Induction of WT1 specific human CD8+ T cells from human HSCs in HLA class I Tg NOD/SCID/Il2rgKO mice

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Induction of WT1 specific human CD8+ T cells from human HSCs in HLA class I Tg NOD/SCID/Il2rgKO mice

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Key points
1. Vaccination using WT1 peptides and DCs induced WT1-specific human CTLs in vivo in HLA class I Tg NSG mice.
2. Human HSCs transduced with human TCR generated HLA class I-restricted, WT1-specific CTLs in vivo.

Abstract
Induction of specific immune response against therapy-resistant tumor cells can potentially improve clinical outcomes in malignancies. To optimize immune-therapy in the clinic, we aim to create an in vivo model enabling us to analyze human CTL responses against human malignancies. To this end, we developed NOD/SCID/Il2rgKO (NSG) mice expressing HLA class I molecules HLA-A*0201 and A*2402. In the BM and spleen of HLA class I Tg NSG mice transplanted with cord blood (CB) hematopoietic stem cells (HSCs), we found human memory CD8+ T cells and APCs. To evaluate antigen-specific human CTL responses, we immunized HLA class I Tg NSG using poly(I:C) mixed Wilms' Tumor 1 (WT1) peptides, with or without WT1 peptide-loaded autologous DCs. After immunization, the frequencies of HLA-restricted WT1-specific CTLs increased significantly in the spleen. Next we transplanted the WT1-specific T cell receptor (WT1-TCR) gene transduced human HSCs into HLA class I Tg NSG newborns. WT1 tetramer+CD8+T cells differentiated from WT1-TCR transduced HSCs in the recipient BM, spleen, and thymus. Upon stimulation with WT1 peptide in vitro, these CTLs produced IFN-γ and showed lytic activity against leukemia cells in antigen-specific, HLA-restricted manner. HLA class I Tg NSG xenografts may serve as a pre-clinical model to develop effective immune-therapy against human malignancies.
Introduction

The immune system prevents infectious disease initiation and progression and functions in multiple homeostatic processes. However, dysfunctional immunity is observed in patients with malignancies contributing to neoplastic progression. Therefore, reconstitution of immunity by allogeneic stem cell transplantation or activation of specific and non-specific immunity targeting diseases improves clinical outcomes in patients with solid cancers and those with hematologic malignancies. Such treatment can be carried out by vaccination and by adoptive immunotherapy.

Vaccinations aim to elicit antigen-specific effector cell-mediated immune responses in vivo. Among several candidates, peptide vaccines and DC vaccines were two widely selected protocols. In the last two decades, however, administration of these vaccines has not significantly improved the prognosis of patients with solid cancers including melanoma and other types of solid tumors. Although several recent trials reported encouraging clinical outcomes using gp100 peptides in combination with IL-2 for melanoma or patient-derived APCs (Sipuleucel-T) for prostate cancer, cancer vaccination appears to require modifications based on increased understanding of in vivo biology of human APCs and T cells.

In contrast, immunotherapy based on adoptive transfer of ex vivo expanded tumor reactive T cell has achieved promising results. In metastatic melanoma, adoptive transfer of tumor infiltrating lymphocytes (TILs) in combination with chemotherapy or irradiation has improved cure rates up to 20 - 40%. Because anti-tumor effect of TILs has not been confirmed in malignancies other than melanoma, genetically engineered T cells which express tumor-antigen specific TCR genes or chimeric antigen receptors (CARs) have been developed. Recent clinical trials showed improved clinical outcomes in patients treated with genetically engineered T cells while adverse effects were observed immediately after the transfusion of T cells expressing CAR.
In several clinical trials of vaccination therapies for hematologic malignancies, promising responses were observed using various antigens including proteinase 3 (PR3)\textsuperscript{16} and WT1\textsuperscript{16,17} for AML, BCR-ABL for chronic myelogenous leukemia\textsuperscript{18} as well as patient-specific idiotypes derived from malignant B cell clones for follicular lymphoma\textsuperscript{19}. In particular, for AML with poor prognostic factors, development of immune-therapy targeting minimal residual disease or leukemia stem cells (LSCs) should play an essential role in achieving long-term patient survival.

We recently reported that WT1, a transcription factor expressed in variety of malignant tissues, is highly expressed by CD34+CD38- AML cells\textsuperscript{20}. WT1 is considered as one of the best antigens to be used for immune-therapy against malignancies based on the multiple criteria such as therapeutic function, immunogenicity and specificity\textsuperscript{21}. Using WT1 peptide or full-length mRNA for WT1, clinical trials against hematological malignancies detected increased frequencies of WT1-specific CD8+ T cells in the patient blood after the treatment\textsuperscript{16,17,22,23}. Nevertheless, to accomplish significant improvement in clinical outcomes of AML patients, we need to better understand the biology of human immune system leading to efficient activation of human acquired immunity against tumor antigens.

In the present study, we aimed to develop an in vivo system for induction of antigen specific, HLA-restricted human CD8+ T cells following vaccination. HLA class I expressing NSG mice supported the development of human T cells and APCs following engraftment with human CB HSCs. We detected high frequencies of WT1 specific CD8+ T cells in the BM and spleen of HLA class I expressing NSG mice following vaccination. Moreover, we confirmed the development of WT1 specific CD8+ T cells in vivo following engraftment human HSCs transduced with WT1 specific TCR V\textalpha and V\textbeta genes. The antigen specific human CD8+ T cells expanded in response to WT1 antigen and were functional both in cytokine production and cytotoxicity. Development of immunotherapy protocols in HLA class I expressing NSG mice may facilitate development and optimization of antigen-specific immune-therapy against
malignancies.
Materials and Methods

Detailed experimental methods are described in a supplementary file. Work using human cord blood was approved by RIKEN IRB.

