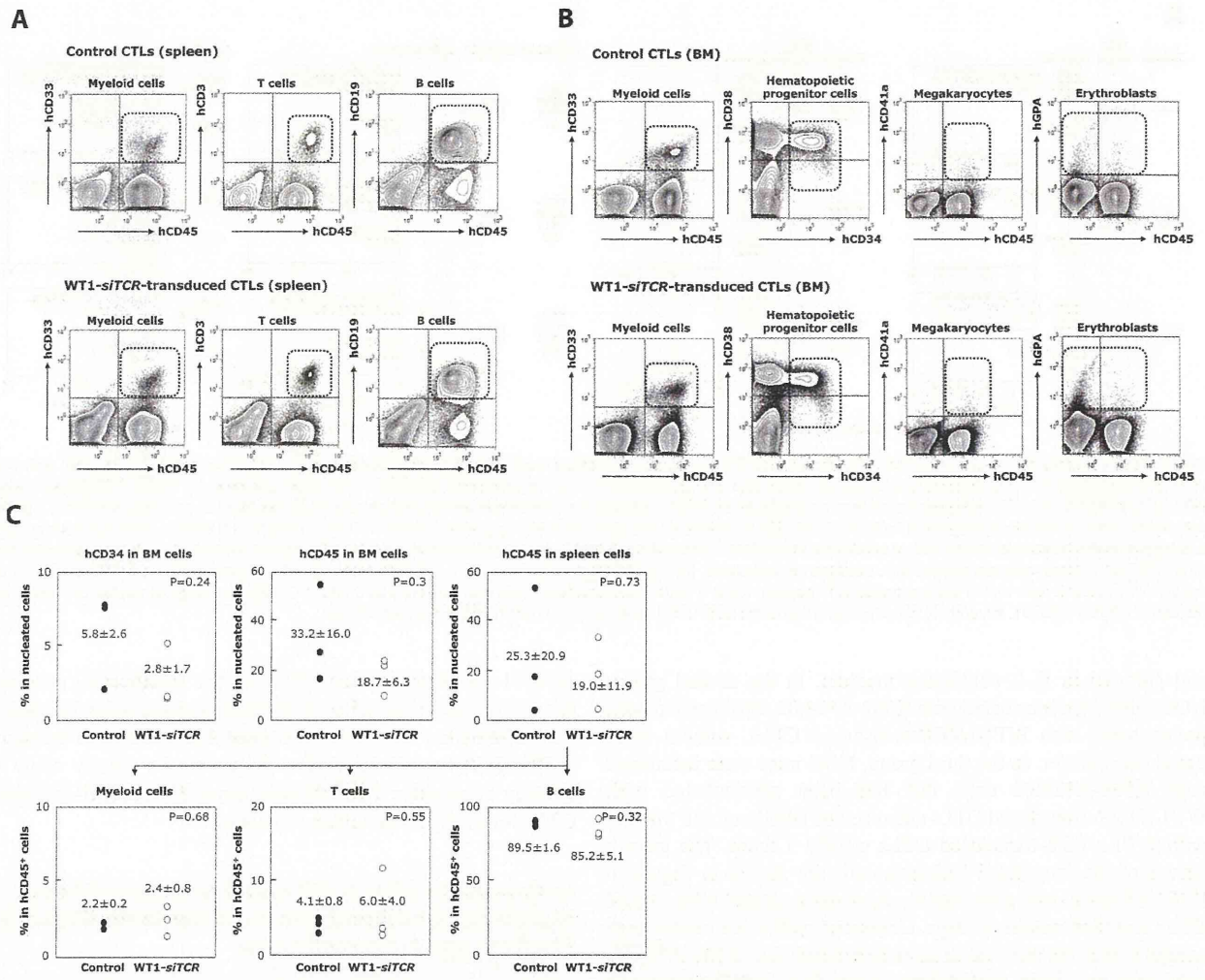


**Figure 6. Lack of an inhibitory effect of WT1-*siTCR*–transduced CTLs on human hematopoiesis in a humanized mouse model.** HLA-A*24:02-positive hCB-CD34$^+$ cells preincubated with autologous WT1-*siTCR*–transduced CTLs or non–gene-modified autologous CTLs (as a negative control) were transplanted into NOG mice. Three months later, the mice were killed and examined for engraftment and differentiation of human hematopoietic progenitor cells in the spleen and bone marrow. (A) Representative data for 3 experiments. (B) Summary of long-term hematopoiesis of engrafted human hematopoietic progenitor cells in the bone marrow and spleen of mice transplanted with hCB-CD34$^+$ cells that had been preincubated with control CTLs or WT1-*siTCR*–transduced CTLs.

WT1-*siTCR*–transduced CTLs never damage human CD34$^+$ hematopoietic progenitor cells (Figure 6B).

## Discussion

In our previous study, we developed a novel retroviral vector system that can express antigen-specific TCR more efficiently based on the concept of siRNA-targeting of the constant regions of the endogenous *TCR-α* and *TCR-β* genes and siRNA-resistant codon-optimization of exogenous *TCR* genes.[25] To apply the basic concept of WT1-*siTCR* clinically for treatment of human leukemia, we investigated in detail the efficacy and safety of this strategy. Consequently, we demonstrated the marked advantages of WT1-*siTCR* gene transfer for adoptive immunotherapy in terms of both enhancement of the antileukemia effect and safety. First, we clearly demonstrated that up-regulated expression of introduced WT1-specific TCR and sufficient inhibition of endogenous TCR could be achieved using an experimental system in which the WT1-*siTCR* gene was transduced into a HBZ-specific T-cell clone. Enhanced expression of the introduced WT1-TCR on WT1-*siTCR*–transduced CTLs resulted in augmentation of WT1-specific cytotoxicity as compared with that mediated by WT1-*coTCR*–transduced CTLs. In addition, through repetitive stimulation with cognate peptide in vitro, WT1-*siTCR*–transduced CTLs showed marked expandability while maintaining their antigen specificity. Furthermore, the WT1-*siTCR*–transduced CTLs were able to successfully lyse autologous leukemia cells but not normal hematopoietic progenitor cells. Importantly, experiments using a xenograft mouse model revealed that adoptively transferred WT1-*siTCR*–transduced CTLs effectively inhibited leukemia cell growth in vivo. In contrast, the engraftment and differentiation abilities of normal hematopoietic progenitors showed no deterioration in the presence of WT1-*siTCR*–transduced CTLs, thus negating the possibility that WT1-specific CTLs might mediate severe bone marrow failure.

