Development of a Wilms' tumor antigen-specific T-cell receptor for clinical trials: engineered patient's T cells can eliminate autologous leukemia blasts in NOD/SCID mice

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ABSTRACT

Background
The Wilms' tumor antigen (WT1) is an attractive target for immunotherapy of leukemia. In the past, we isolated and characterized the specificity and function of a WT1-specific T-cell receptor. The goal of this translational study was to develop a safe and efficient WT1-T-cell receptor retroviral vector for an adoptive immunotherapy trial with engineered T cells.

Design and Methods
We generated a panel of retroviral constructs containing unmodified or codon-optimized WT1-T-cell receptor α and β genes, linked via internal ribosome entry sites or 2A sequences, with or without an additional inter-chain disulfide bond in the T-cell receptor constant domains. These constructs were functionally analyzed *in vitro*, and the best one was tested in an autologous primary leukemia model *in vivo*.

Results
We identified a WT1-T-cell receptor construct that showed optimal tetramer staining, antigen-specific cytokine production and killing activity when introduced into primary human T cells. Fresh CD34⁺ cells purified from a patient with leukemia were engrafted into NOD/SCID mice, followed by adoptive immunotherapy with patient's autologous T cells transduced with the WT1-T-cell receptor. This therapeutic treatment evidently decreased leukemia engraftment in mice and resulted in a substantial improvement of leukemia-free survival.

Conclusions
This is the first report that patient's T cells, engineered to express the WT1-T-cell receptor, can eliminate autologous leukemia progenitor cells in an *in vivo* model. This study provides a firm basis for the planned WT1-T-cell receptor gene therapy trial in leukemia patients.

Key words: WT1, TCR, gene therapy, immunotherapy, leukemia.

Citation: Xue SA, Gao L, Thomas S, Hart DP, Xue JZ, Gillmore R, Voss RH, Morris E, and Stauss HJ. Development of a Wilms' tumor antigen-specific T-cell receptor for clinical trials: engineered patient's T cells can eliminate autologous leukemia blasts in NOD/SCID mice. Haematologica. 2010; 95.126-134. doi:10.3324/haematol.2009.006486

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S-AX and LG contributed equally to this paper.

Funding: this study was supported by Leukemia Research, UK

Manuscript received on February 5,2009. Revised version arrived on June 12, 2009. Manuscript accepted on July 3, 2009.

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The online version of this article has a supplementary appendix.

Introduction

In efforts to identify leukemia-associated antigens, the Wilms' tumor antigen (WT1) has emerged as an attractive target for immunotherapy of leukemia. WT1 is overexpressed in many types of leukemia, such as acute myeloid leukemia, chronic myeloid leukemia and myelodysplastic syndromes,[1-5] and the levels of WT1 mRNA expression at diagnosis correlate inversely with prognosis.[4,6] The levels of WT1 expression have, therefore, been used for monitoring minimal residual disease in patients with leukemia.[7-15] WT1-specific cytotoxic lymphocytes (CTL) were detected in patients with leukemia[16,17] and breast cancer,[18] and a correlation between graft-*versus*-leukemia effects and detectable WT1-specific CTL was observed after allogeneic stem-cell transplantation for chronic myeloid leukemia[19] and acute lymphocytic leukemia.[20] Clinical trials in which leukemia patients were vaccinated with WT1-derived peptides have produced clinical responses, and these responses were associated with an increased frequency of WT1-specific CTL.[21-24]

In the past years our group has focused on developing adoptive T-cell therapy approaches for the treatment of WT1-expressing malignancies. We used the allo-restricted approach to isolate from healthy donors high avidity CTL specific for the WT1-derived peptide pWT126 presented by HLA-A2 class I molecule.[25] We isolated the T-cell receptor (TCR) genes and demonstrated that retroviral TCR gene transfer can redirect the specificity of primary human T cells. Functional analysis of the redirected T cells demonstrated that they displayed cytotoxicity and produced cytokines in a pWT126-specific fashion. The redirected T cells were able to kill fresh leukemia CD34$^+$ cells *in vitro*, and prevent engraftment of a leukemia cell line, BV173, in non-obese diabetic-severe combined immunodeficient (NOD/SCID) mice.[26]

The goal of this study was to develop a safe and efficient retroviral WT1-TCR construct, and validate it in an *in vivo* autologous primary leukemia model for the planned clinical phase I/II trial of leukemia. The retroviral vectors used in our previous studies contained a post-transcriptional regulatory element (PRE) of the woodchuck hepatitis virus, which has been implicated in causing transformation in animal models.[27] Here, we explored to what extent the PRE element is necessary for efficient TCR gene transfer. To enhance TCR expression, we also used codon optimization and introduction of an inter-chain disulfide bond between the WT1-TCR α and β constant domains, which also reduces the risk of mis-pairing between the WT1-TCR and endogenous TCR chains.[28,29]

With our previous vectors, freshly transduced T cells contained only low frequencies of WT1-specific T cells, necessitating antigen-driven *in vitro* expansion. Prolonged *in vitro* culture is associated with the differentiation of transduced cells into end-stage effector T cells that are functionally impaired when adoptively transferred into recipients.[30] Therefore, in this study we aimed to develop a WT1-TCR construct that can achieve high transduction efficacy and generate a high frequency WT1-specific CTL, which will allow us to adoptively transfer freshly transduced T cells. Finally, we designed experiments to test the therapeutic efficacy of WT1-TCR gene transfer in an autologous setting. We were able to purify sufficient numbers of leukemia CD34$^+$ cells and T lymphocytes from a patient with chronic myeloid leukemia in blast crisis and demonstrated that introduction of the WT1-TCR into the patient's T cells enabled rejection of autologous leukemia progenitor cells in immunodeficient NOD/SCID mice.