Flow cytometry

For phenotypical analysis, cells labeled with the monoclonal antibodies (mAbs) (Table S1) were analyzed using FACSaria and FACSCanto-II (BD Biosciences).

Immunization of humanized mice

In the DC vaccine group, HSCs reconstituted HLA class I Tg NSG recipients were injected with WT1 peptide-loaded DCs via retro-orbital plexus on day 0, followed by intraperitoneal and subcutaneous immunization with 300 μg WT1_{126-134} or WT1_{235-243} synthetic peptide mixed with 150 μg polyinosinic:polycytidylic acid (poly I:C, InvivoGen) on days 0, 10 and 20. In the vaccine group without DC, the same doses of WT1_{126-134} or WT1_{235-243} synthetic peptide mixed with poly I:C were administered intraperitoneally and subcutaneously on days 0, 10 and 20. One week after the last immunization, mice were necropsied for the evaluation of human immune cells in peripheral blood (PB), BM, spleen, lymph nodes and thymuses.

In vitro expansion of human CD8+ T cells derived from mice engrafted with human HSCs

Human CTLs were expanded from HLA Class I Tg NSG humanized mice as described previously with some modifications. Splenocytes, thymocytes and BM cells from recipient mice were stimulated with irradiated autologous LCLs pulsed with 40 μg/ml WT1_{126-134} or WT1_{235-243} synthetic peptide at responder to stimulator ratio of 1:1.
Lentiviral transduction

Purified CB CD34+CD38- cells were incubated with concentrated lentiviral supernatant at a
MOI of 100 to 200. After 5 days of incubation, GFP+ cells were sorted using FACS Aria (BD biosciences) and transplanted into irradiated HLA Class I Tg NSG newborns.

ELISPOT assay

WT1-specific T cell responses were quantified by the IFN-γ ELISPOT assay as described previously. Pictures of each well were captured using AlphalMager Image Analysis System (Alpha Innotech) and individual spots were counted per well. Using the same protocol for IFN-γ assay, production of human TNFα by human WT1-specific CD8+ T cells was also confirmed by ELISPOT assay (Mabtech).

51Cr release assay

Cytotoxic activity of WT1 specific CTLs was evaluated by 51Cr release assay as described previously. To examine the HLA restricted cytotoxicity, target cells were incubated with anti-HLA class I framework mAb (w6/32; American Type Culture Collection) or anti-HLA-DR mAb (L243; American Type Culture Collection) at 10 μg/ml for 1 hour, followed by adding the effector cells. The percentage of specific lysis was calculated as (experimental release cpm – spontaneous release cpm) / (maximal release cpm – spontaneous release cpm) x 100 (%).

In vivo cytotoxicity assay

HLA A24-expressing K562 cells were cultured with or without WT1-specific CTLs for five hours. After harvesting all the cultured cells, the cells were inoculated subcutaneously into female NSG mice. WT1-specific CTLs were adoptively transferred weekly. At 24 - 28 days post-inoculation, the mice were sacrificed to evaluate tumor size and weight.

Analysis of TCR repertoire of CTLs

Following in vitro expansion, WT1 specific CTLs were purified by FACS. As a control, 2.4 x
$10^4 - 1.5 \times 10^5$ hCD45+CD3+CD8+ cells were purified from spleens of NSG mice engrafted with non-transduced human HSCs. Total RNA was extracted using TRIzol reagent (Invitrogen) and reverse transcribed for synthesis of first strand cDNA using SMARTer RACE cDNA amplification kit (Clontech). Both universal mix primer and primers specific for the T-cell receptor $\alpha$ (TCR$\alpha$) or $\beta$ (TCR$\beta$) constant region sequence were used for 5′-end specific PCR amplifications, resulting in both TCR$\alpha$ and TCR$\beta$ PCR products of high purity which were then sequenced on a Miseq (Illumina). All reads of both the TCR$\alpha$ and the TCR$\beta$ repertoire sequence were analyzed using Perl scripts based on the USEARCH algorithm (http://drive5.com/usearch/). V-region consensus sequences in each cluster were searched on the ImMunoGeneTics (IMGT) sites (www.imgt.org/IMGT_vquest/share/textes/).
Results

Human CB HSCs generate both human T cells and APCs in HLA Class I Tg NSG mice

We developed immunodeficient NSG mice expressing HLA class I antigens by backcrossing HLA-A*0201 or HLA-A*2402 transgenes onto the NSG background. Both transgenes also encode covalently bound human β2m. By RT-PCR, HLA transgenes were detected in the BM, spleen and CD45-EpCAM+ TECs derived from both NSG-HLA-A2/HHD mice and NSG-HLA-A24/HHD mice (Figure 1A). We then transplanted 4.5 x 10^3 – 5.6 x 10^4 purified human HLA matched CD3-CD4-CD8-CD34+CD38- CB HSCs into HLA Class I Tg NSG newborns. At 15 - 37 weeks post-transplantation, we confirmed engraftment of human CD45+ leukocytes in the PB, BM, spleen, mesenteric and axillary lymph nodes (MLN and AxLN), and thymus (Figure 1B, 1C, Table 1, n = 11 for PB 72.7 +/- 6.9%, spleen 91.5 +/- 2.0%, BM 72.4 +/- 9.2%, MLN 97.3 +/- 0.7% and Thymus 97.6 +/- 1.2%; n = 10 for AxLN 96.7 +/- 1.4%). Human CD3+ T cells were 65.9 +/- 7.2% of engrafted hCD45+ leukocytes in the recipient spleen. CD4+CD8+ double positive (DP) T cells accounted for 34.2 +/- 12.5% in the thymus (n = 6), while CD4+ or CD8+ single positive (SP) T cells were 26.2 +/- 5.9% or 31.7 +/- 11.3% of engrafted human CD3+ T cells. Within the human CD8+ fraction, we identified CD45RA+CCR7+ naïve, CD45RA-CCR7+ central memory (CM), CD45RA-CCR7- effector memory (EM) and CD45RA+CCR7- terminally differentiated (EMRA) T cell subsets in the recipient spleen (Figure 1D and 1E, naïve 20.8 +/- 10.0%, CM 13.7 +/- 4.9%, EM 60.9 +/- 10.3% and EMRA 4.9 +/- 1.8%, n = 10). Cytoplasmic expression of Granzyme A was the highest in EM CD8+ T cells (Figure 1F and S1, naïve 10.0 +/- 2.1%, CM 42.0 +/- 8.9%, EM: 75.4 +/- 10.7%, EMRA: 55.1 +/- 10.3%, n = 4).