One of the major advantages of *TCR* gene-engineered T-cell immunotherapy revealed by our present series of experiments is the establishment of augmented antigen-specific cytotoxicity and safety through silencing of endogenous *TCR* gene expression. Recent clinical studies using *TCR* gene-transduced T cells have indicated

that almost all of these cells disappeared in patients within 2 months after infusion.[8,37] Moreover, there appeared to be a significant correlation between clinical response and the persistence of infused T cells in the peripheral blood of patients.[9] Therefore, it would be important to maintain a sufficient number of *TCR* gene-engineered T cells with adequate antigen specificity in patients for a long period to achieve a good clinical response. In our present study, the antigen specificity of WT1-*coTCR*–transduced CTLs declined rapidly during culture, even though they were stimulated repeatedly with WT1 peptide. This might have been because of the formation of mispaired TCRs that had acquired nonspecific reactivity. In contrast, WT1-*siTCR*–transduced CTLs appeared to be markedly expanded while maintaining WT1 specificity and showing enhanced WT1-specific cytotoxicity for more than 2 months as a result of repetitive stimulation with WT1 peptide. Recently, it has been reported that adoptively transferred gp100-specific murine T cells were expandable up to 1000-fold after cognate peptide vaccination, resulting in an effective antitumor response in vivo.[38] These data strongly support the practical value of WT1-*siTCR* for maintaining the WT1 specificity of *TCR* gene-modified CTLs for a long period in vivo and also suggest that WT1 peptide vaccination after adoptive transfer of WT1-*siTCR*–transduced CTLs would facilitate the expansion of WT1-specific CTLs in human patients.

One of the major concerns related to adoptive transfer of *TCR* gene-engineered T cells is the possibility of evoking severe autoimmunity mediated by mispaired TCR. Recently, an elegant study of *TCR* gene therapy using a mouse model has revealed that mispairing of introduced and endogenous TCR chains in *TCR* gene-modified T cells leads to the formation of self-reactive TCRs that are responsible for lethal graft-versus-host disease.[19] Furthermore, it has been reported that adjustments in the design of gene therapy vectors for preventing the formation of mispaired TCRs could reduce the risk of *TCR* gene therapy-induced lethal autoimmunity.[19] This evidence obtained from basic research strongly supports the clinical advantage of our WT1-*siTCR* vector.

It is also notable that autologous HLA-restricted and foreign antigen-derived peptide-specific TCRs can exert allogeneic HLA responsiveness. Recently, the frequent incidence of allogeneic HLA reactivity mediated by redirected T cells against predefined virus antigens has been reported.[39] Using an LCL panel, we similarly observed that our HLA-A*24:02-restricted and WT1 peptide-specific *TCR* gene-transduced CTLs responded to the HLA-B*57:01 molecule in the absence of cognate WT1 peptide.[40] Therefore, *TCR* gene-engineered T cells should be tested for their allogeneic HLA reactivity against recipient cells before administration.

Another notable finding in the present study was that WT1-*siTCR*-transduced CTLs never exerted a cytotoxic effect on normal hematopoietic progenitor cells. Although it has been proposed that WT1 is an ideal tumor-associated antigen,[41] previous reports have indicated that WT1 expression is certainly detectable in normal hematologic progenitor cells,[21,22,36] suggesting a risk of bone marrow failure mediated by WT1-specific CTLs. Furthermore, the occurrence of severe leukocytopenia after WT1 peptide vaccination in 2 patients with myelodysplastic syndrome has been reported.[42] Therefore, we examined in detail the inhibitory effect of WT1-*siTCR*–transduced CTLs on normal human hematopoiesis in a humanized mouse model. We clearly demonstrated that WT1-*siTCR*–transduced CTLs never damaged normal human hematopoietic progenitor cells. There are 3 possible explanations for the mechanism underlying the resistance of normal hematopoietic progenitors to WT1-specific CTL-mediated cytotoxicity. The first is that the amount of WT1 expressed in normal hematopoietic progenitors is not enough to be recognized by CTLs. This possibility seems likely because quantitative analysis has revealed that the expression level of *WT1* mRNA in normal hematopoietic progenitor cells is relatively low compared with that in leukemia cells.[43,44] The second is that normal hematopoietic progenitors have the potential to resist CTL-mediated cytotoxicity. We previously reported that, although the levels of WT1 expression in myeloma and lymphoma cells were almost the same, only myeloma cells were lysed efficiently by WT1-specific CTLs. The extent of membrane damage induced by purified perforin appeared to be significantly higher in myeloma cells than in lymphoma cells.[32] Therefore, the susceptibility of membranes to perforin is an important factor determining the sensitivity of target cells to CTL-mediated cytotoxicity, and normal hematopoietic cells are relatively resistant to CTL-mediated granule exocytosis. The third is that the introduced WT1-specific TCR in this study might have had an optimal range of avidity for recognition of leukemia cells but that spare normal hematopoietic progenitor cells physiologically expressed WT1, as avidity enhancement of the introduced TCR was able to evoke "on-target" adverse events against normal tissues.[9]

In conclusion, the present study has revealed that our WT1-*siTCR* retrovirus vector system shows considerable promise in terms of efficacy and safety for adoptive immunotherapy for leukemia using *TCR* gene-engineered T cells. On the basis of our data, we intend to begin clinical trials of adoptive WT1-*siTCR*–transduced T-cell therapy with WT1 peptide vaccination for chemotherapy-resistant leukemia.

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## Authorship

Contribution: T.O. designed and performed the research and wrote the paper; H.F. designed and performed the research, wrote and edited the paper, and provided financial support; S.O., J.A., K.N., T.S., J.M., and H.S. discussed and interpreted the experimental results and provided materials; K.K. made and supplied the tetramer; and M.Y. discussed and interpreted the experimental results, wrote and edited the paper, and provided financial support.

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# Intratumoral Injection of *Propionibacterium acnes* Suppresses Malignant Melanoma by Enhancing Th1 Immune Responses

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## Abstract

Malignant melanoma (MM) is an aggressive cutaneous malignancy associated with poor prognosis; many putatively therapeutic agents have been administered, but with mostly unsuccessful results. *Propionibacterium acnes (P. acnes)* is an aerotolerant anaerobic gram-positive bacteria that causes acne and inflammation. After being engulfed and processed by phagocytes, *P. acnes* induces a strong Th1-type cytokine immune response by producing cytokines such as IL-12, IFN-γ and TNF-α. The characteristic Th2-mediated allergic response can be counteracted by Th1 cytokines induced by *P. acnes* injection. This inflammatory response induced by *P. acnes* has been suggested to have antitumor activity, but its effect on MM has not been fully evaluated. We analyzed the anti-tumor activity of *P. acnes* vaccination in a mouse model of MM. Intratumoral administration of *P. acnes* successfully protected the host against melanoma progression *in vivo* by inducing both cutaneous and systemic Th1 type cytokine expression, including TNF-α and IFN-γ, which are associated with subcutaneous granuloma formation. *P. acnes*-treated tumor lesions were infiltrated with TNF-α and IFN-γ positive T cells. In the spleen, TNF-α as well as IFN-γ producing $CD8^+$T cells were increased, and interestingly, the number of monocytes was also increased following *P. acnes* administration. These observations suggest that *P. acnes* vaccination induces both systemic and local antitumor responses. In conclusion, this study shows that *P. acnes* vaccination may be a potent therapeutic alternative in MM.