Design and Methods

Construction of retroviral T-cell receptor constructs

The dual pMP71 vectors for WT1-TCR, Vα1.5-MP71 and Vβ2.1-MP71, have already been described.[26] To construct a single vector expressing both TCR α and β chains, the coding sequences of the WT1-TCR Vβ2.1 and Vα1.5 were amplified by high fidelity polymerase chain reaction and cloned into the original full length-PRE-containing pMP71 vector (WT1-TCR-PRE, Figure 1A). The insertion of Vβ2.1 was through Not1 at the 5'-end, and Sal1 at the 3'-end; and the insertion of Vα1.5 was via Nco1 at the 5'-end, and EcoR1 at the 3'-end. TCR Vβ2.1 and Vα1.5 were linked via an internal ribosome entry site (IRES). To make a short PRE-vector expressing the TCR Vβ2.1 and Vα1.5 (WT1-TCR-sPRE, Figure 1B), the whole TCR cassette Vβ2.1-IRES-Vα1.5 was amplified by polymerase chain reaction and transferred into a truncated short-PRE vector via Not1 at the 5'-end and EcoR1 at the 3'-end. The ΔPRE vector expressing the Vβ2.1-IRES-Vα1.5 cassette (WT1-TCR-ΔPRE, Figure 1C) was constructed by removing the whole PRE element from the original PRE-containg vector via EcoR1 restriction. To make a codon-optimized WT1-TCR, the coding sequences of the Vβ2.1 and Vα1.5 were synthesized by Geneart (Germany) according to optimized codon usage, and then assembled into the ΔPRE-MP71-vector (WT1-Opt-TCR, Figure 1D). WT1-MuR-TCR was constructed by mutating the ATG codon at the end of the IRES into ATC (Figure 1E) so that the translation of Vα1.5 initiated exactly from the ATG codon at the beginning of the TCR-Vα1.5 sequence. To make a TCR with an additional inter-chain disulfide bond in the constant domains, the cysteine molecule was introduced into both TCR α and β chains as described previously,[29] then the WT1-TCR α and β genes were linked via a viral p2A sequence,[31] and the whole cassette Vα1.5-p2A-Vβ2.1 was then cloned into the ΔPRE-MP71-vector (WT1-SS-TCR, Figure 1F). The control Epstein-Barr virus (EBV)-TCR construct was described previously.[32]

Transduction of retroviral T-cell receptor constructs into Jurkat and human peripheral blood mononuclear cells

Retroviral transduction of TCR genes into human peripheral blood mononuclear cells was carried out as described elsewhere.[26] The Jurkat-76 cell line, a cloned human T-cell leukemia line deficient in endogenous TCR expression,[33] was transduced in the same way as the peripheral blood mononuclear cells, but without the need for activation. Forty-eight hours after transduction, expression of TCR transgenes was analyzed by flow cytometry on a LSR II flow cytometer (BD Biosciences), and FACS data were analyzed using FACSDiva software.

Intracellular cytokine detection assays.

Assays were performed in 96-well round-bottom plates in

which 2×10^5 TCR-transduced T cells were incubated with 2×10^5 T2 stimulator cells coated with 100 μM relevant (pWT126: RMFPNAPYL) or irrelevant (pCLG: CLGGLLTMV) peptide in 200 μL of culture medium containing brefeldin A (Sigma-Aldrich) at 1 μg/mL. After incubation for 18 h at 37°C with 5% CO_2, the cells were first stained for surface CD8 and then fixed, permeabilized, and stained for intracellular interferon-γ, interleukin-2 and tumor necrosis factor-α using the Fix & Perm kit (Caltag) according to the manufacturer's instructions. Samples were acquired on a LSR II flow cytometer and the data were analyzed using FACSDiva software (BD Biosciences).

Expansion of T-cell receptor-transduced T cells

TCR freshly transduced bulk T cells were either used directly in functional assays or expanded by antigen-specific stimulation as described previously.[26]

Cytotoxic T lymphocyte assays

CTL assays were performed as described elsewhere.[26] Briefly, 10^6 T2 cells were incubated at 37°C for 1 h in 200 μL assay medium (RPMI 1640 containing 5% heat inactivated fetal calf serum) with 100 μM synthetic peptides (pWT126, pCLG or pWT235: CMTWNQMNL). Tumor cells or peptide-coated T2 cells were then labeled with ^{51}chromium for l h, washed, and added to serial 2-fold dilutions of effector cells in round-bottomed, 96-well plates to obtain a total volume of 200 μL/well. Assay plates were incubated at 37°C in 5% CO_2. After 4 h, 50 μL of supernatants were harvested, diluted with 150 μL of scintillation fluid, and the radiation counted using a Wallac 1450 Microbeta Plus counter. The specific killing was calculated by the equation:

(experimental ^{51}Cr-release – spontaneous ^{51}Cr-release)/(maximum ^{51}Cr-release – spontaneous ^{51}Cr-release) x 100%.