To elucidate whether human APCs developed in the HLA class I Tg NSG mice, we analyzed the expression of CD33 and other myeloid-associated antigens using flow cytometry. In the NSG-HLA-A2/HHD mice (n = 4) and NSG-HLA-A24/HHD mice (n = 6), we found that CD14+CD33+HLA-DR+ monocytes, CD123highCD11c- plasmacytoid DCs (pDCs)
and CD33+CD11c+HLA-DR+ conventional DCs (cDCs) differentiated in the BM and spleen
of HLA class I Tg NSG recipients (Figure 1G and 1H, frequency out of human CD45+ cells:
BM, n = 8, monocyte 3.4 +/- 0.8%, pDC 2.6 +/- 0.7%, cDC 5.2 +/- 1.6%; spleen, n = 7,
monocyte 0.9 +/- 0.5%, pDC 0.4 +/- 0.2%, cDC 1.0 +/- 0.4%). BDCA1+DC and BDCA3+DC
were detected within cDC fraction (Figure 1I, frequency out of human CD33+ cells: BM, n = 8,
BDCA1+ DC 9.2 +/- 4.7%, BDCA3+ DC 1.0 +/- 0.3%; spleen, n = 7, BDCA1+ DC 7.6 +/- 4.4%,
BDCA3+ DC 0.9 +/- 0.2%). Consequently, subsets of human CTLs and human myeloid APCs,
required for the establishment of acquired immunity following vaccination, were present for
long-term in HLA class I Tg NSG recipients.

WT1 is expressed in the CD34+CD38- leukemic stem cell fraction of human AML

Based on the previous report showing that WT1 is highly expressed in AML stem cells 20, we
examined the expression levels of WT1 in the purified AML CD34+CD38- cells and normal
BM CD34+CD38- cells using qRT-PCR. Expression of WT1 in the CD34+CD38- stem cell
fraction was higher in AML samples compared to BM samples from healthy donors (Figure
S2 A, relative mRNA expression of WT1 corrected by GAPDH: Healthy donors 2.84 +/- 2.02 x
10^-4, n = 7; AML patients 1.07 +/- 0.25 x 10^-2, n = 38, *p = 0.0002 by two-tailed t test). Western
blotting analysis confirmed the expression of WT1 in several leukemia cell lines and
CD34+CD38- cells from AML patient samples at the protein level (Figure S2B, upper and
lower).

Immunization induced WT1 specific human CTLs in the HLA class I Tg NSG recipients

We then performed immunization experiments to evaluate the induction of antigen-specific
human CTLs in the HLA class I Tg NSG mice. To do so, we chose WT1 as an antigen and
poly I:C as an adjuvant. In addition, we prepared autologous or HLA class I matched
allogeneic WT1 peptide-loaded DCs. In immunized HLA class I Tg recipients (Table 1), we
found significant increase of WT1 specific tetramer+ cells in the recipient PB following
immunization (Figure 2A and 2B, n = 7, 0.044 +/- 0.017% and 0.399 +/- 0.110% for before
and after immunization, respectively, *p < 0.05 by two-tailed paired t test). Compared with
unvaccinated mice, the frequencies of WT1 tetramer+ CTLs in spleen were increased
significantly in the recipients of the vaccine groups both with and without WT1 peptide-loaded
DCs (Figure 2C and 2D, no immunization control, 0.041 +/- 0.007%, n = 10; immunized
without DC, 0.170 +/- 0.043%, n = 10, *p = 0.0146; immunized with DC, 0.230 +/- 0.044%, n =
9, **p = 0.0027). In the recipients vaccinated with WT1 peptide-loaded DCs, WT1 specific
CTLs were also increased in the PB, BM and MLN, whereas the increases in these organs
were not significant in the group immunized without DCs (Figure 2D, WT1 tetramer+ / CD8+
T cells (%): PB, no immunization control, 0.038 +/- 0.014%, n = 10; immunized without DC,
0.072 +/- 0.024%, n = 7; immunized with DC, 0.361 +/- 0.088%, n = 9, **p = 0.0061; BM, no
immunization control, 0.090 +/- 0.043%, n = 7; immunized without DC, 0.429 +/- 0.136%, n =
5; immunized with DC, 0.658 +/- 0.203%, n = 5, *p = 0.0473; MLN no immunization control,
0.013 +/- 0.006%, n = 6; immunized without DC, 0.071 +/- 0.032%, n = 6; immunized with DC,
0.146 +/- 0.030%, n = 7, **p = 0.0040).
To evaluate the antigen-specific proliferation of CTLs, 2 - 10 x 10^6 splenocytes of recipients
were further stimulated with WT1 peptide-pulsed autologous LCLs in vitro. After 4 weeks, the
frequency of WT1 tetramer+ CTLs derived from splenocytes was higher in the vaccinated
recipients than in the unvaccinated mice (Figure 2E and 2F, WT1 tetramer+ / CD8+ (%)
before and after in vitro expansion: No immunization group, 0.050 +/- 0.006% and 0.168 +/-
0.042%, n = 3; immunized group, 0.194 +/- 0.032% and 0.511 +/- 0.095%, n = 12; **p =
0.0057 for no immunization vs immunized after expansion). The degree of increase in WT1
specific CTL cell numbers after expansion was also higher in the immunized recipients
(Figure 2G, fold increase of WT1 tetramer+ CD8+ cell counts before and after in vitro
expansion: No immunization group 4.06 +/- 2.34, n = 3; immunized group 42.13 +/- 12.1, n =
Transplantation of WT1 specific TCR transduced HSCs into NSG-HLA-A24/HHD reconstituted WT1 specific CTL in vivo