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## Introduction

Malignant melanoma (MM) is a life-threatening disease that is commonly resistant to treatment. Early diagnosis followed by surgical resection improves the prognosis of patients with MM. However, despite careful follow-up and treatment with combination chemotherapy or adjuvant therapy, patients with MM frequently develop both local and distant metastases. Patients with distant metastases almost always have a poor clinical outcome. Due to the high frequency of spontaneous recurrence of MM lesions, adjuvant therapy is generally recommended. Starting with Coley toxins more than one hundred ago, adjuvant therapy or immunotherapy has come to be regarded as one of the effective methods for boosting anti-tumor immunity [1], and various compounds and therapeutic modalities have been tested against melanoma using experimental mouse models [2,3,4]. However, the precise mechanisms driving the response have not been elucidated.

*Propionibacterium acnes (P. acnes)* is a component of the normal bacterial flora of the skin; it is aerotolerant, anaerobic gram-positive bacteria that plays an important role in the pathogenesis of acne [5]. When dendritic cells (DC) phagocyte and process *P. acnes*, a powerful Th1-type cytokine immune response is elicited, leading to an increased production of IL-12, IFN-γ and TNF-α. Injection of *P. acnes* has been reported to shift a dominant Th2 response to a Th1 type response, with an associated improvement in skin symptoms [6]. Clinically, acne vulgaris, a *P. acnes*-associated disease, often occurs in patients after a recovery from severe atopic dermatitis [7].

In the present study, we developed a mouse model of MM and investigated the clinical and immunological effects of *P. acnes* vaccination.

## Materials and Methods

### Ethics Statement

Animal care was performed according to standard ethical guidelines, and all of the experimental protocols were approved by the Institutional Board Committee for Animal Care and Use of Mie University (Permit Number 21-27-1).

### Mice

Female C57BL/6J(B6) mice were purchased from Japan SLC Co. (Shizuoka, Japan) and were bred under specific-pathogen-free conditions.

### P. acnes

*P. acnes* was purified from normal healthy volunteers and cultured in brain-heart infusion medium supplemented with L-cysteine and Tween-80, as reported previously [8]. Cultured bacteria were washed with sterile distilled water, killed by heating at 60°C for 60 min, and then lyophilized. A strain of *P. acnes* that effectively promotes inflammatory cytokine production was selected (*P. acnes-Mie1*) and used in the subsequent experiments [9].

### Melanoma cell and injection

The B16 melanoma cell line was purchased from ATCC (Manassas, VA), and cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (HyClone Laboratories, INC., South Logan, UT) at 37°C in a humidified incubator containing 5% CO2 and 95% air. $1\times10^6$ melanoma cells were injected in the dorsal skin of 8 week-old mice.

### Vaccination schedule

3.3 mg of heat-killed *P. acnes* were injected into the dorsal skin at the site where MM cells had been injected in 8–10 week old mice. Mice were divided into five groups: 1) MM-bearing mice treated with PBS (20 µl (MM, n = 14), 2) a group of MM-bearing mice treated with *P. acnes* at 10 weeks old (PMM1, n = 15), 3) another group treated with *P. acnes*-treated at 8 and 10 weeks old (PMM2, n = 15), 4) a control group of mice treated with *P. acnes* (P1, n = 11) at 10 weeks old, and 5) another control group treated with *P. acnes* at 8 and 10 weeks old (P2, n = 13).

### Clinical manifestations and histopathological study

Tumor growth was measured by the longest (L) point and the perpendicular diameter (W) of the dorsal skin tumor mass at 12 weeks of age, and the tumor volume was calculated according to the following formula: $4\pi W^2L/3$ [10]. The tumor tissue, lung, liver, and spleen were excised from the animals of each group, and samples were embedded in paraffin to prepare sections for haematoxylin & eosin staining.

### Analysis of cytokine mRNA expression

The RNA was extracted from skin lesions using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions and as reported previously [9,11]. Briefly, 1 ml of homogenate was mixed with 200 µl of chloroform and then centrifuged. The aqueous phase was separated and mixed with 0.5 ml of 2-propanol (Nakalai Tesque, Kyoto, Japan) to precipitate RNA. After centrifugation, the precipitate was washed with 70% ethanol (Nakalai Tesque) and the RNA was suspended in 40 µl RNase-free water. The RNA concentration was measured at 260 nm, and the quality was confirmed by electrophoresis. cDNA was synthesized from 2 µg of RNA using an Archive Kit (ABI, Foster City, CA, USA) according to the manufacturer's protocol. Real time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to determine the transcriptional activity in the tumor lesions. A 25-µl reaction mixture containing 1 µg of cDNA, 900 nmol of each primer, and 250 nmol of TaqMan probe was mixed with 12.5 µl of TaqMan Master Mix (ABI). Quantitative RT-PCR for cytokine transcripts was performed using prequalified primers and probes corresponding to IFN-γ T-bet, IL-12p35, IL-12p40, TNF-α IL-17A, IL-10, MIP-2, and GAPDH (ABI). The ΔδCt method was used to standardize the transcripts to GAPDH.

### Cell isolation and preparation from spleen and skin tumor tissue

The spleens were sampled and single cell suspensions was prepared by mechanical mincing, as reported previously [6]. For the characterization of tumor infiltrating lymphocytes (TIL), skin tumor tissue from the dorsal region was removed, minced gently with scissors and then single cell suspensions were prepared. After passing through a 70-µm-pore mesh, the cells were washed and resuspended with PBS. After Ficoll (SIGMA, St. Louis, MO) separation, cells were washed and resuspended in RPMI1640 medium containing 10% FBS.

### FoxP3 intracellular Staining

The spleen cells were initially stained with FITC-labeled anti-mouse CD4 antibody and PE/Cy5 anti-CD25 antibody, and then fixed in FoxP3 Fix/Perm solution (BioLegend); the cells were then stained with PE conjugated anti-mouse FoxP3 antibody (BioLegend). The fluorescence profile was analyzed by flow cytometry using FACSCalibur (BD Biosciences, San Jose, CA).