Purification of hematopoietic CD34+ cells

Informed consent was obtained before collecting normal CD34+ cells from bone marrow of adult healthy donors and leukapheresis products of mobilized stem cell donors. As a source of leukemic CD34+ cells, bone marrow or peripheral blood was obtained from patients with acute or chronic myeloid leukemia. Samples were diluted 1:2 in RPMI medium and enriched for mononuclear cells by density-gradient centrifugation (Lymphoprep 1.077 g/mL; Nycromed Pharma AS, Oslo, Norway). CD34+ cells were isolated from the recovered mononuclear fraction by magnetic bead selection using a CD34 microbead kit (Miltenyi Biotec, Germany) according to the manufacturer's instructions. The purity of the cell population ranged from 80% to 95% as determined by FACS analysis using anti-human CD34-phycoerythrin monoclonal antibody (Miltenyi Biotec, Germany).

In vivo *inhibition of engraftment of CD34+ leukemia progenitor cells*

The *in vivo* animal experiments were carried out in accordance with University College London and national guidelines on the care and use of laboratory animals. CD34+/CD19+ leukemia progenitor cells were isolated from a leukapheresis sample taken from a patient with chronic myeloid leukemia in lymphoid blast crisis. Purified primary leukemia progenitor cells (3×10^6) were injected intravenously into each of the 3-month old immunodeficient NOD/SCID mouse. On the following day, the mice were randomly divided into a treatment group and a control group.

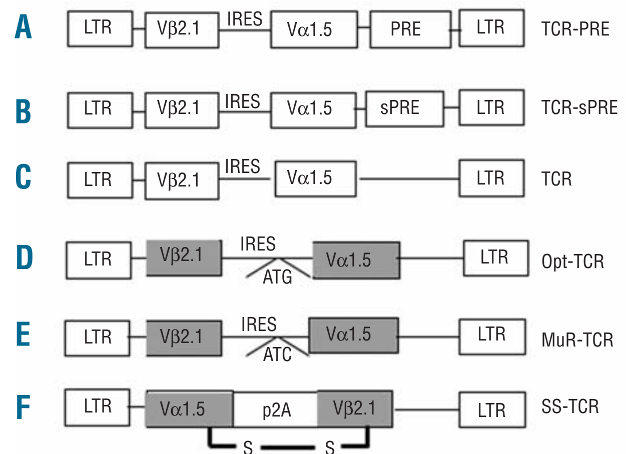

Figure 1. Development of a WT1-TCR construct for clinical trial application. (A). The WT1-TCR Vβ2.1 and Vα1.5, previously cloned in two separate vectors, were linked together with an IRES sequence and transferred into one single vector with the full length post-transcriptional regulatory element (TCR-PRE). To minimize the oncogenic potential of this woodchuck hepatitis virus-derived PRE, the X protein coding sequence was deleted by shortening the PRE (B), or the PRE sequence was removed completely in the TCR construct (C). To improve TCR gene expression, the wild-type TCR sequence was codon-optimized (indicated by gray background) to generate the Opt-TCR construct (D). To initiate the translation of Vα1.5 at exactly the start codon, an ATG codon within the IRES sequence was mutated into ATC to generate the MuR-TCR construct (E). To reduce the risk of mis-pairing, an extra inter-chain disulfide bond was introduced into the TCR constant domains to generate the SS-TCR, and the porcine teschovirus 2A sequence was used to link the TCR α and β genes to favor equimolar expression of the modified TCR chains (F).

Each mouse in the treatment group was injected intravenously with 20×10^6 autologous T cells transduced with WT1-SS-TCR, while each mouse in the control group was given an intravenous injection of 20×10^6 autologous T cells transduced with EBV-TCR. The mice were given irradiated food and water, and monitored for leukemia burden. When the mice showed signs of ill health, they were sacrificed and bone marrows were harvested for FACS analysis to detect engrafted human leukemia cells using triple staining with anti-human HLA-class I-fluoroscein isothiocyanate, CD19-allophycocyanin and CD8-phycoerythrin monoclonal antibodies (BD Biosciences).