As another way of inducing antigen-specific human CTLs in vivo, we evaluated differentiation of human T cells from antigen-specific TCR transduced HSCs. We constructed a lentiviral vector encoding WT1 specific TCR Vα and Vβ genes. We transduced the TCR Vα and Vβ genes into purified human CB HSCs using the lentiviral vector (Figure 3A, Figure S4A), and purified CD34+GFP+ cells by FACS. Both HLA-matched and -mismatched CBs were used as HSC sources (Table 2). Then we transplanted GFP-labeled gene-transduced CD34+ cells into NSG-HLA-A24/HHD newborns. After 4 - 6 months, multi-lineage human hematopoietic cells developed (Figure 3B and 3C, Figure S4B: Spleen, n = 7 for non-transduced and GFP control recipients, B cells 56.9 +/- 12.2%, T cells 34.4 +/- 13.1%; n = 15 for WT1 TCR gene transduced HSC recipients, B cells 71.5 +/- 4.3%, T cells 9.8 +/- 4.0%. Thymus, n = 6 for recipients of non-transduced or control vector transduced HSCs, B cells 12.2 +/- 4.9%, T cells 53.1 +/- 9.2%; n = 15 for WT1 TCR gene transduced HSC recipients, B cells 5.2 +/- 2.3%, T cells 28.0 +/- 6.0%). CD4+ and CD8+ T cells were identified in the spleen of control NSG recipients and NSG recipient transplanted with WT1 TCR-transduced HSCs. In the recipient thymus, double positive T cells as well as single positive T cells were found in both recipients (Figure 3D). Engraftment levels of human CD45+ cells were not statistically different between control mice and TCR transduced mice (Table 2).

At the day of sacrifice, WT1 tetramer+ CD8+ cells were present at frequencies of 0.316 +/- 0.070 % (n=9), 1.158 +/- 0.513 % (n=11) and 0.565 +/- 0.200 % (n=3) in the spleen, thymus and BM of WT1 TCR transduced HSC recipients, respectively (Figure 4A and 4B).

Human CTLs developed in the NSG-HLA-A24/HHD mice were stimulated in vitro using HLA-A*2402(+) WT1 peptide–pulsed LCLs for further expansion (FigureS5). When
HLA-A*2402-mismatched CB HSCs were transplanted into NSG-HLA-A24/HHD newborns. WT1 peptide-pulsed, HLA-A*2402-matched allogeneic CB LCLs were used as stimulators to expand WT1-specific CTLs in vitro. After 2 - 3 weeks, the frequency of WT1 tetramer+ CTLs derived from splenocytes of the recipients was significantly increased (Figure 5A and 5B, WT1 tetramer+CD8+ T cells (%): BM, n = 5, before 0.626 +/- 0.140%, after 11.57 +/- 10.23%, p = 0.3880; spleen, n = 9, before 0.316 +/- 0.070%, after 10.12 +/- 7.781%, *p = 0.0197; thymus, n = 4, before 1.533 +/- 1.282%, after 23.95 +/- 14.10%, p = 0.0561). The cell numbers of WT1 tetramer+ CTLs derived from BM, spleen and thymus were significantly increased after in vitro expansion (Figure 5C, cell numbers of WT1 tetramer+CD8+ T cells: BM, n=5, before 8.1 x 10^1 +/- 4.5 x 10^1, after 1.5 x 10^6 +/- 1.5 x 10^6, *p = 0.0253; spleen, n=9, before 2.7 x 10^2 +/- 1.1 x 10^2, after 9.4 x 10^5 +/- 8.9 x 10^5, **p = 0.0013; thymus, n=4, before 4.0 x 10^2 +/- 2.4 x 10^2, after 1.9 x 10^6 +/- 1.8 x 10^6, *p = 0.0102). To elucidate whether the GFP+ WT1 tetramer+ CD8+ T cells retain specific TCRs, we performed a repertoire analysis of TCR using a Miseq. The result of repertoire sequences demonstrated that FACS-purified GFP+ WT1 tetramer+ CD8+ T cells displayed skewing of both Vα and Vβ repertoires and that the majority of Vα and Vβ chains of the T cells were the transduced Vα and Vβ chains (Figure 6). After the expansion of WT1 specific CTLs for 4 - 5 weeks, we found that the majority of GFP+CD8+ T cells were CD45RA(+)CCR7(-) effector T cells (Figure S6). We also evaluated the antigen-specific cytokine production by the CTLs with ELISPot assay. Recipient-derived WT1-specific CTLs produced IFN-γ against WT1 peptide-pulsed but not peptide-unpulsed HLA-A*2402(+) LCLs. The addition of anti-HLA class I antibody significantly reduced the number of IFN-γ producing cells against WT1 peptide-pulsed LCLs (Figure 5D and 5E). WT1-specific CTLs also produced TNF-α (Figure S7). Using 51Cr release assay, we evaluated cytotoxicity by the amplified CTLs derived from a WT1 TCR transduced NSG-HLA-A24/HHD recipient BM (A24-TCR-2) (Figure 5F and G). Since this donor CB sample used for the transplantation of WT1 TCR transduced HSC was
HLA-A*2402-mismatched (Table 2, CB23-1), allogeneic HLA-A*2402-matched LCLs were used for in vitro stimulation. Cytotoxic activities were observed against allogeneic WT1 peptide-pulsed HLA-A*2402-matched LCLs (Figure 5F, LCL#1, derived from CB23-3), but not against peptide-unpulsed LCL#1 and autologous HLA-A*2402-mismatched LCLs (Figure 6F upper, LCL#2, derived from CB23-1) with or without peptide-loading. WT1(+) HLA-A*2402(+) KAZZ leukemia cell lines were lysed by these CTLs. While HLA-A*2402(-) K562 leukemia cell lines were not lysed, HLA-A*2402 gene transduced K562 cells became susceptible to lysis by these CTLs (Figure 5G left). The cytotoxic activities of CTLs against WT1 peptide-pulsed LCL#1 and HLA-A*2402 expressing leukemic cell lines were inhibited by anti-HLA class I mAb, not by anti-HLA class II mAb (Figure 5F lower and Figure 5G right). We further aimed to determine the effect of WT1-specific CTLs on the in vivo growth of leukemic cells. To this end, we used as effector cells WT1-specific CTLs obtained from spleens of NSG-HLA-A24/HHD recipients engrafted with human HSCs transduced with WT1-specific TCR genes. HLA A24 expressing K562 cells were used as target cells. We cultured the target K562 cells with WT1-specific CTLs ex vivo then inoculated the cultured K562 cells subcutaneously into NSG mice. At 3-7 days after three weekly transfers of WT1-specific CTLs, we found inhibition of subcutaneous tumor formation (Table 3, Figure S8). These results indicate that WT1 TCR transduced human HSCs can induce specific and functional CTLs as well as multi-lineage immune subsets in HLA Class I Tg NSG recipients.
Discussion