### *In vitro* stimulation for cytokine production

Spleen cells were seeded at $2\times10^6$ cells/ml (1 ml/well) and cultured in 24-well plates (Costar, NY, USA) in RPMI 1640 medium containing 10% (v/v) FBS (5% of the murine serum was used for IL-10 analysis), 2.0 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. TIL cells were cultured in 96-well culture plates (Costar) under the same conditions. Cells were stimulated with 1 µg/ml of anti-mouse CD3e (BD Pharmingen, San Jose, CA), 2 µg/ml anti-mouse CD28 (BD Pharmingen) and 1 µg/ml brefeldin A (Biolegend, San Diego, CA). The cells were incubated for 8 h at 37°C under a 5% $CO_2$/95% air atmosphere.

### Flow cytometric immunofluorescence analysis

The cell surface antigen and the intracellular cytokines were stained according to the formal Cell Surface Immunofluorescence Staining Protocol and Intracellular Cytokine Staining Protocol (BioLegend), as previously reported [6,11]. Briefly, for the detection of IFN-γ, IL-17, IL-10 and TNF-α, the cells were first stained with PE anti-mouse CD8 and PE/Cy5 anti-mouse CD3e antibodies (BioLegend). After treatment with the fixation buffer (BioLegend) and the permeabilization wash buffer (BioLegend), cells were stained with FITC-conjugated anti-mouse IFN-γ, IL-17A, IL-10 and TNF-α antibodies (BioLegend). For the characterization of monocytes, the cells were similarly stained with PE-conjugated anti-mouse CD14 monoclonal antibody, followed by intracellular cytokine staining. The fluorescence profiles were analyzed by flow cytometry using FACSCalibur.

### Statistical analyses

Statistical analysis was performed by using the Kruskal-Wallis nonparametric analysis of variance with *post hoc* analysis using the Dunn multiple comparison test. A *P*-value of less than 0.05 was considered to be statistically significant.

## Results

### Cutaneous manifestations ~Dramatic regression of the tumor by P. acnes treatment~

We assessed the tumor size 4 weeks after the injection of the B16 melanoma cells. PBS-treated control mice (MM) had developed

large tumors. The tumor size in the *P. acnes*-treated melanoma-bearing mice, PMM1 and PMM2, was significantly smaller than in the control mice (Fig. 1A, B).

## Histopathological findings

In the MM control mice, melanoma cells proliferated massively in the subcutaneous region and invaded the underlining muscular layers. Inflammatory cell infiltration was sparsely detected both inside and around the tumors (Fig. 1B). In contrast, histopathological study in PMM1 revealed subcutaneous granuloma formation with only a small number of melanoma cells around the muscular layer. In PMM2, there was enhanced granuloma formation and mononuclear cell infiltration compared with that detected in PMM1. Surprisingly, melanoma cells were almost undetectable in PMM2. No distant melanoma cell metastasis was detected.

## Flow cytometric analysis of spleen cells

The percentage of $CD14^+$ monocytes in the total number of spleen cells was significantly increased in PMM2 and PMM1 compared with the MM and control groups. This increase was also observed in the *P. acnes*-treated control mice (P1 and P2) (Fig. 2A).