Results

Improving the safety of the retroviral vector

Previously we used two separate retroviral vectors to transfer the WT1-TCR α and β genes into doubly infected T lymphocytes.[26] These vectors contained the PRE derived from the woodchuck hepatitis virus, which was thought to be required for efficient TCR gene transfer and expression.[34] As recent studies suggested that this PRE might be oncogenic,[27,35] we aimed to modify or remove it from our vectors. We first assembled a retroviral construct containing the full-length PRE with the WT1-TCR α and β chains linked by an IRES sequence (Figure 1A). To reduce the potential oncogenic activity of this PRE, we then generated a vector variant containing a truncated PRE sequence (Figure 1B) that was unable to encode the X-protein,

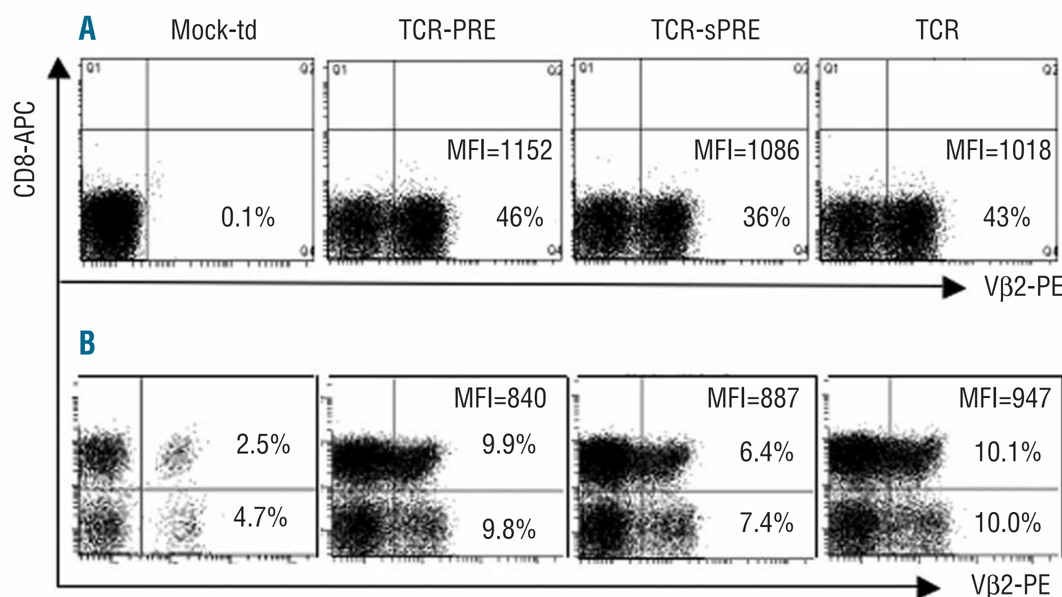

Figure 2. The PRE is not required for efficient TCR gene transfer and expression. (A) The PRE-variant vectors (indicated at the top) were transduced into Jurkat-76 cells. WT1-TCR expression was detected by Vβ2 antibody staining. Mock transduced (Mock-td) Jurkat-76 cells were stained as a control. The percentage of TCR-Vβ2 expressing cells is shown, and the level of Vβ2 expression is indicated by mean fluorescent intensity (MFI). (B) The PRE-variant vectors (indicated at the top) were transduced into peripheral blood derived human T lymphocytes. WT1-TCR expression was detected by Vβ2 antibody staining. Mock transduced lymphocytes were stained to determine the level of endogenous Vβ2 expression. The percentage of Vβ2 expressing cells is shown, and the level of Vβ2 expression is indicated by the MFI. The experiment was repeated three times with similar results, and a typical example is shown here.

which was implicated in the transforming activity of PRE.[27] In order to abolish the oncogenic potential of this PRE completely, we also deleted the whole PRE sequence from the vector, and generated a WT1-TCR retroviral construct without the PRE (Figure 1C).

When these three vector variants containing the same wild-type WT1-TCR were transduced into Jurkat-76 cells, the transduction efficiency and the TCR expression levels were similar, as determined by the percentage of Jurkat-76 cells expressing the Vβ2.1 chain of the WT1-TCR and the mean fluorescence intensity (MFI) of Vβ2 staining (Figure 2A). Similarly, transduction experiments with primary human T cells revealed that the average transduction efficiency and Vβ2 expression levels were again similar for the three vector variants (Figure 2B). These experiments clearly demonstrated that removal of the PRE did not impair either transduction efficiency or WT1-TCR expression level. The PRE-deleted vector variant was, therefore, used for all subsequent experiments.

Improving the functionality of the Wilms' tumor antigen-T-cell receptor

To improve the expression of the WT1-TCR, we first codon-optimized the WT1 Vα1.5 and Vβ2.1 gene sequences. Rare codons were replaced by codon sequences that are frequently used in humans to facilitate t-RNA binding and protein translation. Since the 'generic' IRES sequence separating the TCR-β and the TCR-α genes contains an ATG start codon 12 nucleotides upstream of the ATG start codon of the TCR-α chain (Figure 1D), this may result in the production of a TCR-α chain with an N-terminal extension of four amino acids derived from the IRES element, which could impair the endoplasmic reticulum import of the TCR-α chain. To test directly whether the ATG in the IRES element can impair TCR expression, we generated a vector variant in which the ATG was mutated to ATC (Figure 1E). Finally, to reduce the mispairing between the introduced WT1-TCR and endogenous TCR chains, we introduced an extra inter-chain disulfide bond between the WT1-TCRα and β chains, and linked the two genes with a viral 2A sequence in an attempt to achieve equimolar expression of the cysteine-modified TCR chains (Figure 1F). We used the Jurkat-76 cell line to compare the transduction efficiency and expression of the retroviral vectors carrying the wild type WT1-TCR sequence (Figure 1C), or the three codon-optimized variants (Figure 1D-F). As shown in Figure 3A, Jurkat-76 cells were efficiently transduced by all the constructs (44-56% Vβ2$^+$ cells); analysis of the MFI of Vβ2 indicated that the level of expression of the codon-optimized TCR containing the additional disulfide bond and the 2A sequence (SS-TCR) was approximately 1.4-fold that of the wild-type TCR. The levels of expression of the codon-optimized IRES variants (Opt-TCR and MuR-TCR) were higher than that of the non-codon-optimized IRES construct (WT-TCR), but lower than that of the codon-optimized 2A construct (SS-TCR) (Figure 3A).

