

1 **Induction of WT1 specific human CD8+ T cells from human HSCs in HLA class I Tg**

2 **NOD/SCID/Il2rgKO mice**

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1 **Key points**

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

6

7 **Abstract**

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

24

1 **Introduction**

2 The immune system prevents infectious disease initiation and progression and functions in
3 multiple homeostatic processes. However, dysfunctional immunity is observed in patients
4 with malignancies contributing to neoplastic progression. Therefore, reconstitution of
5 immunity by allogeneic stem cell transplantation or activation of specific and non-specific
6 immunity targeting diseases improves clinical outcomes in patients with solid cancers and
7 those with hematologic malignancies ^{1,2}. Such treatment can be carried out by vaccination
8 and by adoptive immunotherapy.

9 Vaccinations aim to elicit antigen-specific effector cell-mediated immune responses in vivo
10 ³. Among several candidates, peptide vaccines and DC vaccines were two widely selected
11 protocols. In the last two decades, however, administration of these vaccines has not
12 significantly improved the prognosis of patients with solid cancers including melanoma and
13 other types of solid tumors ^{4,5}. Although several recent trials reported encouraging clinical
14 outcomes using gp100 peptides in combination with IL-2 for melanoma ⁶ or patient-derived
15 APCs (Sipuleucel-T) for prostate cancer ⁷, cancer vaccination appears to require
16 modifications based on increased understanding of in vivo biology of human APCs and T
17 cells.

18 In contrast, immunotherapy based on adoptive transfer of ex vivo expanded tumor reactive
19 T cell has achieved promising results. In metastatic melanoma, adoptive transfer of tumor
20 infiltrating lymphocytes (TILs) in combination with chemotherapy or irradiation has improved
21 cure rates up to 20 - 40% ⁸. Because anti-tumor effect of TILs has not been confirmed in
22 malignancies other than melanoma, genetically engineered T cells which express
23 tumor-antigen specific TCR genes or chimeric antigen receptors (CARs) have been
24 developed ⁹. Recent clinical trials showed improved clinical outcomes in patients treated with
25 genetically engineered T cells ¹⁰⁻¹³ while adverse effects were observed immediately after the
26 transfusion of T cells expressing CAR ^{14,15}.

1 In several clinical trials of vaccination therapies for hematologic malignancies, promising
2 responses were observed using various antigens including proteinase 3 (PR3)¹⁶ and WT1
3^{16,17} for AML, BCR-ABL for chronic myelogenous leukemia¹⁸ as well as patient-specific
4 idiotypes derived from malignant B cell clones for follicular lymphoma¹⁹. In particular, for AML
5 with poor prognostic factors, development of immune-therapy targeting minimal residual
6 disease or leukemia stem cells (LSCs) should play an essential role in achieving long-term
7 patient survival.

8 We recently reported that WT1, a transcription factor expressed in variety of malignant
9 tissues, is highly expressed by CD34+CD38- AML cells²⁰. WT1 is considered as one of the
10 best antigens to be used for immune-therapy against malignancies based on the multiple
11 criteria such as therapeutic function, immunogenicity and specificity²¹. Using WT1 peptide or
12 full-length mRNA for WT1, clinical trials against hematological malignancies detected
13 increased frequencies of WT1-specific CD8+ T cells in the patient blood after the treatment
14^{16,17,22,23}. Nevertheless, to accomplish significant improvement in clinical outcomes of AML
15 patients, we need to better understand the biology of human immune system leading to
16 efficient activation of human acquired immunity against tumor antigens.

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

1 malignancies.

2

3

1 **Materials and Methods**

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

3

4 **Flow cytometry**

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

7

8 **Immunization of humanized mice**

9 In the DC vaccine group, HSCs reconstituted HLA class I Tg NSG recipients were injected
10 with WT1 peptide-loaded DCs via retro-orbital plexus on day 0, followed by intraperitoneal
11 and subcutaneous immunization with 300 µg WT1₁₂₆₋₁₃₄ or WT1₂₃₅₋₂₄₃ synthetic peptide mixed
12 with 150 µg polyinosinic:polycytidylic acid (poly I:C, InvivoGen) on days 0, 10 and 20. In the
13 vaccine group without DC, the same doses of WT1₁₂₆₋₁₃₄ or WT1₂₃₅₋₂₄₃ synthetic peptide
14 mixed with poly I:C were administered intraperitoneally and subcutaneously on days 0, 10
15 and 20. One week after the last immunization, mice were necropsied for the evaluation of
16 human immune cells in peripheral blood (PB), BM, spleen, lymph nodes and thymuses.

17

18 **In vitro expansion of human CD8⁺ T cells derived from mice engrafted with human** 19 **HSCs**

20 Human CTLs were expanded from HLA Class I Tg NSG humanized mice as described
21 previously with some modifications²⁴. Splenocytes, thymocytes and BM cells from recipient
22 mice were stimulated with irradiated autologous LCLs pulsed with 40 µg/ml WT1₁₂₆₋₁₃₄ or
23 WT1₂₃₅₋₂₄₃ synthetic peptide at responder to stimulator ratio of 1:1.

24

25 **Lentiviral transduction**

26 Purified CB CD34+CD38- cells were incubated with concentrated lentiviral supernatant at a

1 MOI of 100 to 200. After 5 days of incubation, GFP+ cells were sorted using FACS Aria (BD
2 biosciences) and transplanted into irradiated HLA Class I Tg NSG newborns.

3

4 **ELISPOT assay**

5 WT1-specific T cell responses were quantified by the IFN- γ ELISPOT assay as described
6 previously²⁴. Pictures of each well were captured using AlphaMager Image Analysis System
7 (Alpha Innotech) and individual spots were counted per well. Using the same protocol for
8 IFN- γ assay, production of human TNF α by human WT1-specific CD8+ T cells was also
9 confirmed by ELISPOT assay (Mabtech).

10

11 **⁵¹Cr release assay**

12 Cytotoxic activity of WT1 specific CTLs was evaluated by ⁵¹Cr release assay as described
13 previously²⁴. To examine the HLA restricted cytotoxicity, target cells were incubated with
14 anti-HLA class I framework mAb (w6/32; American Type Culture Collection) or anti-HLA-DR
15 mAb (L243; American Type Culture Collection) at 10 μ g/ml for 1 hour, followed by adding the
16 effector cells. The percentage of specific lysis was calculated as (experimental release cpm –
17 spontaneous release cpm) / (maximal release cpm – spontaneous release cpm) x 100 (%).

18

19 **In vivo cytotoxicity assay**

20 HLA A24-expressing K562 cells²⁵ were cultured with or without WT1-specific CTLs for five
21 hours. After harvesting all the cultured cells, the cells were inoculated subcutaneously into
22 female NSG mice. WT1-specific CTLs were adoptively transferred weekly. At 24 - 28 days
23 post-inoculation, the mice were sacrificed to evaluate tumor size and weight.

24

25 **Analysis of TCR repertoire of CTLs**

26 Following in vitro expansion, WT1 specific CTLs were purified by FACS. As a control, 2.4 x

1 10^4 - 1.5×10^5 hCD45+CD3+CD8+ cells were purified from spleens of NSG mice engrafted
2 with non-transduced human HSCs. Total RNA was extracted using TRIzol reagent
3 (Invitrogen) and reverse transcribed for synthesis of first strand cDNA using SMARTer RACE
4 cDNA amplification kit (Clontech). Both universal mix primer and primers specific for the
5 T-cell receptor α (TCR α) or β (TCR β) constant region sequence were used for 5'-end specific
6 PCR amplifications, resulting in both TCR α and TCR β PCR products of high purity which
7 were then sequenced on a Miseq (Illumina). All reads of both the TCR α and the TCR β
8 repertoire sequence were analyzed using Perl scripts based on the USEARCH algorithm
9 (<http://drive5.com/usearch/>). V-region consensus sequences in each cluster were searched
10 on the ImMunoGeneTics (IMGT) sites (www.imgt.org/IMGT_vquest/share/textes/).
11