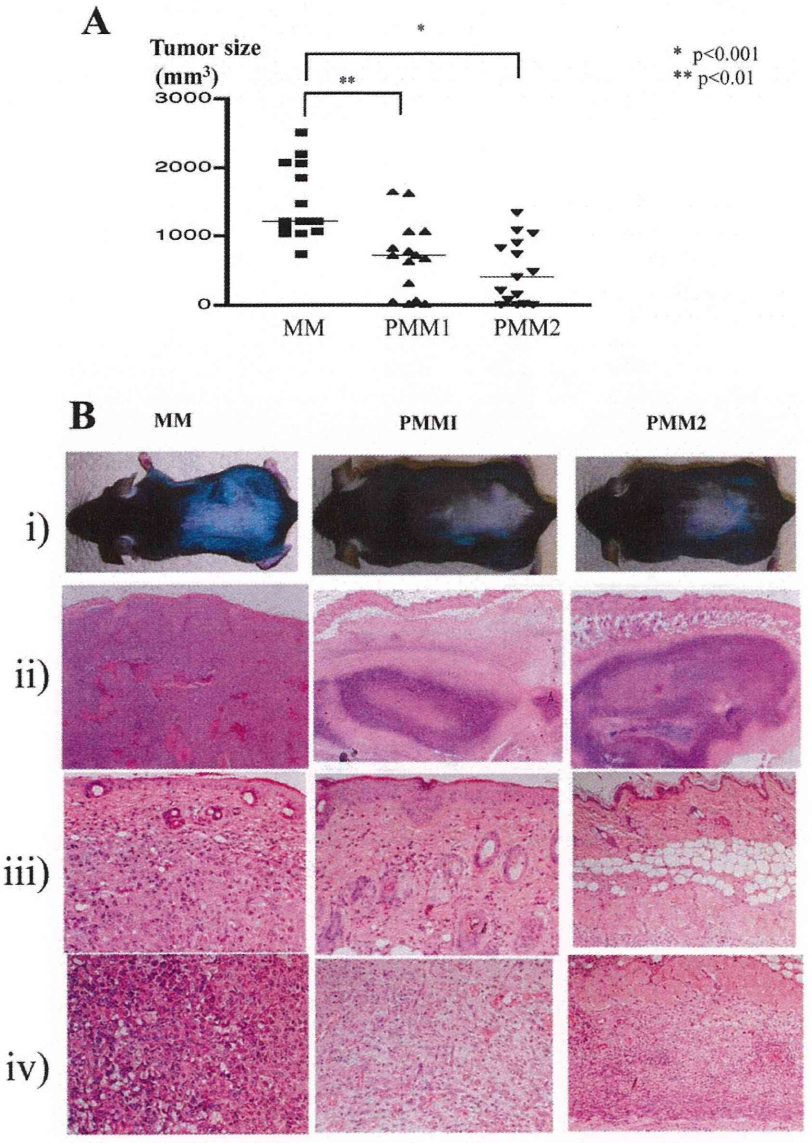


**Figure 1. Tumor size, clinical phenotype, and histological findings. A.** Tumor size at the end of observation period: 4 weeks after B16 melanoma implantation. PBS-treated control mice (MM) developed a large tumor. Treatment with *P. acnes* led to a significant decrease of tumor size in both PMM1 ($p<0.01$) and PMM2 ($p<0.001$). **B.** i) Clinical picture of the tumors implanted on the dorsal skin. ii) Massive melanoma cell proliferation in the subcutis with invasion of the surrounding muscular tissues in MM. By contrast, PPM1 and PMM2 displayed subcutaneous granuloma formation (×40). iii) Close up of the upper dermis (×200). iv) Close up of the muscular tissue levels (×200). In MM mice, melanoma cell growth was observed without inflammatory cell infiltration. In PMM1 mice, melanoma cells were detected around the muscular lesion, but melanoma cells were almost undetectable, and abundant mononuclear cell infiltration was found in the PMM2 mice.
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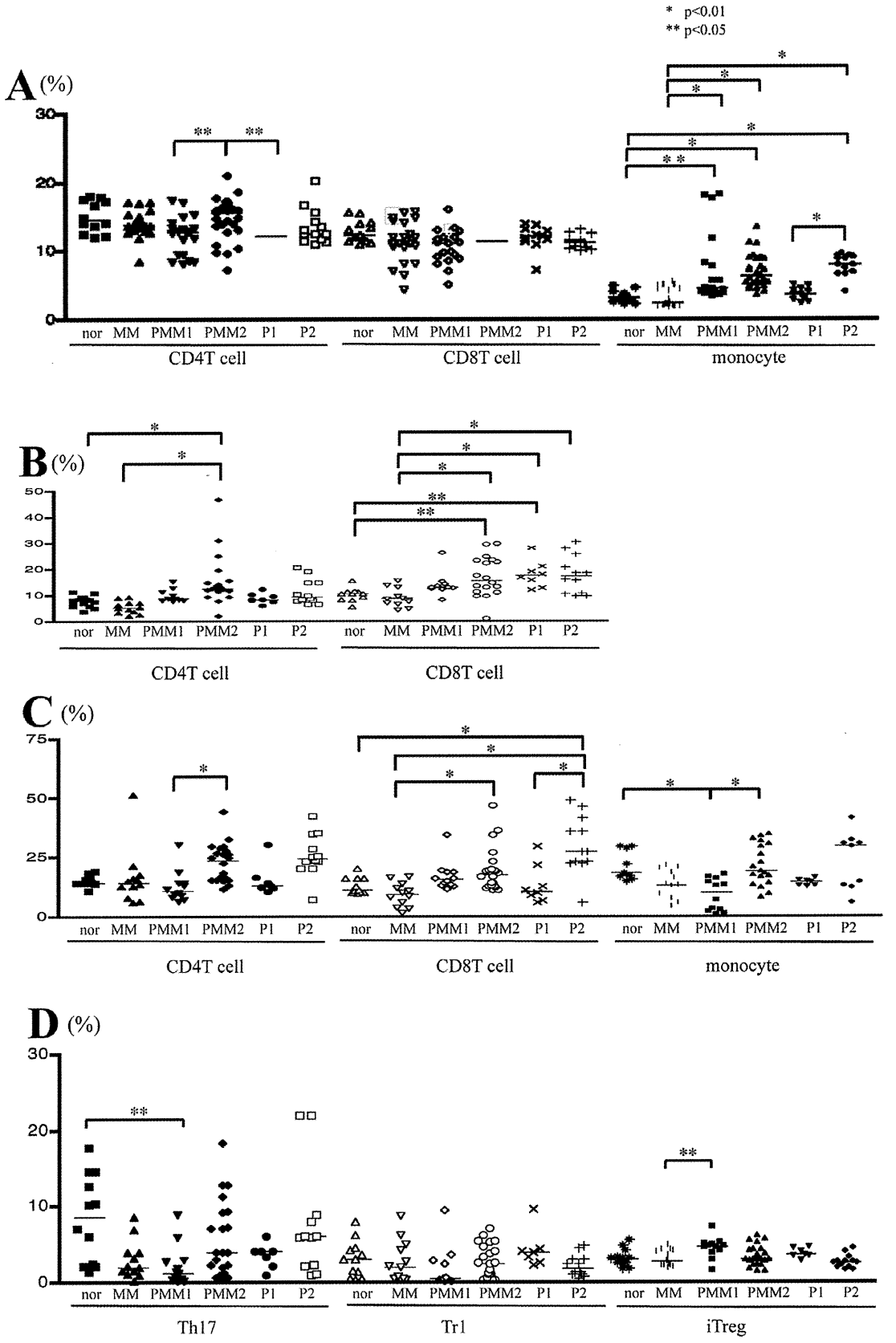
* p<0.01
** p<0.05
A (%)
B (%)
C (%)
D (%)
nor MM PMM1 PMM2 P1 P2
CD4T cell
CD8T cell
monocyte
Th17
Tr1
iTreg

**Figure 2. Intracellular cytokine and Foxp3 expression in spleen cells. A,** Phenotypical analysis: the ratios of the $CD4^+$ T cells to total lymphocytes, $CD8^+$ T cells to total lymphocytes and $CD14^+$ monocytes to total splenic mononuclear cells are shown. The percentage of $CD14^+$ monocytes was significantly increased in the PMM2, PMM1, and P2 groups compared to the MM and control groups, respectively. **B,** The percentage of IFN-$\gamma^+CD4^+$ and IFN-$\gamma^+CD8^+$ T cells among the total $CD4^+$ and $CD8^+$ T cells, respectively. In PMM2 mice, the percentage of both populations was significantly elevated compared to the MM and control mice. $CD8^+$IFN-$\gamma^+$ T cells were also increased in each of the P1 and P2 groups. **C,** The percentage of TNF-$\alpha^+CD4^+$, TNF-$\alpha^+CD8^+$ T cells and TNF-$\alpha^+$ monocytes among the total $CD4^+$, $CD8^+$ T cells, and monocytes, respectively. In PMM2 mice, the percentage of TNF-$\alpha^+CD8^+$ T cells was significantly elevated compared to MM. TNF-$\alpha^+CD8^+$ T cells were also increased in the P2 group. **D,** The percentages of Th17, Tr1 and iTreg in $CD4^+$ T cells. The percentages of IL-$17^+CD4^+$ (Th17) cells and IL-$10^+CD4^+$ (Tr1) cells among the total $CD4^+$ cells, and the ratios of the Foxp$3^+CD4^+CD25^{high}$ T cells (iTreg) to $CD4^+$ T cells, were unchanged by *P. acnes* treatment.
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The percentages of IFN-$\gamma^+CD4^+$ and IFN-$\gamma^+CD8^+$ T cells among the total number of $CD4^+$ and $CD8^+$ T cells were significantly elevated in PMM2 mice compared with MM and control mice (Fig. 2B). $CD8^+$IFN-$\gamma^+$ T cells were also increased in the P1 and P2 groups. Similar results were observed in TNF-$\alpha$ expressing $CD8^+$ T cells (Fig. 2C). The ratio of IL-$17^+CD4^+$ (Th17) cells and IL-$10^+CD4^+$ (Tr1) cells among the total number of $CD4^+$ cells remained unchanged. The ratio of Foxp$3^+CD4^+CD25^{high}$ T (iTreg) cells was also not changed by *P. acnes* treatment (Fig. 2D).