Transduction experiments with primary human T cells

showed that all four constructs substantially increased the percentage of Vβ2+-expressing T cells over that seen in mock transduced T cells (Figure 3B). We used HLA-A2 tetramers containing the pWT126 peptide to identify the number of freshly transduced T cells capable of binding the tetramer. The cells transduced with the codon-optimized SS-TCR contained 4- to 10-fold more tetramer binding T cells than the cells transduced with the other WT1-TCR constructs (Figure 3C). This was not due to greater transduction efficiency, as the percentage of Vβ2+ T cells was similar for the four TCR constructs (Figure 3B). The improved tetramer binding is most likely due to the additional disulfide bond, which facilitates correct pairing between the introduced WT1-TCR chains, while at the same time reducing mis-pairing with endogenous chains. As a result, the density of correctly paired WT1-TCR capable of binding tetramer is expected to be highest in cells expressing the SS-TCR construct.

We next determined the percentage of antigen-responsive T cells immediately after transduction. Using our previously published vector system this was usually below 1%, necessitating antigen-driven expansion to increase the frequency of WT1-specific T cells.[26] In contrast, transduction of the TCR constructs developed in this study into primary T cells produced a respectable frequency of antigen-specific T cells. Figure 4A shows the frequency of interleukin-2 and interferon-γ-producing T cells in the freshly transduced T cells when stimulated with pWT126 peptide or an EBV-derived control peptide (pCLG). Following transduction with WT1-SS-TCR, 24.6% of the CD8 T cells produced interleukin-2 and/or interferon-γ after pWT126 peptide stimulation (Figure 4A). The frequency of antigen-responsive T cells was substantially lower after transduction with other WT1-TCR constructs (3.8-5.1% responsive cells). Figure 4B shows a summary of the ability of primary T cells transduced with the indicated TCR constructs to produce interferon-γ, interleukin-2 or tumor necrosis factor-α, demonstrating again that the highest responses were seen with the SS-TCR. Finally, T cells transduced with the SS-TCR had the highest antigen-specific killing activity while maintaining the lowest background killing (Figure 4C).

The WT1-SS-TCR can protect against autologous leukemia

The experiments described above demonstrated that the vector containing the codon-optimized TCR with the additional disulfide bond and the 2A sequence was superior to the other constructs. However, the antigen-specific activity was demonstrated by using peptide-loaded target