Evoking immune responses against specific antigens requires HLA-restricted interaction between APCs and effector cells. In this study, we examined whether active immunization with vaccines can prime HLA-restricted antigen-specific human CTLs in mice expressing HLA Class I molecules HLA-A*0201 or HLA-A*2402, the most common alleles in Caucasian or Japanese populations, respectively. First, we confirmed HLA class I expression in murine TECs from the previously reported NSG-HLA-A2/HHD as well as from the newly developed NSG-HLA-A24/HHD strains. In HLA class I Tg NSG mice transplanted with HLA class I matched CB-HSCs, human memory T cells expressing cytotoxic molecules and human HLA-DR positive antigen-presenting myeloid cells including BDCA1+ or BDCA3+ cDCs were detected in the recipient BM and spleen. Following vaccination using adjuvant mixed 9-mer WT1 peptides, both with or without the support of ex vivo prepared antigen-loaded autologous DCs, antigen-specific CTLs increased in vivo. The frequencies of WT1 specific tetramer+ CD8+ T cells in the vaccinated recipients were comparable to those in the AML patients who received vaccination using WT1 antigen. When we compared the peptide vaccination group and DC vaccination group, we found more efficient induction of WT1 specific CD8+ T cells with the support of autologous DCs. That might be accounted for by the incomplete DC development in the lymph nodes of HSC-engrafted NSG mice.

TLR3-expressing human BDCA3+ cDCs are regarded as a functional equivalent of murine CD8a+ DCs which play crucial role in the induction of CTL responses against cancer cells and viruses. The TLR3 agonist poly I:C has been shown to function as an adjuvant and induces cellular immune responses in a mouse model and in clinical trials. Since we confirmed the expression of TLR3 in dendritic cells in human HSC-engrafted NSG mice, we chose poly I:C as adjuvant. The frequency of BDCA3+ DCs were increased after the vaccination of poly I:C mixed with WT1 peptides (Figure S3).
Although adoptive immune-therapy with peripheral T cells that express antigen specific TCR genes has been evaluated in clinical trials ⁹, there are potential problems such as limited ex vivo expansion of peripheral T cells ³⁵ and a risk of generating autoreactive T cell clones due to mis-pairing between transgenic and endogenous TCR chains ³⁶. Recently, using a retroviral vector encoding small interfering RNAs targeting endogenous TCR genes ³⁷, we transduced WT1 specific TCR genes into peripheral T cells and demonstrated their in vivo cytotoxicity against leukemia cells ³⁸.

In the current study, we aimed to transfer WT1 specific TCR genes into HSCs, to generate transgenic T cells in vivo for long-term function. It was reasoned that these T cells would undergo negative selection resulting in the elimination of autoreactive T cells ³⁹. During a median follow-up of 26 weeks, the recipients of WT1 TCR gene transduced HSCs did not show any signs of severe graft-versus-host-diseases (GVHD), such as weight loss, skin inflammation and diarrhea. The recipient-derived WT1-specific CTLs retained the capacity to proliferate in vitro and exerted antigen-specific cytokine response and lytic activity, indicating the HSCs generated functional transgenic CTLs in vivo.