## Cytokine mRNA expression in skin lesions

Quantitative RT-PCR was performed to investigate the cytokine mRNA expression in the tumor lesions. The RNA expression of IFN-$\gamma$ T-bet, IL-12p35, TNF-$\alpha$ and MIP2 was significantly increased in PMM2 and P2 mice compared with the MM group. No significant change was found in the mRNA expression of IL-12p40, IL-17 or IL-10 among the six groups (Fig. 3).

## Characterization of tumor infiltrating lymphocytes

Single cell suspensions of TIL from PMM2 were analyzed. Abundant IFN-$\gamma^+CD8^+$ and IFN-$\gamma^+CD4^+$ cells were detected (Fig. 4). There was also an infiltration of TNF-$\alpha^+CD4^+$ and TNF-$\alpha^+CD8^+$ cells. On the other hand, Th17 and Tr1 cells were fewer in the TILs. The number of infiltrating cells was very limited in non-treated MM and could not be assessed.

# Discussion

In the present study, the effects of intra-tumor *P.acnes* vaccination (ITPV) on the growth of melanoma skin lesions was evaluated, and found that the growth of seeded melanoma cells was suppressed. ITPV successfully controlled melanoma progression *in vivo* by an induction of Th1 type cytokines, including TNF-$\alpha$ and IFN-$\gamma$ in both the skin and the systemic circulation. The clinical benefit of vaccination was associated with subcutaneous granuloma formation. Tumor cells were not detected in the granulomas. The measured tumor size was significantly decreased in the vaccine-treated group compared with the control groups. The tumor size may have been underestimated because of the granuloma volume. However, granuloma formation is an immunological event that is related to augmented phagocytic activity as well as cellular cytotoxic activity. Granuloma formation thus plays an important role in effective anti-tumor immunotherapy. In the present study, we found that ITPV promotes the activation of TNF-$\alpha$ and IFN-$\gamma$ producing cells in the skin tumor lesions. IL-12, TNF-$\alpha$ and IFN-$\gamma$ are known to be effective anti-tumor cytokines. However, individual cytokines are reported to exert only limited clinical effects, and thus they have been most commonly used in combination with chemotherapy. Unlike the *in vitro* study results, the effect of recombinant cytokine therapy *in vivo* is limited, in large part due to the very short biological half-life of recombinant cytokines. A branched-chain polyethylene glycol moiety attached interferon alfa-2a (peginterferon alfa-2a) has been used to prolong the biological half-life [12]. Previous studies have shown that *P. acnes* enhances the anti-tumor activity of monocytes/macrophages [13,14,15] and the tumoricidal function of NKT and NK against melanoma [15]. In ITPV, *P. acnes* is phagocytosed and processed by monocytes/macrophages, which are present inside and around the tumors, and the persistent secretion of cytokines and chemokines from these cells leads to granuloma formation.

IL-12 is an antitumor cytokine that activates NK and cytotoxic T cells, thereby promoting strong anti-tumor activity by inducing IFN-$\gamma$ [16,17]. In the present study, we found increased local expression of IL-12p35 in ITPV. IL-12 is a heterodimeric cytokine containing IL-12p35 and IL-12p40 that binds to a specific receptor. On the other hand, free IL-12p40 forms sulfide-linked homodimers that block IL-12 function both *in vitro* and *in vivo* [18]. Enhanced expression of IL-12p35, but not IL-12p40, is suggested to have the potential to exert favorable therapeutic effects against tumors.

*P. acnes* binds to TLR2 on monocytes and dendritic cells, leading to activation of the IL-12 promoter [19]. IL-12 activates STAT-4 and T-bet transcription factors in T cells and NK cells. T-bet binds to the IFN-$\gamma$ gene promoter and increases the production of IFN-$\gamma$ [7]. In this study we found that IFN-$\gamma$ induces cytotoxic effects by activating $CD8^+$ T cells, NK cells and B cells. It also induces chemokines, including CXCL9 (MIG) and CXCL10 (IP-10) that suppress vascular proliferation.

Recombinant IL-12 has been used as an anti-cancer therapy, but with unsuccessful results, eliciting systemic side effects and only limited clinical benefit. Local administration of IL-12 for therapeutic purposes has been suggested to improve the outcome in certain cancers. To limit the expression of IL-12 and prolong local IL-12 secretion, IL-12 plasmid vaccination has been administered for metastatic melanomas [20]. In this regard, ITPV has the advantage of persistently inducing IL-12 expression at the site of injection; this is followed by infiltration of TNF-$\alpha$ and IFN-$\gamma$ producing T cells into the lesions, resulting in tumor suppression.

Granuloma formation is a characteristic feature of *P. acnes* vaccination, with the accumulation of monocytes being required for this activity. Potent IFN-$\gamma$ expression occurs after *P. acnes* administration and leads to granuloma formation. Granuloma is a persistent source of Th1 type cytokines *in vivo*. An increase in MIP2 (CXCL2) was detected in *P. acnes*-treated skin lesions. This chemokine is secreted by monocytes and macrophages, and is chemotactic for polymorphonuclear leukocytes and hematopoietic stem cells. MIP2 is one of the chemokines involved in granuloma formation. On the other hand, granuloma formation and ulceration have been considered as side effects in systemic anti-cancer vaccine trials. Since melanoma is a cutaneous malignancy, no special technique is required for accurate intralesional administration of the vaccine. Accumulated phagocytes in the granuloma may additionally contribute to the effective removal of tumor cells in combination with cytotoxic lymphocytes. Based on these observations, granuloma or ulceration is still considered to be relevant to successful cutaneous tumor immunotherapy.