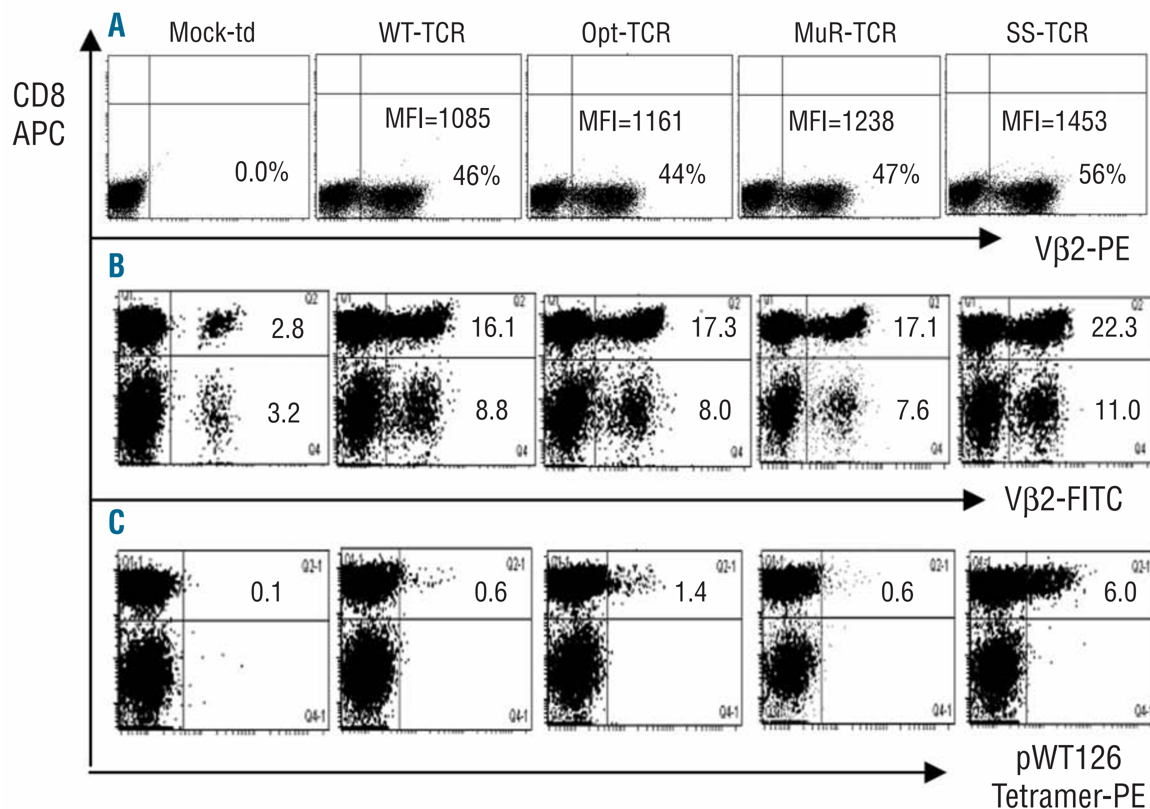

Figure 3. Expression of WT1-TCR variants in Jurkat-76 cells and human peripheral blood mononuclear cells (PBMC). The WT1-TCR variants (indicated at the top) were transduced into Jurkat-76 cells (A) and into human PBMC (B,C), and their expression was analyzed by anti-CD8, Vβ2 antibody and pWT126 tetramer staining (C). Mock transduced cells were also stained as a control. The percentage of Vβ2 expressing cells is shown, and the levels of Vβ2 expression in Jurkat 76 cells are indicated by mean florescent intensity. The experiment was repeated more than three times with similar results, and a typical example is shown here.

cells, and not target cells endogenously expressing WT1 antigen. In preparation for possible clinical application, the specificity profile and anti-leukemia activity of this WT1-SS-TCR construct was further analyzed by using target cells endogenously expressing WT1 antigen. T cells from a healthy donor were transduced with this TCR and expanded *in vitro* then tested against a leukemia cell line, K562-A2, and a panel of CD34$^+$ and CD34$^-$ target cells. As shown in Figure 5A, WT1-SS-TCR-transduced T cells were able to kill K562-A2 tumor cells, but not the K562 cell line, demonstrating the recognition of endogenously processed tumor antigen in a MHC-restricted manner. Specific killing activity was also observed against CD34$^+$ cells isolated from a patient with leukemia, but not against CD34$^-$ cells from the same patient (CML9) or CD34$^+$ cells from a healthy donor (N8) or a cord blood (CB2) sample (Figure 5B). This is in agreement with the observation that WT1 is over-expressed in CD34$^+$ leukemia stem/progenitor cells, while its expression is low in differentiated CD34$^-$ cells from patients and in CD34$^+$ cells from healthy donors.[25] This correlated with detectable killing of CD34$^+$ cells from patients and lack of killing of the other cell populations. It is important to note that normal CD34$^+$ cells were not killed by T cells expressing the WT1-SS-TCR (Figure 5B).

Next, we transduced the T cells isolated from a patient with chronic myeloid leukemia in blast crisis with the WT1-SS-TCR or with a control TCR specific for a peptide epitope of the EBV virus (pCLG). The patient's T cells transduced with the two TCR constructs displayed the expected reciprocal killing activity against target cells coated with pWT126 or pCLG peptides (Figure 5C). The patient's transduced T cells were then tested against autologous CD34$^+$ and CD34$^-$ cells. Only the patient's T cells expressing the WT1-SS-TCR, but not those expressing the EBV-specific control TCR, selectively killed CD34$^+$ leukemia cells (Figure 5D).

Having shown that WT1-SS-TCR-transduced patient's T cells were functionally active *in vitro*, we then explored whether they were able to inhibit the engraftment of autologous leukemia progenitor cells in the NOD/SCID mouse model. The mice were first injected intravenously with CD34$^+$ leukemia progenitor cells isolated from the patient, then, 24 h later, with autologous T cells expressing the WT1-SS-TCR or the control EBV-TCR. As shown in Figure 6A, 80% of the mice treated with the control TCR died of leukemia within 12 weeks, while 80% of the WT1-SS-TCR treated mice survived. When mice died dur-