Our results supported previous reports describing development of transgenic CTLs specific for Melanoma Antigen Recognized by T cells (MART-1) ⁴⁰-⁴². Transgenic MART-1 specific CD8⁺ T cells were detected in bone marrow/liver/thymus (BLT) humanized mice ⁴³ transplanted with TCR gene transduced fetal liver-derived CD34⁺ HSPCs ⁴⁰ or in HLA-A*0201 Tg NSG mice transplanted with TCR transduced CB CD34⁺ HSPCs ⁴². Through the TCR Vβ repertoire analysis of MART-1 specific tetramer positive T cells in the recipients, both groups demonstrated that the allelic exclusion by exogenous TCRs suppressed rearrangement of endogenous human TCR genes during thymocyte differentiation and reduced the risk of generating autoreactive CTL clones.

The frequency of WT1 specific transgenic CTLs in our recipients were relatively lower compared to that of MART-1 specific CTLs detected in those reports ⁴⁰-⁴². Several differences
in experimental settings might explain this disparity. First, we isolated CD34+CD38-
CB-HSCs for lentiviral TCR transfer and transduction marker (GFP) positive cells for
transplantation to exclude the possibility of residual preformed human T cells in the graft.
Purified WT1-TCR transferred HSCs still retained the multi-lineage differentiation capacity.
Second, the variances of gene expression patterns between WT1 and MART-1 might affect
their immunogenicity. WT1 is expressed in normal tissues including podocytes of normal
kidney, Sertoli cells of testis and granulosa cells of ovary, while the expression of
MART-1 is localized to melanocytes of skin and retina.
Without expression of human HLA class II molecules, appropriate CD4 T-cell help might
be lacking in our recipients, causing a failure of T cell memory formation as well as
incomplete priming of CD8+ cells in vivo. In addition, current xenograft recipient lymph
nodes do not fully recapitulate human lymph node architecture (Figure S9). In the future, we
aim to humanize lymph node microenvironment in the recipient and support development of
lymph node architecture through transgenic expression of human cytokines and a more
physiological repopulation of human immune cells.
As previously reported, we confirmed high expression of WT1 in CD34+CD38- LSC
fraction from patients with AML at mRNA and protein levels. As a minimal residual disease
marker, WT1 mRNA expression in patients with AML has been assessed for the prediction of
relapse after chemotherapy and HSC transplantation (HSCT). Our results might provide
some motivation to perform immunotherapeutic targeting of WT1 against persistent LSCs in
hematological remission. Regardless of the HLA-A locus genotype of the donor, we detected
functional WT1 specific CTLs in the BM and spleen of the recipients transplanted with
TCR transduced CB HSCs, suggesting the application for wide variety of donor-graft
combinations in the clinical settings of HSCT.
In the current study, we were able to evaluate two different options of human
immune-therapy in a mouse model. Based on these results, the HLA class I Tg NSG
xenograft system might serve as a pre-clinical tool for immune-therapy against human malignancies.

**Author contribution**

Y.N. designed and performed experiments, analyzed data, and wrote the manuscript. M.T-M. performed sorting experiments and T cell functional assays. T.O. and H.F. performed \(^{51}\)Cr releasing assay and analyzed the data. R.O. analyzed the data. N.S. performed sorting experiments and analyzed myeloid differentiation. Y.S., O.O., L.D.S., M.Y, and F.I. designed the research, analyzed the data, and wrote the manuscript.

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**Conflict of interest**

The authors declare no competing interests.

**References**


35. Klebanoff CA, Gattinoni L, Restifo NP. CD8+ T-cell memory in tumor immunology and


44. Hastie ND. Life, sex, and WT1 isoforms: three amino acids can make all the difference. *Cell.* 2001;106(4):391-394.


49. Cilloni D, Renneville A, Hermitte F, et al. Real-time quantitative polymerase chain reaction detection of minimal residual disease by standardized WT1 assay to enhance risk


Figure Legend

Figure 1
HLA expression and multi-lineage human immune subsets in HLA Class I Tg NSG humanized mice.

(A) RT-PCR for HLA-A*0201, HLA-A*2402, human B2M and murine B2m using cDNA from BM, spleen and msCD45-EpCAM+ thymic epithelial cells derived from non-engrafted mice. A2Tg and A24Tg indicate NSG-HLA-A2/HHD (left panel) and NSG-HLA-A24/HHD (right panel), respectively. (B) Representative contour plots indicating the engraftment of human CD45+ leukocytes, hCD3+ T cells, hCD19+ B cells in the spleen of an NSG-HLA-A24/HHD recipient at 18 weeks after transplantation. (C) Engraftment levels of human CD45+ cells in each organ (n = 11 for PB, BM, MLN and Thymus; n = 10 for AxLN). (D) In the CD8+ cell fraction of the spleen of HLA Class I Tg NSG recipients, naïve, CM, EM and EMRA T cell subsets were identified. (E) The frequencies of CD8+ memory T-cell subsets in the spleen of HLA Class I Tg NSG recipients (n = 10). (F) Cytoplasmic expression of granzyme A in CD8+ T-cell subsets from naïve to effector phenotype in the spleen of HLA Class I Tg NSG recipients (n = 4). (G) Representative contour plots of human myeloid subsets in the BM of an HLA Class I Tg NSG recipient. (H) The frequencies of monocytes (CD14+CD33+HLA-DR+), pDCs (CD123+CD11c-) and cDCs (CD33+HLA-DR+CD11c+) among human CD45+ cells are shown. (I) cDCs were further divided into BDCA1+DCs and BDCA3+DCs (BM, n = 8, left; spleen, n = 7, right). Bars indicate the mean value.

Figure 2
Immunization of HLA Class I Tg NSG recipients using a WT1 antigen.