In addition to its local effects, ITPV may also exert systemic anti-tumor activity. After ITPV, TNF-$\alpha$ and IFN-$\gamma$ producing

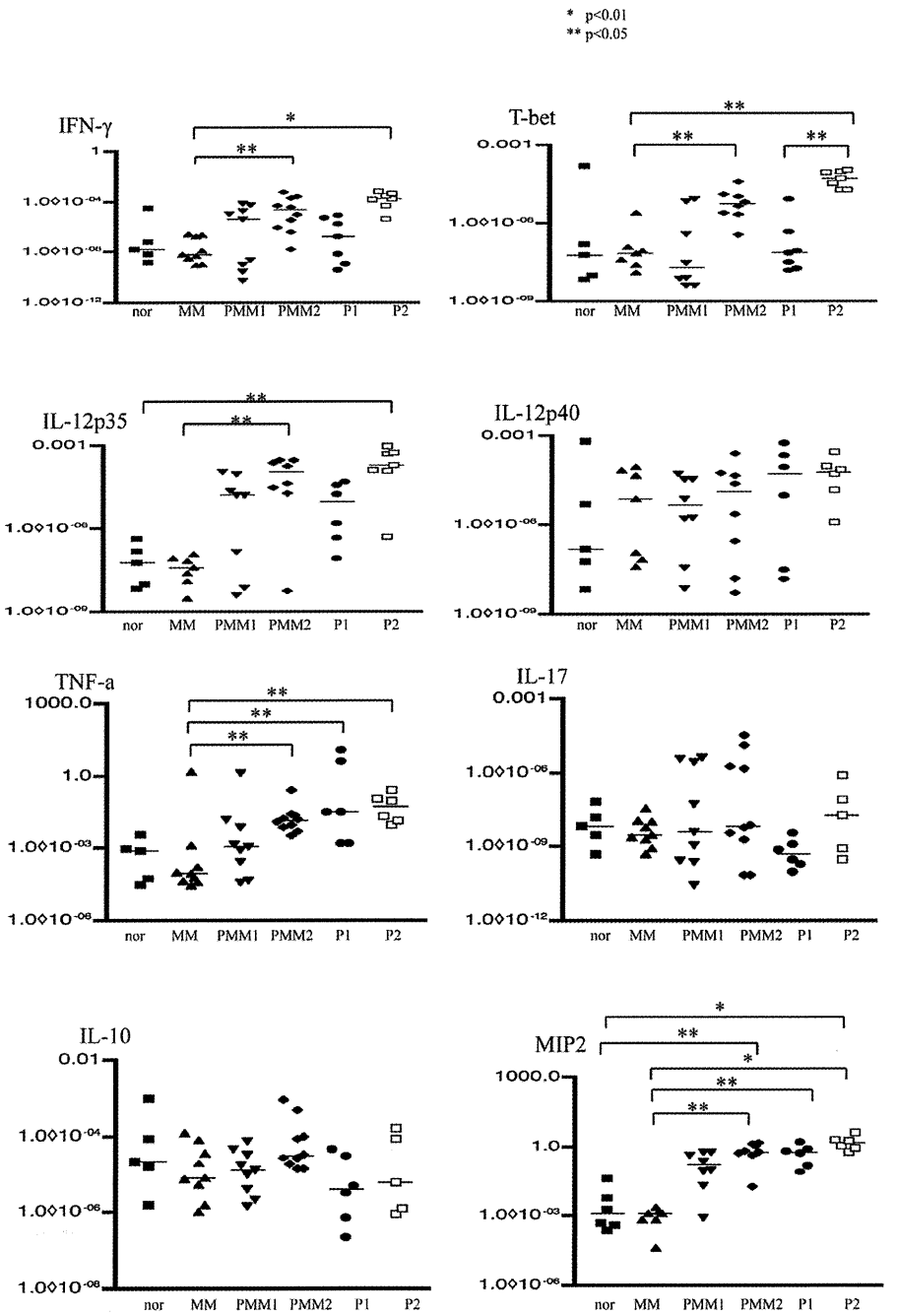


**Figure 3. Cytokine mRNA expressions in the skin lesions.** Quantitative RT-PCR was performed to determine the cytokine mRNA expression in the tumor lesions. The IFN-γ T-bet, IL-12p35, TNF-α and MIP2 mRNA levels in PMM2 and P2 mice were significantly increased compared to MM mice. No significant change was found in the IL-12p40, IL-17 or IL-10 mRNA levels among the six groups of mice.
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$CD8^+$ T cells were increased in the spleen and skin. However, melanoma-specific cytotoxicity of CD8 T cells was not increased in the spleen or draining lymph nodes in *P. acnes* injected melanoma-bearing mice (Fig. S1), suggesting that most of the cytotoxic CD8 T cells may be recruited into the injected skin lesions. Consistent with the previous reports [21,22], the number of splenic monocytes was also increased by ITPV. The role of the systemic immune response in the mechanism of distant metastasis remains unclear. Previous studies have suggested that augmentation of anti-tumor cytokine expression in spleen cells have preventive effects against distant metastasis.

In the present study, mice received either a single dose or two doses of vaccination. We injected *P. acnes* on day 0 and/or on day 14 into the cutaneous tumor lesions. Even the single therapy on