1 **Results**

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

3 We developed immunodeficient NSG mice expressing HLA class I antigens by backcrossing
4 HLA-A*0201²⁴ or HLA-A*2402 transgenes onto the NSG background. Both transgenes also
5 encode covalently bound human β 2m. By RT-PCR, HLA transgenes were detected in the BM,
6 spleen and CD45-EpCAM⁺ TECs derived from both NSG-HLA-A2/HHD mice and
7 NSG-HLA-A24/HHD mice (Figure 1A). We then transplanted 4.5×10^3 – 5.6×10^4 purified
8 human HLA matched CD3-CD4-CD8-CD34⁺CD38⁻ CB HSCs into HLA Class I Tg NSG
9 newborns. At 15 - 37 weeks post-transplantation, we confirmed engraftment of human
10 CD45⁺ leukocytes in the PB, BM, spleen, mesenteric and axillary lymph nodes (MLN and
11 AxLN), and thymus (Figure 1B, 1C, Table 1, n = 11 for PB 72.7 +/- 6.9%, spleen 91.5 +/-
12 2.0%, BM 72.4 +/- 9.2%, MLN 97.3 +/- 0.7% and Thymus 97.6 +/- 1.2%; n = 10 for AxLN 96.7
13 +/- 1.4%). Human CD3⁺ T cells were 65.9 +/- 7.2% of engrafted hCD45⁺ leukocytes in the
14 recipient spleen. CD4⁺CD8⁺ double positive (DP) T cells accounted for 34.2 +/- 12.5% in the
15 thymus (n = 6), while CD4⁺ or CD8⁺ single positive (SP) T cells were 26.2 +/- 5.9% or 31.7
16 +/- 11.3% of engrafted human CD3⁺ T cells. Within the human CD8⁺ fraction, we identified
17 CD45RA⁺CCR7⁺ naïve, CD45RA⁺CCR7⁺ central memory (CM), CD45RA⁺CCR7⁻ effector
18 memory (EM) and CD45RA⁺CCR7⁻ terminally differentiated (EMRA) T cell subsets in the
19 recipient spleen (Figure 1D and 1E, naïve 20.8 +/- 10.0%, CM 13.7 +/- 4.9%, EM 60.9 +/-
20 10.3% and EMRA 4.9 +/- 1.8%, n = 10). Cytoplasmic expression of Granzyme A was the
21 highest in EM CD8⁺ T cells (Figure 1F and S1, naïve 10.0 +/- 2.1%, CM 42.0 +/- 8.9%, EM:
22 75.4 +/- 10.7%, EMRA: 55.1 +/- 10.3%, n = 4).

23 To elucidate whether human APCs developed in the HLA class I Tg NSG mice, we
24 analyzed the expression of CD33 and other myeloid-associated antigens using flow
25 cytometry. In the NSG-HLA-A2/HHD mice (n = 4) and NSG-HLA-A24/HHD mice (n = 6), we
26 found that CD14⁺CD33⁺HLA-DR⁺ monocytes, CD123^{high}CD11c⁻ 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 cells²⁰, 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 $2.84 \pm 2.02 \times 10^{-4}$, n = 7; AML patients $1.07 \pm 0.25 \times 10^{-2}$, 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

1 found significant increase of WT1 specific tetramer+ cells in the recipient PB following
2 immunization (Figure 2A and 2B, $n = 7$, $0.044 \pm 0.017\%$ and $0.399 \pm 0.110\%$ for before
3 and after immunization, respectively, $*p < 0.05$ by two-tailed paired t test). Compared with
4 unvaccinated mice, the frequencies of WT1 tetramer+ CTLs in spleen were increased
5 significantly in the recipients of the vaccine groups both with and without WT1 peptide-loaded
6 DCs (Figure 2C and 2D, no immunization control, $0.041 \pm 0.007\%$, $n = 10$; immunized
7 without DC, $0.170 \pm 0.043\%$, $n = 10$, $*p = 0.0146$; immunized with DC, $0.230 \pm 0.044\%$, $n =$
8 9 , $**p = 0.0027$). In the recipients vaccinated with WT1 peptide-loaded DCs, WT1 specific
9 CTLs were also increased in the PB, BM and MLN, whereas the increases in these organs
10 were not significant in the group immunized without DCs (Figure 2D, WT1 tetramer+ / CD8+
11 T cells (%): PB, no immunization control, $0.038 \pm 0.014\%$, $n = 10$; immunized without DC,
12 $0.072 \pm 0.024\%$, $n = 7$; immunized with DC, $0.361 \pm 0.088\%$, $n = 9$, $**p = 0.0061$; BM, no
13 immunization control, $0.090 \pm 0.043\%$, $n = 7$; immunized without DC, $0.429 \pm 0.136\%$, $n =$
14 5 ; immunized with DC, $0.658 \pm 0.203\%$, $n = 5$, $*p = 0.0473$; MLN no immunization control,
15 $0.013 \pm 0.006\%$, $n = 6$; immunized without DC, $0.071 \pm 0.032\%$, $n = 6$; immunized with DC,
16 $0.146 \pm 0.030\%$, $n = 7$, $**p = 0.0040$).

17 To evaluate the antigen-specific proliferation of CTLs, $2 - 10 \times 10^6$ splenocytes of recipients
18 were further stimulated with WT1 peptide-pulsed autologous LCLs in vitro. After 4 weeks, the
19 frequency of WT1 tetramer+ CTLs derived from splenocytes was higher in the vaccinated
20 recipients than in the unvaccinated mice (Figure 2E and 2F, WT1 tetramer+ / CD8+ (%)
21 before and after in vitro expansion: No immunization group, $0.050 \pm 0.006\%$ and $0.168 \pm$
22 0.042% , $n = 3$; immunized group, $0.194 \pm 0.032\%$ and $0.511 \pm 0.095\%$, $n = 12$; $**p =$
23 0.0057 for no immunization vs immunized after expansion). The degree of increase in WT1
24 specific CTL cell numbers after expansion was also higher in the immunized recipients
25 (Figure 2G, fold increase of WT1 tetramer+ CD8+ cell counts before and after in vitro
26 expansion: No immunization group 4.06 ± 2.34 , $n = 3$; immunized group 42.13 ± 12.1 , $n =$

1 12, **p = 0.0097).

2

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

4 **reconstituted WT1 specific CTL in vivo**

5 As another way of inducing antigen-specific human CTLs in vivo, we evaluated differentiation
6 of human T cells from antigen-specific TCR transduced HSCs. We constructed a lentiviral
7 vector encoding WT1 specific TCR V α and V β genes. We transduced the TCR V α and V β
8 genes into purified human CB HSCs using the lentiviral vector (Figure 3A, Figure S4A), and
9 purified CD34+GFP+ cells by FACS. Both HLA-matched and -mismatched CBs were used
10 as HSC sources (Table 2). Then we transplanted GFP-labeled gene-transduced CD34+ cells
11 into NSG-HLA-A24/HHD newborns. After 4 - 6 months, multi-lineage human hematopoietic
12 cells developed (Figure 3B and 3C, Figure S4B: Spleen, n = 7 for non-transduced and GFP
13 control recipients, B cells 56.9 +/- 12.2%, T cells 34.4 +/- 13.1%; n = 15 for WT1 TCR gene
14 transduced HSC recipients, B cells 71.5 +/- 4.3%, T cells 9.8 +/- 4.0%. Thymus, n = 6 for
15 recipients of non-transduced or control vector transduced HSCs, B cells 12.2 +/- 4.9%, T cells
16 53.1 +/- 9.2%; n = 15 for WT1 TCR gene transduced HSC recipients, B cells 5.2 +/- 2.3%, T
17 cells 28.0 +/- 6.0%). CD4+ and CD8+ T cells were identified in the spleen of control NSG
18 recipients and NSG recipient transplanted with WT1 TCR-transduced HSCs. In the recipient
19 thymus, double positive T cells as well as single positive T cells were found in both recipients
20 (Figure 3D). Engraftment levels of human CD45+ cells were not statistically different between
21 control mice and TCR transduced mice (Table 2).

22 At the day of sacrifice, WT1 tetramer+ CD8+ cells were present at frequencies of 0.316 +/-
23 0.070 % (n=9), 1.158 +/- 0.513 % (n=11) and 0.565 +/- 0.200 % (n=3) in the spleen, thymus
24 and BM of WT1 TCR transduced HSC recipients, respectively (Figure 4A and 4B).