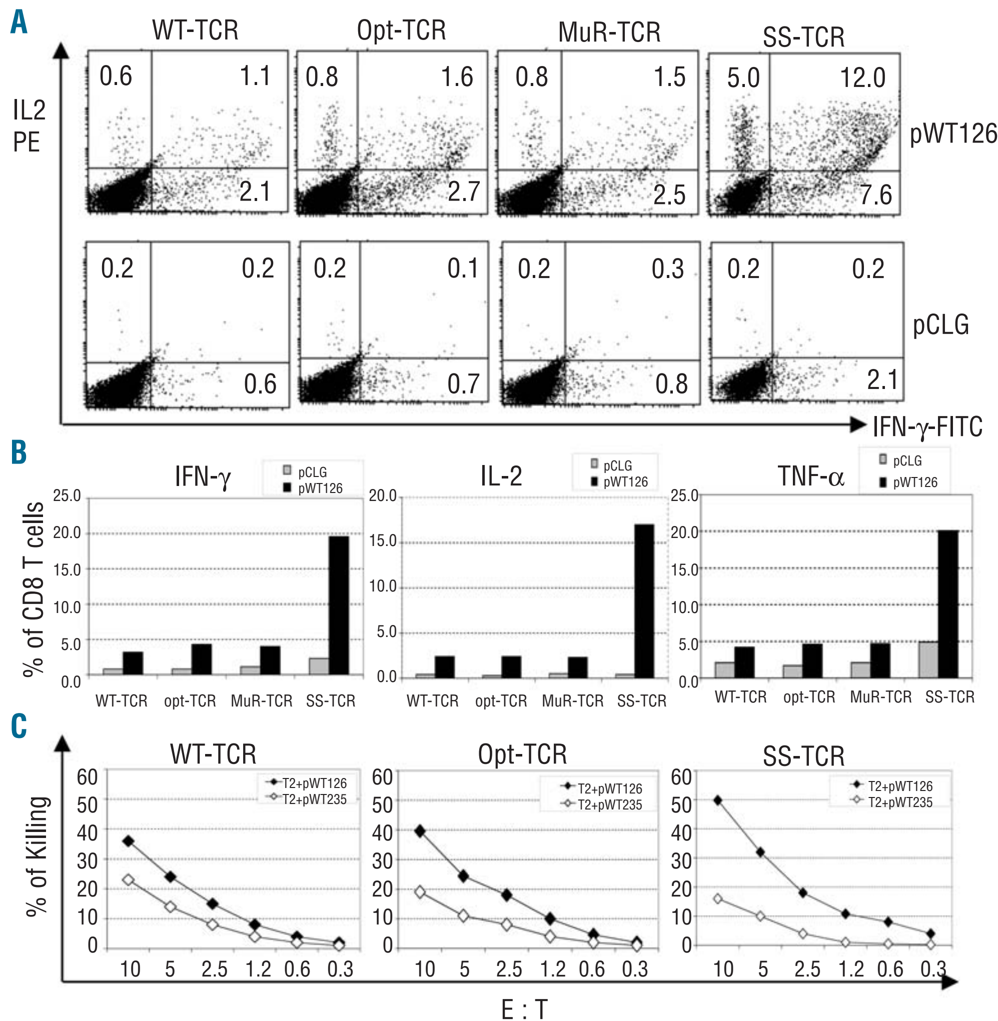

Figure 4. Antigen-specific responses of freshly transduced human T cells. The WT1-TCR constructs (indicated at the top) were transduced into primary human T cells and their functional activities were determined. (A). Antigen-specific interleukin-2 (IL-2) and interferon γ (IFNγ) production was determined after overnight stimulation with specific pWT126 peptide or control pCLG peptide. Representative FACS plot data of intracellular IL2 and IFNγ staining are shown. (B). A summary of all three cytokines (including tumor necrosis factor-α; TNFα) produced by WT1-TCR freshly transduced T cells after stimulation with specific pWT126 or control pCLG peptide. (C). Antigen- specific killing activity of the indicated WT1-TCR variants against target cells coated with the relevant pWT126 peptide or a control pWT235 peptide. The experiment was repeated three times with similar results, and a typical example is shown here.

ing the experiment or when they were killed at the end of the experiment (week 16), the bone marrow was harvested and stained with antibodies against human CD19 and HLA class I. As shown in Figure 6B, four out of five mice that were treated with the control EBV-TCR had human leukemia cells in the bone marrow. The one mouse in the control group whose bone marrow did not contain human leukemia cells was the animal that survived a long time (Figure 6A). In contrast, four animals treated with the WT1-SS-TCR had no detectable leukemia cells in the bone