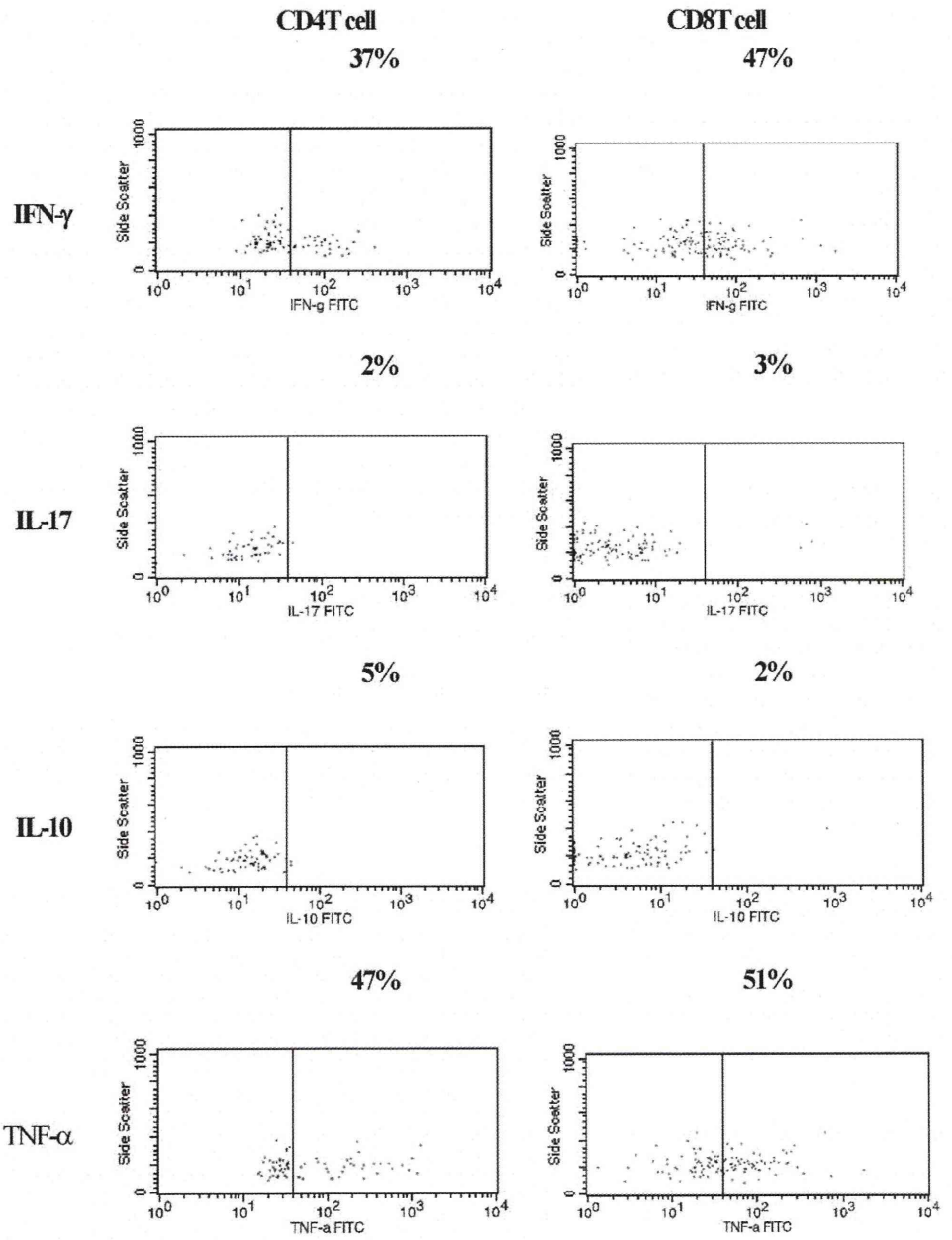


**Figure 4. Characterization of the tumor infiltrating lymphocytes.** One half of tumor infiltrating T cells from PMM2 tumor lesion were TNF-α producing cells. IFN-$\gamma^+CD4^+$ and IFN-$\gamma^+CD8^+$ T cells were also present in the tumor. By contrast, very few Th17 cells or Tr1 cells were detected in TILs. The number of infiltrating cells in the non-treated melanoma tumors was very limited, and thus was not analyzable. Representative results from five experiments are shown.
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day14 significantly suppressed the growth of melanoma cells. However, priming on day 0 followed by a second vaccination on day 14 resulted in a more potent growth inhibitory activity compared to the single vaccination. Early intervention before full tumor development may have the advantage of inducing enhanced Th1 type anti-tumor activity compared to vaccination after established melanoma growth. A second vaccination induced a booster effect on the activation of the cytokine network.

Th17, Tr1 and iTreg cells play critical roles in the regulation of the immune system. In the present study, *P. acnes* vaccination shifted the Th1/Th2 balance toward a dominant Th1 immune response. Th17 is involved in Th1-associated diseases such as psoriasis. However, we found no changes in the protein or RNA expression of IL-17 in the present immunotherapy, as IL-17 was undetectable in TIL. Therefore, it is unlikely that Th17 was involved in the beneficial effect of *P. acnes* vaccination therapy.

Tr1 and iTreg cells are known to regulate the inflammatory response. Tr1 regulatory cells produce IL-10 and play a critical role in the suppression of allergic diseases [11,23]. It was reported that *P. acnes* therapy increases iTreg cells by stimulating TLR2 in

Th2-mediated diseases [19,24]. By contrast, suppression of iTreg cells has been associated with successful cancer immunotherapy [25]. Interestingly, neither Tr1 cells nor iTreg cells were elevated in the present study, suggesting that they are not involved in the mechanistic effect of this therapy. Unlike allergic mice, which have a Th2 dominant response, cancer-bearing mice may have different immunological backgrounds in response to *P. acnes* vaccination. Further investigation is required to clarify the precise mechanism of the *P. acnes* mediated immune responses.

In conclusion, the results of this study showed that ITPV successfully suppresses MM, and that the beneficial effect of this therapy depends on the induction of granuloma formation along with the secretion of IL-12, IFN-γ and TNF-α. Further investigation is required before this treatment comes into use in clinical practice. *P. acnes* vaccine is a promising candidate as an adjuvant therapy of melanoma.

## Supporting Information

**Figure S1 The cytotoxicity of $CD8^+$ T cells prepared from spleen or draining lymph node was analyzed using three methods.** The first method is chromium release assay, and the second is viability detection by flow cytometry using Live/Dead cell-mediated cytotoxicity kit (Molecular probes, Carlsbad, CA). Finally DHL cell cytotoxicity assay kit (AnaSpec Corporate Headquarter, San Jose, CA) was used to detect the release of Lactate Dehydrogenase (LDH) from targeted melanoma cells. Spleen and draining lymph node samples were taken from melanoma and *P. acnes*-injected mice: melanoma cell was free in the dorsal skin, *P. acnes* only injected mice, and normal control mice. Single cell suspensions were prepared by mechanical mincing, and after passing through a 70-μm-pore mesh, the cells are washed and resuspended in PBS. After Ficoll separation, the cells were washed and resuspended in RPMI1640 medium containing 10% FBS. CD8 T cells were purified using magnetic beads, and co-cultured with B16 melanoma cells at three different effector cell/target cell ratio (12.5:1, 25:1, 50:1) according to previous reports. Chromium release assay **A,** 6 hours incubation LN CD8 T cells. **B,** 6 hours incubation splenic CD8 T cells. **C,** 15 hours incubation LN CD8 T cells. **D,** 15 hours incubation splenic CD8 T cells. When there was injury of targeted melanoma cells, chromium was released. Analysis of apoptotic melanocytes using live/dead viability detection system by flow cytometry **E,** 8 hours incubation LN CD8 T cells. **F,** 8 hours incubation splenic CD8 T cells. Analysis of lactate dehydrogenase (LDH) released from targeted melanoma cells **G,** 8 hours incubation LN CD8 T cells **H,** 8 hours incubation splenic CD8 T cells. Melanoma and *P. acnes*-injected mice: ●, *P. acnes* only injected mice: ■, and normal control mice: ▲. CD8 T cells melanoma-specific cytotoxicity was not increased in *P. acnes* injected melanoma-bearing mice, suggesting that most cytotoxic CD8 T cells was recruited into the injected skin lesions.
(TIFF)

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## Author Contributions

Conceived and designed the experiments: KY HM. Performed the experiments: KT WL YM TA TN HK. Analyzed the data: MK IK. Contributed reagents/materials/analysis tools: KT WL YM TA TN HK. Wrote the paper: KY ECG HS HM.

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Cancer Research

# Intracellular Tumor-Associated Antigens Represent Effective Targets for Passive Immunotherapy

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