25 Human CTLs developed in the NSG-HLA-A24/HHD mice were stimulated in vitro using
26 HLA-A*2402(+) WT1 peptide-pulsed LCLs for further expansion (FigureS5). When

1 HLA-A*2402-mismatched CB HSCs were transplanted into NSG-HLA-A24/HHD newborns,
 2 WT1 peptide-pulsed, HLA-A*2402-matched allogeneic CB LCLs were used as stimulators to
 3 expand WT1-specific CTLs in vitro. After 2 - 3 weeks, the frequency of WT1 tetramer+ CTLs
 4 derived from splenocytes of the recipients was significantly increased (Figure 5A and 5B,
 5 WT1 tetramer+CD8+ T cells (%): BM, n = 5, before 0.626 +/- 0.140%, after 11.57 +/- 10.23%,
 6 p = 0.3880; spleen, n = 9, before 0.316 +/- 0.070%, after 10.12 +/- 7.781%, *p = 0.0197;
 7 thymus, n = 4, before 1.533 +/- 1.282%, after 23.95 +/- 14.10%, p = 0.0561). The cell
 8 numbers of WT1 tetramer+ CTLs derived from BM, spleen and thymus were significantly
 9 increased after in vitro expansion (Figure 5C, cell numbers of WT1 tetramer+CD8+ T cells:
 10 BM, n=5, before 8.1×10^1 +/- 4.5×10^1 , after 1.5×10^6 +/- 1.5×10^6 , *p = 0.0253; spleen, n= 9,
 11 before 2.7×10^2 +/- 1.1×10^2 , after 9.4×10^5 +/- 8.9×10^5 , **p = 0.0013; thymus, n=4, before
 12 4.0×10^2 +/- 2.4×10^2 , after 1.9×10^6 +/- 1.8×10^6 , *p = 0.0102). To elucidate whether the
 13 GFP+ WT1 tetramer+ CD8+ T cells retain specific TCRs, we performed a repertoire analysis
 14 of TCR using a Miseq. The result of repertoire sequences demonstrated that FACS-purified
 15 GFP+ WT1 tetramer+ CD8+ T cells displayed skewing of both V α and V β repertoires and that
 16 the majority of V α and V β chains of the T cells were the transduced V α and V β chains (Figure
 17 6). After the expansion of WT1 specific CTLs for 4 - 5 weeks, we found that the majority of
 18 GFP+CD8+ T cells were CD45RA(+)CCR7(-) effector T cells (Figure S6). We also
 19 evaluated the antigen-specific cytokine production by the CTLs with ELISPOT assay.
 20 Recipient-derived WT1-specific CTLs produced IFN- γ against WT1 peptide-pulsed but not
 21 peptide-unpulsed HLA-A*2402(+) LCLs. The addition of anti-HLA class I antibody
 22 significantly reduced the number of IFN- γ producing cells against WT1 peptide-pulsed LCLs
 23 (Figure 5D and 5E). WT1-specific CTLs also produced TNF α (Figure S7). Using ^{51}Cr
 24 release assay, we evaluated cytotoxicity by the amplified CTLs derived from a WT1 TCR
 25 transduced NSG-HLA-A24/HHD recipient BM (A24-TCR-2) (Figure 5F and G). Since this
 26 donor CB sample used for the transplantation of WT1 TCR transduced HSC was

1 HLA-A*2402-mismatched (Table 2, CB23-1), allogeneic HLA-A*2402-matched LCLs were
2 used for in vitro stimulation. Cytotoxic activities were observed against allogeneic WT1
3 peptide-pulsed HLA-A*2402-matched LCLs (Figure 5F, LCL#1, derived from CB23-3), but not
4 against peptide-unpulsed LCL#1 and autologous HLA-A*2402-mismatched LCLs (Figure 6F
5 upper, LCL#2, derived from CB23-1) with or without peptide-loading. WT1(+) HLA-A*2402(+)
6 KAZZ leukemia cell lines were lysed by these CTLs. While HLA-A*2402(-) K562 leukemia cell
7 lines were not lysed, HLA-A*2402 gene transduced K562 cells became susceptible to lysis by
8 these CTLs (Figure 5G left). The cytotoxic activities of CTLs against WT1 peptide-pulsed
9 LCL#1 and HLA-A*2402 expressing leukemic cell lines were inhibited by anti-HLA class I
10 mAb, not by anti-HLA class II mAb (Figure 5F lower and Figure 5G right). We further aimed to
11 determine the effect of WT1-specific CTLs on the in vivo growth of leukemic cells. To this end,
12 we used as effector cells WT1-specific CTLs obtained from spleens of NSG-HLA-A24/HHD
13 recipients engrafted with human HSCs transduced with WT1-specific TCR genes. HLA A24
14 expressing K562 cells were used as target cells. We cultured the target K562 cells with
15 WT1-specific CTLs ex vivo then inoculated the cultured K562 cells subcutaneously into NSG
16 mice. At 3-7 days after three weekly transfers of WT1-specific CTLs, we found inhibition of
17 subcutaneous tumor formation (Table 3, Figure S8). These results indicate that WT1 TCR
18 transduced human HSCs can induce specific and functional CTLs as well as multi-lineage
19 immune subsets in HLA Class I Tg NSG recipients.
20

1 **Discussion**

2 Evoking immune responses against specific antigens requires HLA-restricted interaction
3 between APCs and effector cells. In this study, we examined whether active immunization
4 with vaccines can prime HLA-restricted antigen-specific human CTLs in mice expressing HLA
5 Class I molecules HLA-A*0201 or HLA-A*2402, the most common alleles in Caucasian ²⁶ or
6 Japanese populations, respectively ²⁷. First, we confirmed HLA class I expression in murine
7 TECs from the previously reported NSG-HLA-A2/HHD ^{24,28} as well as from the newly
8 developed NSG-HLA-A24/HHD strains. In HLA class I Tg NSG mice transplanted with HLA
9 class I matched CB-HSCs, human memory T cells expressing cytotoxic molecules and
10 human HLA-DR positive antigen-presenting myeloid cells including BDCA1+ or BDCA3+
11 cDCs were detected in the recipient BM and spleen. Following vaccination using adjuvant
12 mixed 9-mer WT1 peptides, both with or without the support of ex vivo prepared
13 antigen-loaded autologous DCs, antigen-specific CTLs increased in vivo. The frequencies of
14 WT1 specific tetramer+ CD8+ T cells in the vaccinated recipients were comparable to those
15 in the AML patients who received vaccination using WT1 antigen ^{16,17,22,23}. When we
16 compared the peptide vaccination group and DC vaccination group, we found more efficient
17 induction of WT1 specific CD8+ T cells with the support of autologous DCs. That might be
18 accounted for by the incomplete DC development in the lymph nodes of HSC-engrafted NSG
19 mice.

20 TLR3-expressing human BDCA3+ cDCs are regarded as a functional equivalent of murine
21 CD8a+ DCs which play crucial role in the induction of CTL responses against cancer cells
22 and viruses ²⁹⁻³². The TLR3 agonist poly I:C has been shown to function as an adjuvant and
23 induces cellular immune responses in a mouse model ³³ and in clinical trials ³⁴. Since we
24 confirmed the expression of TLR3 in dendritic cells in human HSC-engrafted NSG mice, we
25 chose poly I:C as adjuvant. The frequency of BDCA3+ DCs were increased after the
26 vaccination of poly I:C mixed with WT1 peptides (Figure S3).

1 Although adoptive immune-therapy with peripheral T cells that express antigen specific
2 TCR genes has been evaluated in clinical trials ⁹, there are potential problems such as limited
3 ex vivo expansion of peripheral T cells ³⁵ and a risk of generating autoreactive T cell clones
4 due to mis-pairing between transgenic and endogenous TCR chains ³⁶. Recently, using a
5 retroviral vector encoding small interfering RNAs targeting endogenous TCR genes ³⁷, we
6 transduced WT1 specific TCR genes into peripheral T cells and demonstrated their in vivo
7 cytotoxicity against leukemia cells ³⁸.

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

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

25 The frequency of WT1 specific transgenic CTLs in our recipients were relatively lower
26 compared to that of MART-1 specific CTLs detected in those reports ⁴⁰⁻⁴². Several differences

1 in experimental settings might explain this disparity. First, we isolated CD34+CD38-
2 CB-HSCs for lentiviral TCR transfer and transduction marker (GFP) positive cells for
3 transplantation to exclude the possibility of residual preformed human T cells in the graft.
4 Purified WT1-TCR transferred HSCs still retained the multi-lineage differentiation capacity.
5 Second, the variances of gene expression patterns between WT1 and MART-1 might affect
6 their immunogenicity. WT1 is expressed in normal tissues including podocytes of normal
7 kidney ⁴⁴, Sertoli cells of testis and granulosa cells of ovary ⁴⁵, while the expression of
8 MART-1 is localized to melanocytes of skin and retina ^{46,47}.