(A) Representative contour plots show development of WT1-specific CD8+ CTLs in PB from an HLA class I Tg NSG recipient before and after immunization using HLA-A*2402
WT1(mutant)\textsubscript{235-243} tetramers (left and right). (B) Frequencies of WT1 specific tetramer+ CTLs
in the PB of HLA class I Tg NSG recipients were increased after immunization (n = 7). (C)
Representative flow cytometry plots of tetramer assay in the spleens from HLA class I Tg
NSG recipients at the day of sacrifice. Data of control and the DC vaccine group in
NSG-HLA-A2/HHD (left 2 panels) or NSG-HLA-A24/HHD (right 2 panels) recipients are
shown. (D) Summary of tetramer analysis in each organ at the day of sacrifice. Open inverted
triangles, diamonds and circles indicate the groups of no immunization control, immunized
without DC and with DC, respectively. The frequencies of WT1 tetramer+ cells gated on
CD8+ T cells in spleen, PB, BM and MLN are plotted. (E) Representative contour plots of
tetramer assay before and after in vitro expansion of CTLs derived from spleens of HLA class
I Tg NSG recipients in the group immunized with DC. (F) Frequencies of WT1 tetramer+
spleen-derived CTLs (%) before and after in vitro expansion. (G) Fold increase of tetramer+
CTL numbers before and after in vitro expansion. Bars indicate the mean value. * and **
indicate p < 0.05 and p < 0.01 by two-tailed \( t \) test, respectively.

**Figure 3**

**Transplantation of WT1 specific TCR gene transduced HSCs into NSG-HLA-A24/HHD
recipients**

(A) Purification of CB CD34+CD38- cells using a CD34+ cell enriched CB sample (left panel).
WT1 TCR V\( \alpha \) and V\( \beta \) genes were transduced into the isolated cells with a lentiviral vector at
an MOI of 100. After 5 days of incubation, GFP+ cells were sorted and transplanted into
NSG-HLA-A24/HHD newborns (right panel). (B) Multi-lineage analysis of WT1-TCR gene
transduced NSG-HLA-A24/HHD recipients. Representative plots of spleen and thymus are
shown. (C) Frequencies of B cells and T cells gated on human CD45+ leukocytes in spleen
and thymus of NSG-HLA-A24/HHD recipients transplanted with non-transduced, GFP control
transduced or WT1-TCR gene transduced HSCs. (D) Frequencies of human T cell subsets in
the spleen and thymus of NSG-HLA-A24/HHD WT1 TCR gene transduced HSC recipients
(spleen, n = 6 for non-transduced or GFP control recipients, CD4+ 64.3 +/- 3.1%, CD8+ 28.5 +/- 3.5%, n = 11 for WT1 TCR gene transduced HSC recipients, CD4+ 50.8 +/- 7.4%, CD8+ 29.0 +/- 5.3%. Thymus, n = 5 for non-transduced or GFP control recipients, CD4+ 46.7 +/- 7.1%, CD8+ 29.7 +/- 3.8%, DP 20.4 +/- 5.6%, n = 15 for WT1 TCR gene transduced HSC recipients, CD4+ 25.1 +/- 3.8%, CD8+ 35.2 +/- 4.3%, DP 33.6 +/- 6.6%). Open and closed circles indicate non-transduced and GFP control recipients, respectively. Bars indicate the mean value.

Figure 4
CTL analysis of NSG-HLA-A24/HHD WT1 TCR gene transduced HSC recipients
(A) Representative flow cytometry plots showing tetramer analysis in the spleen and thymus of the recipients transplanted with WT1 TCR gene transduced HSCs. (B) The frequencies of WT1-specific CTLs in spleen were significantly increased in the recipients of WT1 TCR gene transduced HSCs compared to those of non-transduced or control vector transduced HSCs. The frequency of WT1 tetramer+ gated on CD8+ T cells (%) are shown (left, spleen, n=6 for non-transduced or GFP control vector transduced HSC recipients, 0.027 +/- 0.010%; n=9 for WT1 TCR gene transduced HSC recipients, 0.316 +/- 0.070%, **p=0.0055; middle, thymus, n=5 for non-transduced or GFP control vector transduced HSC recipients, 0.074 +/- 0.031%; n=11 for WT1 TCR gene transduced HSC recipients, 1.158 +/- 0.513%; BM, n= 3 for non-transduced or GFP control vector transduced HSC recipients, 0.093 +/- 0.041%, n=3 for WT1 TCR gene transduced HSC recipients, 0.565 +/- 0.200%).

Figure 5
In vitro expanded CTL derived from NSG-HLA-A24/HHD WT1 TCR gene transduced HSC recipients exerted HLA-restricted antigen specific response.
(A) Representative flow cytometry plots showing WT1/HLA-A*2402 tetramer analysis before and after in vitro stimulation of CTL from BM (top), spleen (middle) and thymus (bottom) of NSG-HLA-A24/HHD recipients transplanted with WT1 TCR transduced HSC. (B and C) The frequencies (B) and cell numbers (C) of WT1 specific tetramer+ CTLs derived from WT1 TCR transduced HSC recipients before and 1 week after the second in vitro stimulation. * and ** indicate p < 0.05 and p < 0.01 by ratio t test, respectively. (D) After expansion, recipient-derived WT1-specific CTLs exerted IFN-γ production in response to WT1 peptide-pulsed LCLs (top row), but were not responsive against peptide-unpulsed LCLs (middle row). The addition of anti-HLA class I antibody reduced the WT1-peptide specific cytokine production (bottom row). (E) Spot counts of IFN-γ producing cells out of 1 x 10^4 CTLs after expansion were shown. * and ** indicate p < 0.05 and p < 0.01 by two-tailed t test, respectively. (F) Results of ^{51}Cr release assay showing antigen-specific, HLA-restricted cytotoxicity by amplified CTLs derived from a WT1 TCR transduced NSG-HLA-A24/HHD recipient BM (A24-TCR-2) at indicated effector to target cell ratios (upper). ^{51}Cr release assays at an E:T ratio of 5:1 in the presence or absence of anti-HLA class I or anti-HLA class II mAb (lower). (G) Cytotoxic activity of WT1 specific CTLs from A24-TCR-2 BM against leukemia cell lines (left). The cytotoxicity of these CTLs was inhibited by adding anti-HLA class I but not by anti-HLA class II mAb (right).