9 Without expression of human HLA class II molecules, appropriate CD4 T-cell help might
10 be lacking in our recipients, causing a failure of T cell memory formation as well as
11 incomplete priming of CD8+ cells in vivo ⁴⁸. In addition, current xenograft recipient lymph
12 nodes do not fully recapitulate human lymph node architecture (Figure S9). In the future, we
13 aim to humanize lymph node microenvironment in the recipient and support development of
14 lymph node architecture through transgenic expression of human cytokines and a more
15 physiological repopulation of human immune cells.

16 As previously reported ²⁰, we confirmed high expression of WT1 in CD34+CD38- LSC
17 fraction from patients with AML at mRNA and protein levels. As a minimal residual disease
18 marker, WT1 mRNA expression in patients with AML has been assessed for the prediction of
19 relapse after chemotherapy ⁴⁹ and HSC transplantation (HSCT) ⁵⁰. Our results might provide
20 some motivation to perform immunotherapeutic targeting of WT1 against persistent LSCs in
21 hematological remission. Regardless of the HLA-A locus genotype of the donor, we detected
22 functional WT1 specific CTLs in the BM ^{51,52} and spleen of the recipients transplanted with
23 TCR transduced CB HSCs, suggesting the application for wide variety of donor-graft
24 combinations in the clinical settings of HSCT.

25 In the current study, we were able to evaluate two different options of human
26 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 ⁵¹Cr 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

1. Finn OJ. Cancer immunology. *N Engl J Med*. 2008;358(25):2704-2715.
2. Jenq RR, van den Brink MR. Allogeneic haematopoietic stem cell transplantation: individualized stem cell and immune therapy of cancer. *Nat Rev Cancer*. 2010;10(3):213-221.
3. Palucka K, Banchereau J. Cancer immunotherapy via dendritic cells. *Nat Rev Cancer*. 2012;12(4):265-277.
4. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nat Med*. 2004;10(9):909-915.
5. Klebanoff CA, Acquavella N, Yu Z, Restifo NP. Therapeutic cancer vaccines: are we there yet? *Immunol Rev*. 2011;239(1):27-44.
6. Schwartzentruber DJ, Lawson DH, Richards JM, et al. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. *N Engl J Med*. 2011;364(22):2119-2127.

- 1 7. Kantoff PW, Higano CS, Shore ND, et al. Sipuleucel-T immunotherapy for
2 castration-resistant prostate cancer. *N Engl J Med*. 2010;363(5):411-422.
- 3 8. Rosenberg SA. Raising the bar: the curative potential of human cancer immunotherapy.
4 *Sci Transl Med*. 2012;4(127):127ps128.
- 5 9. Restifo NP, Dudley ME, Rosenberg SA. Adoptive immunotherapy for cancer: harnessing
6 the T cell response. *Nat Rev Immunol*. 2012;12(4):269-281.
- 7 10. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T
8 cells in chronic lymphoid leukemia. *N Engl J Med*. 2011;365(8):725-733.
- 9 11. Brentjens RJ, Riviere I, Park JH, et al. Safety and persistence of adoptively transferred
10 autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory
11 B-cell leukemias. *Blood*. 2011;118(18):4817-4828.
- 12 12. Kochenderfer JN, Dudley ME, Feldman SA, et al. B-cell depletion and remissions of
13 malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19
14 chimeric-antigen-receptor-transduced T cells. *Blood*. 2012;119(12):2709-2720.
- 15 13. Grupp SA, Kalos M, Barrett D, et al. Chimeric antigen receptor-modified T cells for acute
16 lymphoid leukemia. *N Engl J Med*. 2013;368(16):1509-1518.
- 17 14. Brentjens R, Yeh R, Bernal Y, Riviere I, Sadelain M. Treatment of chronic lymphocytic
18 leukemia with genetically targeted autologous T cells: case report of an unforeseen
19 adverse event in a phase I clinical trial. *Mol Ther*. 2010;18(4):666-668.
- 20 15. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of
21 a serious adverse event following the administration of T cells transduced with a chimeric
22 antigen receptor recognizing ERBB2. *Mol Ther*. 2010;18(4):843-851.
- 23 16. Rezvani K, Yong AS, Mielke S, et al. Leukemia-associated antigen-specific T-cell
24 responses following combined PR1 and WT1 peptide vaccination in patients with myeloid
25 malignancies. *Blood*. 2008;111(1):236-242.
- 26 17. Keilholz U, Letsch A, Busse A, et al. A clinical and immunologic phase 2 trial of Wilms
27 tumor gene product 1 (WT1) peptide vaccination in patients with AML and MDS. *Blood*.
28 2009;113(26):6541-6548.
- 29 18. Bocchia M, Gentili S, Abruzzese E, et al. Effect of a p210 multi-peptide vaccine associated
30 with imatinib or interferon in patients with chronic myeloid leukaemia and persistent
31 residual disease: a multicentre observational trial. *Lancet*. 2005;365(9460):657-662.
- 32 19. Schuster SJ, Neelapu SS, Gause BL, et al. Vaccination with patient-specific
33 tumor-derived antigen in first remission improves disease-free survival in follicular
34 lymphoma. *J Clin Oncol*. 2011;29(20):2787-2794.
- 35 20. Saito Y, Kitamura H, Hijikata A, et al. Identification of therapeutic targets for quiescent,
36 chemotherapy-resistant human leukemia stem cells. *Sci Transl Med*. 2010;2(17):17ra9.
- 37 21. Cheever MA, Allison JP, Ferris AS, et al. The prioritization of cancer antigens: a national
38 cancer institute pilot project for the acceleration of translational research. *Clin Cancer*

1 *Res.* 2009;15(17):5323-5337.

2 22. Oka Y, Tsuboi A, Taguchi T, et al. Induction of WT1 (Wilms' tumor gene)-specific cytotoxic
3 T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. *Proc Natl*
4 *Acad Sci U S A.* 2004;101(38):13885-13890.

5 23. Van Tendeloo VF, Van de Velde A, Van Driessche A, et al. Induction of complete and
6 molecular remissions in acute myeloid leukemia by Wilms' tumor 1 antigen-targeted
7 dendritic cell vaccination. *Proc Natl Acad Sci U S A.* 2010;107(31):13824-13829.

8 24. Shultz LD, Saito Y, Najima Y, et al. Generation of functional human T-cell subsets with
9 HLA-restricted immune responses in HLA class I expressing NOD/SCID/IL2r
10 gamma(null) humanized mice. *Proc Natl Acad Sci U S A.* 2010;107(29):13022-13027.

11 25. Demachi-Okamura A, Torikai H, Akatsuka Y, Miyoshi H, Yoshimori T, Kuzushima K.
12 Autophagy creates a CTL epitope that mimics tumor-associated antigens. *PLoS One.*
13 2012;7(10):e47126.

14 26. Ellis J, Steiner N, Kosman C, et al. Seventeen more novel HLA-A locus alleles. *Tissue*
15 *Antigens.* 2000;55(4):369-373.

16 27. Date Y, Kimura A, Kato H, Sasazuki T. DNA typing of the HLA-A gene: population study
17 and identification of four new alleles in Japanese. *Tissue Antigens.* 1996;47(2):93-101.

18 28. Strowig T, Gurer C, Ploss A, et al. Priming of protective T cell responses against
19 virus-induced tumors in mice with human immune system components. *J Exp Med.*
20 2009;206(6):1423-1434.

21 29. Poulin LF, Salio M, Griessinger E, et al. Characterization of human DNGR-1+ BDCA3+
22 leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. *J Exp Med.*
23 2010;207(6):1261-1271.

24 30. Jongbloed SL, Kassianos AJ, McDonald KJ, et al. Human CD141+ (BDCA-3)+ dendritic
25 cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell
26 antigens. *J Exp Med.* 2010;207(6):1247-1260.

27 31. Crozat K, Guiton R, Contreras V, et al. The XC chemokine receptor 1 is a conserved
28 selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells. *J*
29 *Exp Med.* 2010;207(6):1283-1292.

30 32. Bachem A, Guttler S, Hartung E, et al. Superior antigen cross-presentation and XCR1
31 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic
32 cells. *J Exp Med.* 2010;207(6):1273-1281.

33 33. Steinhagen F, Kinjo T, Bode C, Klinman DM. TLR-based immune adjuvants. *Vaccine.*
34 2011;29(17):3341-3355.

35 34. Sabbatini P, Tsuji T, Ferran L, et al. Phase I trial of overlapping long peptides from a
36 tumor self antigen and poly-ICLC shows rapid induction of integrated immune response
37 in ovarian cancer patients. *Clin Cancer Res.* 2012;18(23):6497-6508.

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

1 immunotherapy. *Immunol Rev.* 2006;211:214-224.

2 36. Bendle GM, Linnemann C, Hooijkaas AI, et al. Lethal graft-versus-host disease in mouse
3 models of T cell receptor gene therapy. *Nat Med.* 2010;16(5):565-570, 561p following 570.

4 37. Okamoto S, Mineno J, Ikeda H, et al. Improved expression and reactivity of transduced
5 tumor-specific TCRs in human lymphocytes by specific silencing of endogenous TCR.
6 *Cancer Res.* 2009;69(23):9003-9011.

7 38. Ochi T, Fujiwara H, Okamoto S, et al. Novel adoptive T-cell immunotherapy using a
8 WT1-specific TCR vector encoding silencers for endogenous TCRs shows marked
9 antileukemia reactivity and safety. *Blood.* 2011;118(6):1495-1503.

10 39. Yang L, Baltimore D. Long-term in vivo provision of antigen-specific T cell immunity by
11 programming hematopoietic stem cells. *Proc Natl Acad Sci U S A.*
12 2005;102(12):4518-4523.

13 40. Vatakis DN, Koya RC, Nixon CC, et al. Antitumor activity from antigen-specific CD8 T
14 cells generated in vivo from genetically engineered human hematopoietic stem cells. *Proc*
15 *Natl Acad Sci U S A.* 2011;108(51):E1408-1416.

16 41. Vatakis DN, Arumugam B, Kim SG, Bristol G, Yang O, Zack JA. Introduction of
17 exogenous T-cell receptors into human hematopoietic progenitors results in exclusion of
18 endogenous T-cell receptor expression. *Mol Ther.* 2013;21(5):1055-1063.

19 42. Giannoni F, Hardee CL, Wherley J, et al. Allelic exclusion and peripheral reconstitution
20 by TCR transgenic T cells arising from transduced human hematopoietic stem/progenitor
21 cells. *Mol Ther.* 2013;21(5):1044-1054.

22 43. Melkus MW, Estes JD, Padgett-Thomas A, et al. Humanized mice mount specific adaptive
23 and innate immune responses to EBV and TSST-1. *Nat Med.* 2006;12(11):1316-1322.

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

26 45. Pelletier J, Schalling M, Buckler AJ, Rogers A, Haber DA, Housman D. Expression of the
27 Wilms' tumor gene WT1 in the murine urogenital system. *Genes Dev.*
28 1991;5(8):1345-1356.

29 46. Coulie PG, Brichard V, Van Pel A, et al. A new gene coding for a differentiation antigen
30 recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J Exp Med.*
31 1994;180(1):35-42.

32 47. Kawakami Y, Eliyahu S, Delgado CH, et al. Cloning of the gene coding for a shared
33 human melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc*
34 *Natl Acad Sci U S A.* 1994;91(9):3515-3519.

35 48. Wiesel M, Oxenius A. From crucial to negligible: functional CD8(+) T-cell responses and
36 their dependence on CD4(+) T-cell help. *Eur J Immunol.* 2012;42(5):1080-1088.

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

1 stratification in acute myeloid leukemia: a European LeukemiaNet study. *J Clin Oncol*
2 2009;27(31):5195-5201.

3 50. Lange T, Hubmann M, Burkhardt R, et al. Monitoring of WT1 expression in PB and
4 CD34(+) donor chimerism of BM predicts early relapse in AML and MDS patients after
5 hematopoietic cell transplantation with reduced-intensity conditioning. *Leukemia*.
6 2011;25(3):498-505.

7 51. Gattinoni L, Lugli E, Ji Y, et al. A human memory T cell subset with stem cell-like
8 properties. *Nat Med*. 2011;17(10):1290-1297.

9 52. Gattinoni L, Klebanoff CA, Restifo NP. Paths to stemness: building the ultimate
10 antitumour T cell. *Nat Rev Cancer*. 2012;12(10):671-684.

1 **Figure Legend**

2

3 **Figure 1**

4 **HLA expression and multi-lineage human immune subsets in HLA Class I Tg NSG**
5 **humanized mice.**

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

22

23 **Figure 2**

24 **Immunization of HLA Class I Tg NSG recipients using a WT1 antigen.**

25 (A) Representative contour plots show development of WT1-specific CD8+ CTLs in PB from
26 an HLA class I Tg NSG recipient before and after immunization using HLA-A*2402

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

15

16 **Figure 3**

17 **Transplantation of WT1 specific TCR gene transduced HSCs into NSG-HLA-A24/HHD** 18 **recipients**

19 (A) Purification of CB CD34+CD38- cells using a CD34+ cell enriched CB sample (left panel).
20 WT1 TCR V α and V β genes were transduced into the isolated cells with a lentiviral vector at
21 an MOI of 100. After 5 days of incubation, GFP+ cells were sorted and transplanted into
22 NSG-HLA-A24/HHD newborns (right panel). (B) Multi-lineage analysis of WT1-TCR gene
23 transduced NSG-HLA-A24/HHD recipients. Representative plots of spleen and thymus are
24 shown. (C) Frequencies of B cells and T cells gated on human CD45+ leukocytes in spleen
25 and thymus of NSG-HLA-A24/HHD recipients transplanted with non-transduced, GFP control
26 transduced or WT1-TCR gene transduced HSCs. (D) Frequencies of human T cell subsets in

1 the spleen and thymus of NSG-HLA-A24/HHD WT1 TCR gene transduced HSC recipients
2 (spleen, n = 6 for non-transduced or GFP control recipients, CD4+ 64.3 +/- 3.1%, CD8+ 28.5
3 +/- 3.5%, n = 11 for WT1 TCR gene transduced HSC recipients, CD4+ 50.8 +/- 7.4%, CD8+
4 29.0 +/- 5.3%. Thymus, n = 5 for non-transduced or GFP control recipients, CD4+ 46.7 +/-
5 7.1%, CD8+ 29.7 +/- 3.8%, DP 20.4 +/- 5.6%, n = 15 for WT1 TCR gene transduced HSC
6 recipients, CD4+ 25.1 +/- 3.8%, CD8+ 35.2 +/- 4.3%, DP 33.6 +/- 6.6%). Open and closed
7 circles indicate non-transduced and GFP control recipients, respectively. Bars indicate the
8 mean value.

9

10 **Figure 4**

11 **CTL analysis of NSG-HLA-A24/HHD WT1 TCR gene transduced HSC recipients**

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

23

24 **Figure 5**

25 **In vitro expanded CTL derived from NSG-HLA-A24/HHD WT1 TCR gene transduced**
26 **HSC recipients exerted HLA-restricted antigen specific response.**

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

19

20 **Figure 6**

21 **Skewed TCR repertoire in human CD8+ T cells developed in NSG-HLA-A24/HHD WT1** 22 **TCR gene transduced HSC recipients**

23 CD8+GFP+tetramer+ T cells were purified from an NSG-HLA-A24/HHD mouse transplanted
 24 with WT1-specific TCR transduced human HSCs. Human CD8+ T cells were also purified
 25 from control mice engrafted with non-transduced human HSCs. Pie Charts depict the major
 26 repertoire greater than 1%. The minor repertoires less than 1% are indicated as "others"