Figure 6

Skewed TCR repertoire in human CD8+ T cells developed in NSG-HLA-A24/HHD WT1 TCR gene transduced HSC recipients

CD8+GFP+tetramer+ T cells were purified from an NSG-HLA-A24/HHD mouse transplanted with WT1-specific TCR transduced human HSCs. Human CD8+ T cells were also purified from control mice engrafted with non-transduced human HSCs. Pie Charts depict the major repertoire greater than 1%. The minor repertoires less than 1% are indicated as “others”
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<td>24-7</td>
<td>A3101/-</td>
<td>0.6</td>
<td>62.2</td>
<td>11.0</td>
<td>79.0</td>
<td>3.3</td>
<td>N.A.</td>
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<td>A24-TCR-9</td>
<td>24-13</td>
<td>A2402/-</td>
<td>78.5</td>
<td>62.8</td>
<td>89.4</td>
<td>74.5</td>
<td>9.9</td>
<td>0.636</td>
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<td>A24-TCR-10</td>
<td>24-15</td>
<td>A3101/3303</td>
<td>50.7</td>
<td>94.6</td>
<td>76.4</td>
<td>92.8</td>
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<td>A24-TCR-11</td>
<td>24-21</td>
<td>A2402/-</td>
<td>51.5</td>
<td>2.0</td>
<td>87.2</td>
<td>7.2</td>
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<td>A24-TCR-12</td>
<td>24-16</td>
<td>A0201/3303</td>
<td>11.9</td>
<td>92.1</td>
<td>34.0</td>
<td>95.8</td>
<td>13.5</td>
<td>N.A.</td>
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<td>24-16</td>
<td>A0201/3303</td>
<td>28.3</td>
<td>18.1</td>
<td>74.2</td>
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<td>46.7</td>
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<td>A24-TCR-14</td>
<td>24-15</td>
<td>A3101/3303</td>
<td>10.1</td>
<td>99.0</td>
<td>54.6</td>
<td>98.7</td>
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<td>N.A.</td>
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<td>24-40</td>
<td>A2402/-</td>
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<td>90.2</td>
<td>58.6</td>
<td>95.7</td>
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<td></td>
<td>36.3 +/- 6.2</td>
<td>46.0 +/- 9.2</td>
<td>67.6 +/- 6.1</td>
<td>61.8 +/- 8.2</td>
<td>22.1 +/- 7.1</td>
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1
Table 3. NSG recipients transplanted with HLA A24-expressing K562 cells

<table>
<thead>
<tr>
<th>Mouse</th>
<th>days</th>
<th>Co-culture</th>
<th>IV transfer of CTLs</th>
<th>Size of tumor (g)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td># of K562</td>
<td># of CTLs</td>
<td>cell #</td>
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<tr>
<td>1</td>
<td>24</td>
<td>1.0E+06</td>
<td>5.0E+06</td>
<td>3.4E+06</td>
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<tr>
<td>2</td>
<td>24</td>
<td>1.0E+06</td>
<td>5.0E+06</td>
<td>3.0E+06</td>
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<tr>
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<td>25</td>
<td>4.0E+05</td>
<td>4.0E+06</td>
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<td>24</td>
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<td>5.7E+06</td>
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<tr>
<td>5</td>
<td>28</td>
<td>4.8E+05</td>
<td>2.4E+06</td>
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</tbody>
</table>
Figure 1

A

<table>
<thead>
<tr>
<th>NSG</th>
<th>A2Tg</th>
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<tbody>
<tr>
<td>BM</td>
<td>Spl</td>
</tr>
<tr>
<td>BM</td>
<td>Spl</td>
</tr>
<tr>
<td>A*0201</td>
<td>A*2402</td>
</tr>
<tr>
<td>hu-β2M</td>
<td>hu-β2M</td>
</tr>
<tr>
<td>ms-β2M</td>
<td>ms-β2M</td>
</tr>
</tbody>
</table>

B

- Viable cells
- Human CD45+

C

- Human CD45+ chimerism (%)
- PB, Spl, BM, MLN, AxLN, Thy

D

- Human CD8+
- CCR7
- CD45RA

E

- Frequency in CD8+ (%)
- Naive, CM, EM, E

F

- Granzyme A+ / CD8+ (%)
- Naive, CM, EM, E

G

- Human CD45+
- CD33
- HLA-DR
- CD14
- CD123
- CD11c
- BDCA1
- BDCA3

H

- Frequency in CD45+ (%)
- Mono, pDC, cDC
- BM, Spl

I

- Frequency in CD33+ (%)
- BDCA1+, BDCA3+
- BM, Spl
Figure 4

A

Spl

CD8+ tet+ 0.041%
CD8+ tet+ 0.636%

Thy

CD8+ tet+ 0.058%
CD8+ tet+ 0.896%

B

- Spl -

WT1 Tetramer+/CD8+ (%)

- Thy -

WT1 Tetramer+/CD8+ (%)

- BM -

WT1 Tetramer+/CD8+ (%)

Non-/GFP Ctrl transduced
Non-/GFP Ctrl transduced
Non-/GFP Ctrl transduced
Non-/GFP Ctrl transduced

WT1 TCR transduced
WT1 TCR transduced
WT1 TCR transduced
WT1 TCR transduced