Table 1. Characteristics of HLA class I Tg NSG recipients

Therapeutic Group	Recipient ID	CB ID	HLA	PB Chimerism (%)	Spl Chimerism (%)	Spl CD3+ / hCD45+ (%)	Spl CD33+ / hCD45+ (%)	Spl WT1 tet+ / CD8+ (%)
No Immunization n = 10	A2-1	1-030304	*A2+A24+	39.6	92.2	72.4	4.2	0.039
	A2-2	23-4	A0201/0206	93.4	94.0	57.4	4.3	0.015
	A2-3	23-35	A0201/0206	60.1	84.1	89.2	2.4	0.000
	A2-4	23-35	A0201/0206	65.7	77.1	84.4	2.0	0.029
	A24-1	22-52	*A2-A24+	96.1	99.1	81.0	1.5	0.062
	A24-2	22-58	A0206/2402	76.1	98.3	69.2	2.7	0.071
	A24-3	23-3	A0206/2402	81.2	92.4	39.3	4.6	0.061
	A24-4	23-11	A1101/2402	94.6	98.5	87.7	1.3	0.045
	A24-5	24-13	A2402/-	64.8	88.8	19.2	2.5	0.048
	A24-6	24-19	A2402/3303	30.9	87.9	59.7	4.2	0.041
				70.3 +/- 7.1	91.2 +/- 2.2	65.9 +/- 7.2	3.0 +/- 0.4	0.041 +/- 0.007
Immunized without DC n = 10	A2-5	20-71	A0206/2402	23.7	N.A.	N.A.	N.A.	0.208
	A2-6	22-7	*A2+A24-	78.9	95.2	58.5	5.3	0.050
	A2-7	22-18	*A2+A24+	97.7	99.2	64.4	10.6	0.030
	A24-7	22-58	A0206/2402	47.1	94.5	69.8	3.8	0.353
	A24-8	22-31	A2402/3201	75.2	75.4	79.1	1.5	0.116
	A24-9	22-29	*A2-A24+	44.1	89.8	36.5	4.7	0.022
	A24-10	23-3	A0206/2402	63.5	89.8	22.5	4.3	0.035
	A24-11	23-3	A0206/2402	50.1	74.7	14.7	5.5	0.287
	A24-12	23-11	A1101/2402	15.1	62.7	39.3	4.5	0.252
	A24-13	24-13	A2402/-	58.0	92.6	13.5	1.6	0.349
				55.3 +/- 7.9	86.0 +/- 4.1	44.2 +/- 8.2	4.7 +/- 0.9	0.170 +/- 0.043
Immunized with DC n = 9	A2-8	20-28	A0201/3101	55.8	N.A.	N.A.	N.A.	0.503
	A2-9	07-329	A0202/3303	58.9	N.A.	N.A.	N.A.	0.072
	A2-10	22-63	*A2+A24-	53.8	79.4	44.2	3.3	0.213
	A24-14	22-52	*A2-A24+	66.2	92.5	72.6	4.1	0.201
	A24-15	22-29	*A2-A24+	70.2	93.5	5.9	7.0	0.334
	A24-16	23-3	A0206/2402	39.3	83.1	13.8	1.6	0.248
	A24-17	23-3	A0206/2402	84.5	87.1	78.4	3.8	0.056
	A24-18	23-16	A2402/3303	93.6	95.6	93.0	0.9	0.218
	A24-19	24-13	A2402/-	81.9	94.9	11.9	1.0	0.222
				61.2 +/- 8.3	78.8 +/- 10.3	41.0 +/- 12.0	2.8 +/- 0.7	0.211 +/- 0.046

1 *HLA-A genotypes were not available for these CB-MNCs. Their flow cytometric phenotypes of HLA-A2 and -A24 were shown.

Table 2. NSG-HLA-A24/HHD recipients transplanted with WT1 TCR transduced HSCs

Group	Recipient ID	CB ID	HLA	PB	PB	Spl	Spl	Spl	Spl
				Chimerism	GFP+ /	Chimerism	GFP+ /	CD3+ /	WT1 tet+ /
				(%)	hCD45+ (%)	(%)	hCD45+ (%)	hCD45+ (%)	CD8+ (%)
Non-transduced n = 4	A24-20	23-1	A1101/2601	60.0	-	84.3	-	68.9	0.067
	A24-21	23-44	A0206/3303	87.3	-	97.9	-	35.3	0.021
	A24-22	23-44	A0206/3303	81.2	-	95.3	-	92.8	0.015
	A24-23	24-11	A2402/-	80.3	-	94.3	-	26.7	0.048
				77.2 +/- 5.9	-	93.0 +/- 3.0	-	55.9 +/- 15.3	0.038 +/- 0.012
GFP Ctrl n = 3	A24-GFP-1	24-7	A3101/-	18.5	90.3	64.4	98.8	12.7	0.000
	A24-GFP-2	24-7	A3101/-	1.1	100.0	2.3	99.4	1.2	N.A.
	A24-GFP-3	24-21	A2402/-	57.7	14.0	87.1	22.4	46.7	0.013
				25.8 +/- 16.7	68.1 +/- 27.2	51.3 +/- 25.4	73.6 +/- 25.6	20.2 +/- 13.6	0.006 +/- 0.006
WT1-TCR n = 15	A24-TCR-1	23-1	A1101/2601	47.0	7.5	80.9	31.6	50.2	0.455
	A24-TCR-2	23-1	A1101/2601	63.9	25.7	90.2	33.8	78.6	0.186
	A24-TCR-3	23-44	A0206/3303	45.3	21.3	79.9	32.1	25.4	0.216
	A24-TCR-4	23-44	A0206/3303	51.5	20.2	67.4	27.8	10.9	0.165
	A24-TCR-5	23-44	A0206/3303	53.0	24.7	86.8	36.3	18.4	0.118
	A24-TCR-6	23-44	A0206/3303	27.3	27.2	75.9	73.2	0.7	N.A.
	A24-TCR-7	24-7	A3101/-	9.1	42.7	48.0	83.2	0.3	N.A.
	A24-TCR-8	24-7	A3101/-	0.6	62.2	11.0	79.0	3.3	N.A.
	A24-TCR-9	24-13	A2402/-	78.5	62.8	89.4	74.5	9.9	0.636
	A24-TCR-10	24-15	A3101/3303	50.7	94.6	76.4	92.8	1.4	0.585
	A24-TCR-11	24-21	A2402/-	51.5	2.0	87.2	7.2	68.4	0.073
	A24-TCR-12	24-16	A0201/3303	11.9	92.1	34.0	95.8	13.5	N.A.
	A24-TCR-13	24-16	A0201/3303	28.3	18.1	74.2	64.5	46.7	0.410
	A24-TCR-14	24-15	A3101/3303	10.1	99.0	54.6	98.7	2.3	N.A.
	A24-TCR-15	24-40	A2402/-	16.2	90.2	58.6	95.7	1.0	N.A.
				36.3 +/- 6.2	46.0 +/- 9.2	67.6 +/- 6.1	61.8 +/- 8.2	22.1 +/- 7.1	0.316 +/- 0.070

Table 3. NSG recipients transplanted with HLA A24-expressing K562 cells

Mouse	days	Co-culture		IV transfer of CTLs			Size of tumor (g)	
		# of K562	# of CTLs	cell #	cell #	cell #	With CTLs	w/o CTLs
1	24	1.0E+06	5.0E+06	3.4E+06	5.0E+06	6.0E+06	1.69	3.61
2	24	1.0E+06	5.0E+06	3.0E+06	4.0E+06	2.0E+06	2.49	5.3
3	25	4.0E+05	4.0E+06	2.0E+06	1.3E+06	8.0E+05	0.592	0.615
4	24	7.5E+05	5.7E+06	2.5E+06	1.2E+06	3.1E+06	0.697	1.652
5	28	4.8E+05	2.4E+06	3.0E+06	5.0E+06	5.4E+06	0.107	0.283

1

Figure 1

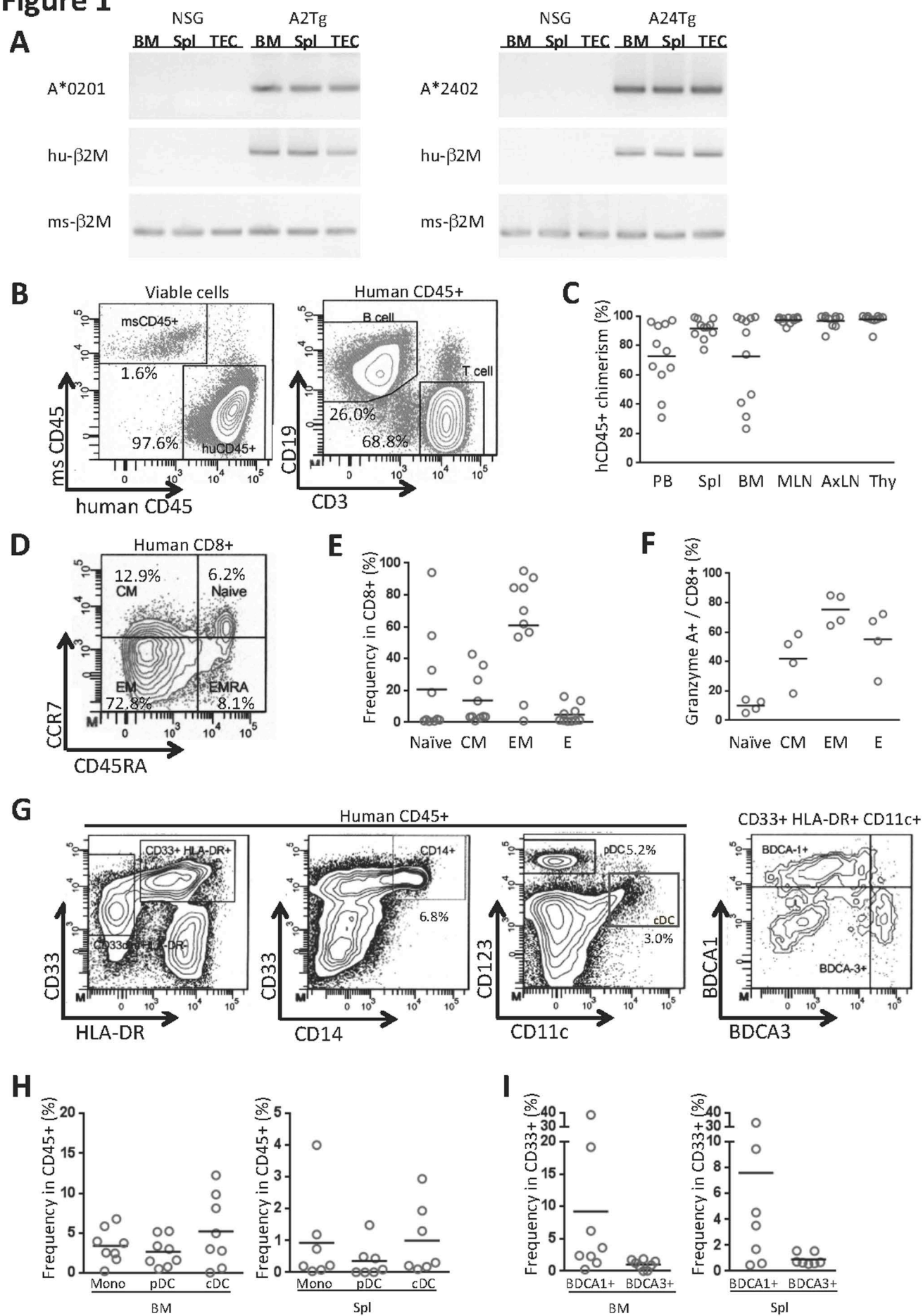


Figure 2

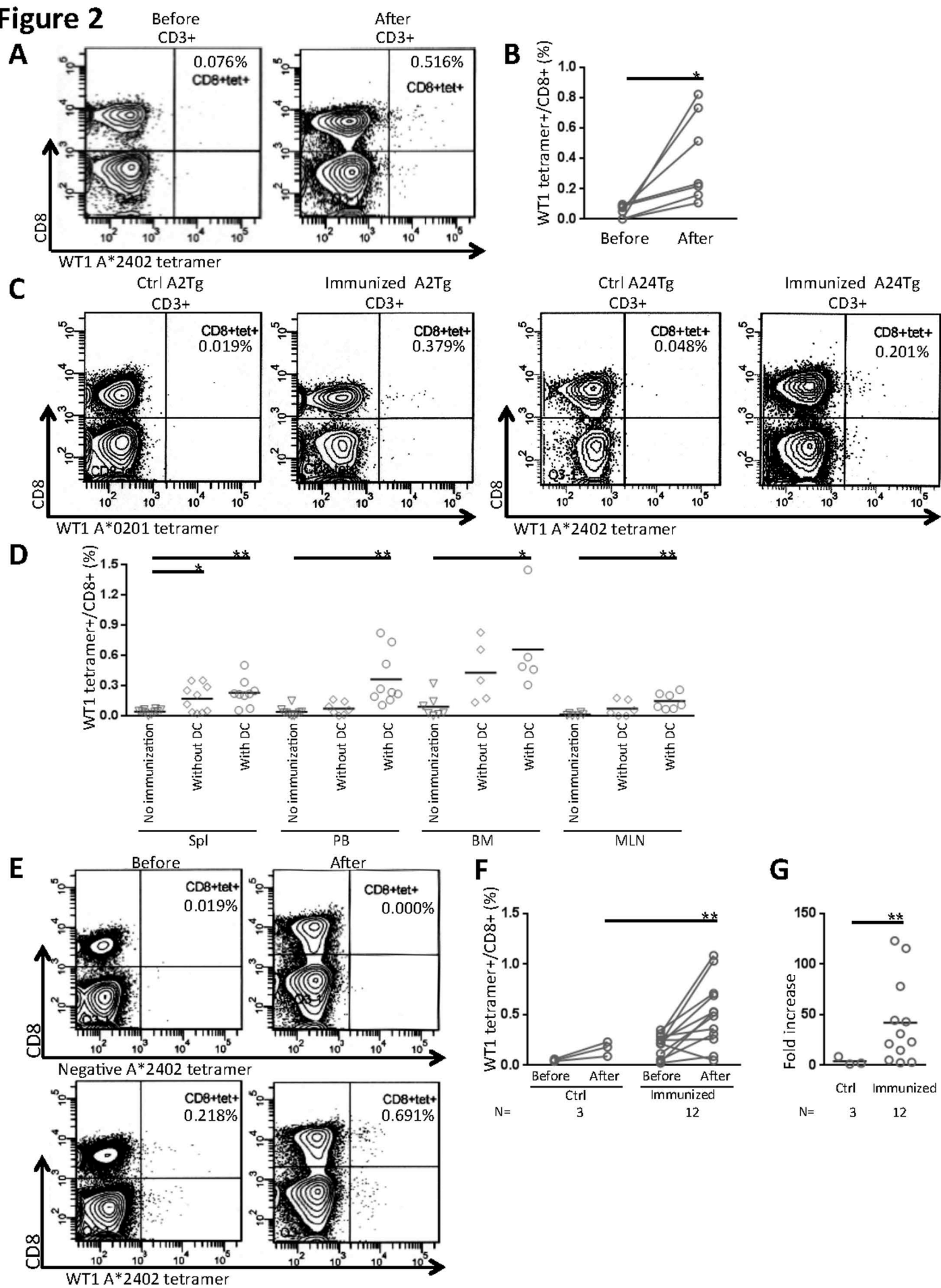


Figure 3

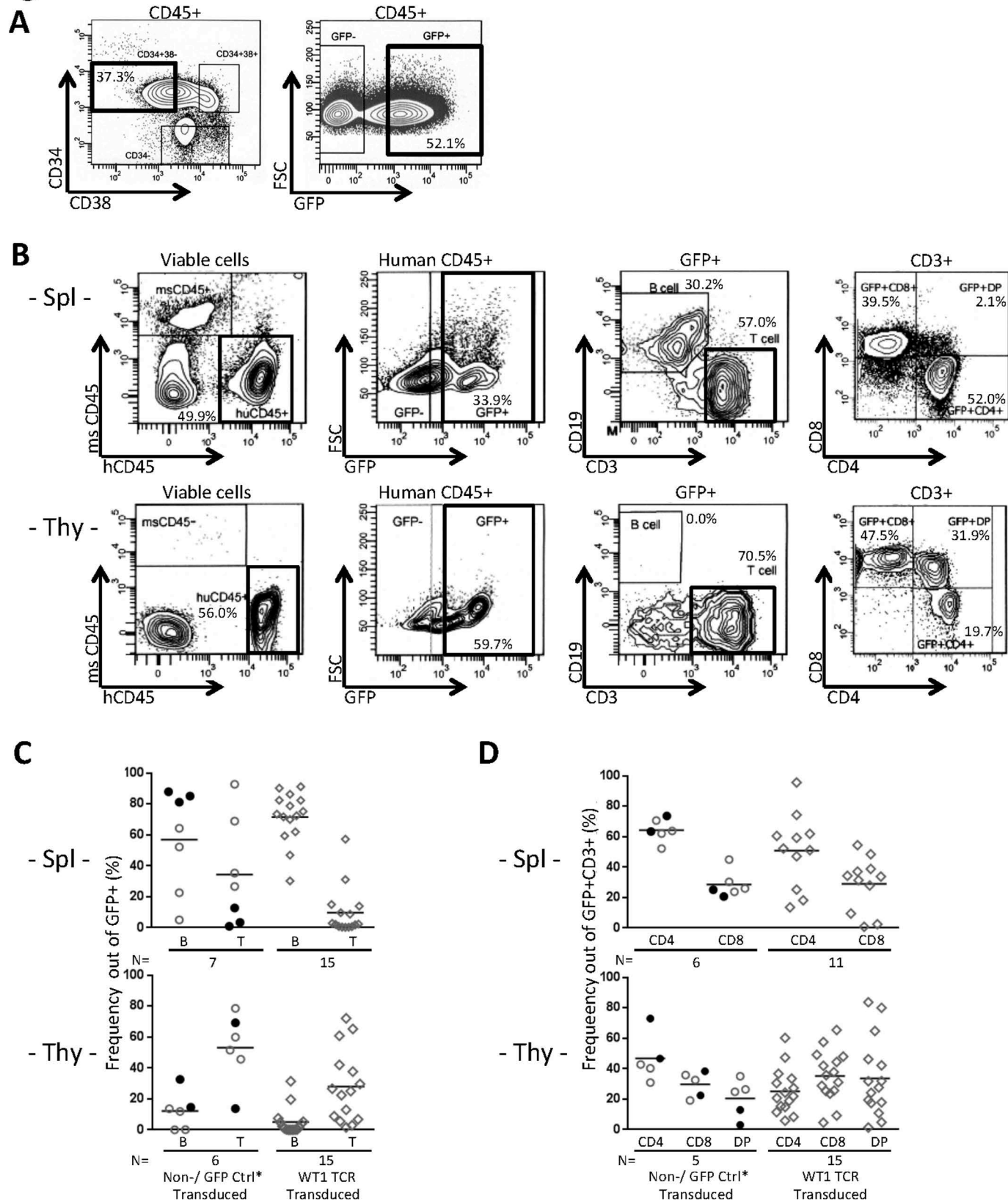


Figure 4

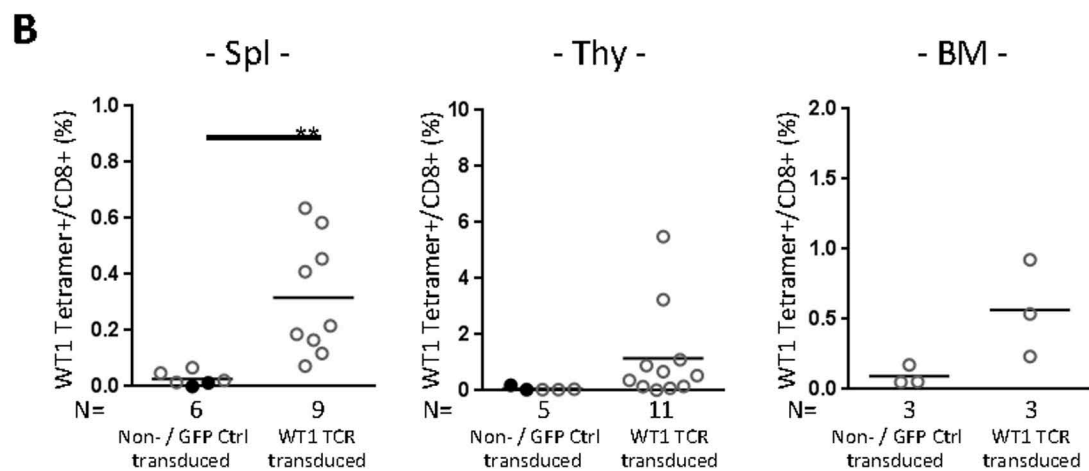
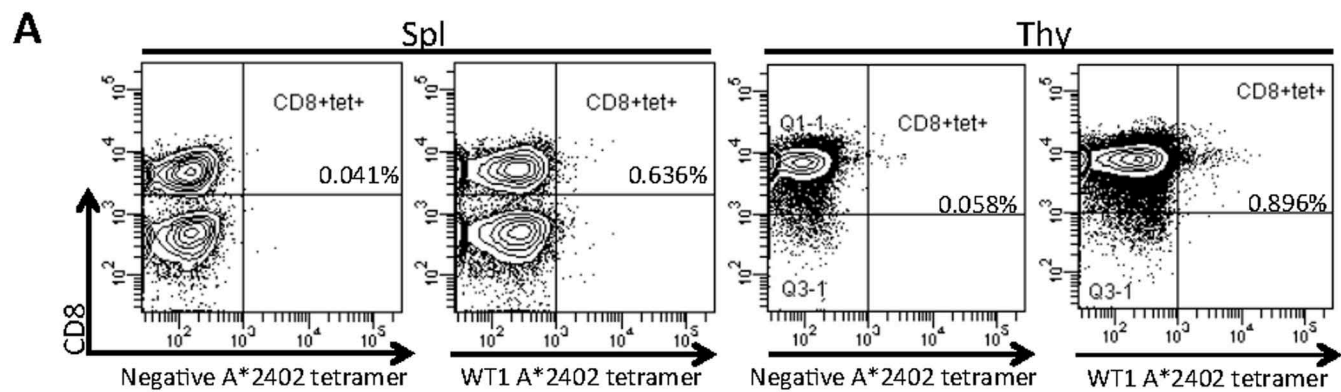


Figure 5

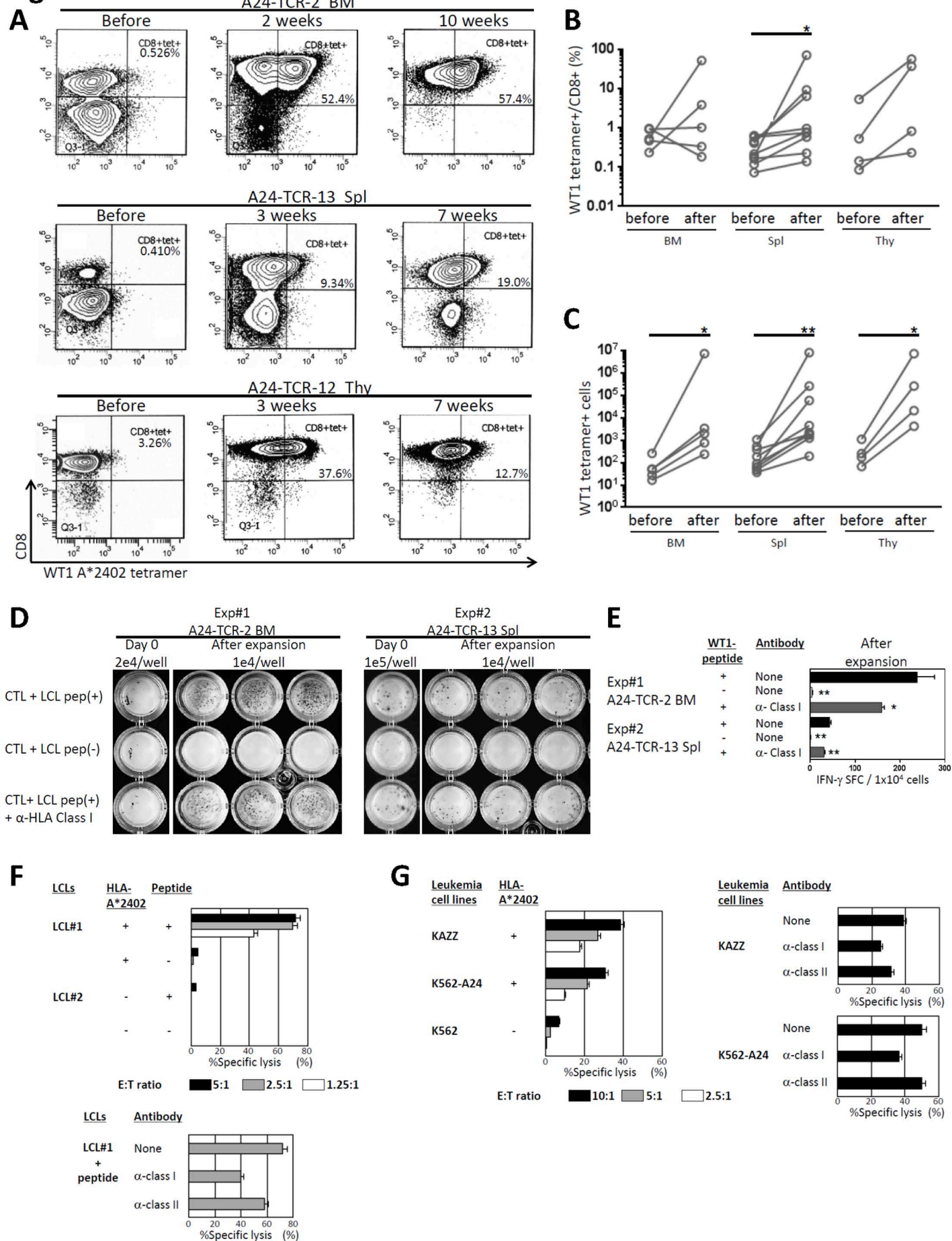


Figure 6