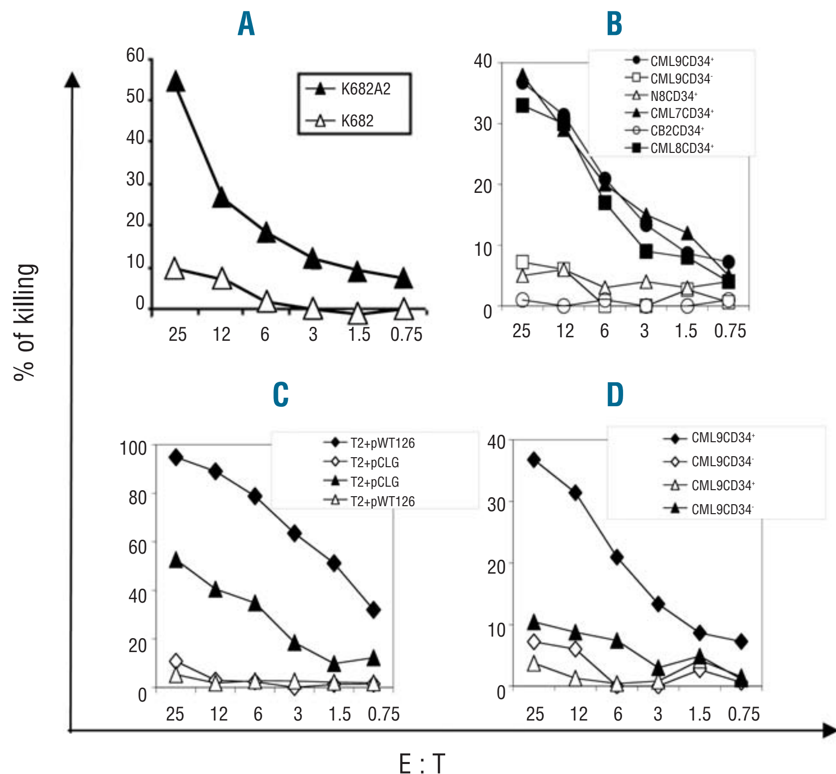

Figure 5. WT1-SS-TCR engineered T cells can recognize endogenously processed tumor antigen and selectively kill autologous CD34+ leukemia progenitor cells *in vitro*. (A). WT1-SS-TCR transduced normal donor T cells killed K562-A2 tumor cells which endogenously express WT1. (B). WT1-SS-TCR transduced normal donor T cells can distinguish CD34+ leukemia progenitor cells from normal CD34+ stem cells or CD34- cells. CD34+ leukemia progenitor cells isolated from three patients with chronic myeloid leukemia (indicated as solid symbols) were killed, but not CD34- cells (from CML9) or CD34+ cells isolated from healthy donor (N8) or a cord blood (CB2) sample. (C). Patient's (CML9) T cells were transduced with WT1-SS-TCR (indicated by diamonds (◆) or EBV-TCR (▲), and their cytolytic activity was tested in a ^{51}Cr release CTL assay. Both TCR transduced patient's T cells can recognize and kill their specific targets (solid symbols) but not control targets (open symbols). (D). WT1-SS-TCR transduced patient's T cells can kill autologous CD34+ leukemia progenitor cells (◆), but not autologous CD34- cells (◇). EBV-TCR transduced patient's T cells (▲) did not kill either target.

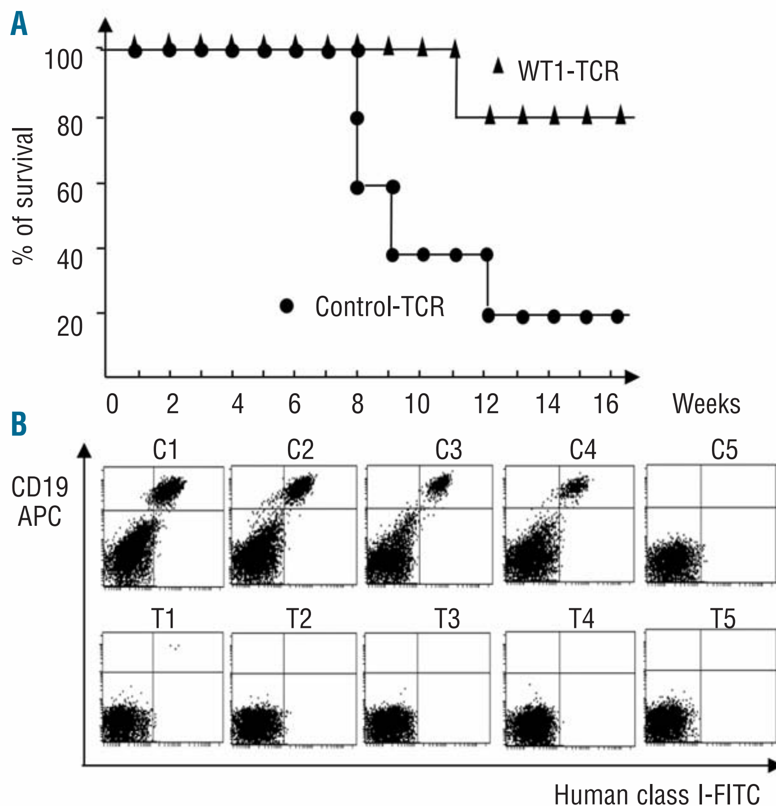

Figure 6. WT1-SS-TCR engineered patient's T cells can inhibit the engraftment of autologous leukemia CD34+ progenitor cells in the NOD/SCID mouse model. (A). WT1-SS-TCR transduced patient's T cells significantly prolonged the survival of mice engrafted with autologous CD34+ leukemia progenitor cells. CD34+ /CD19+ leukemia progenitor cells were isolated from a leukapheresis sample of a patient (CML9) with lymphoid blast crisis, and i.v. injected 3x10^6 into each NOD/SCID mouse. After 24 h, 20x10^6 autologous T cells transduced with WT1-TCR or control TCR were i.v. injected. Mice were monitored for leukemia burden, and were sacrificed when they showed signs of ill health. (B). Elimination of human leukemia progenitor cells in NOD/SCID mice by WT1-SS-TCR transduced autologous T cells. Engraftment of human leukemia progenitor cells was detected by FACS analysis of the harvested bone marrow from control (C) and treated (T) mice after staining with anti-human class I and CD19 antibodies.

marrow (T2-T5). One mouse (T1) that died at 11 weeks (Figure 6A) had a small but detectable number of leukemia cells in the bone marrow. Together, these data indicate that patient's T cells expressing the WT1-SS-TCR were able to protect against the growth of autologous leukemia progenitor cells in the majority of mice.

Discussion

Adoptive immunotherapy of cancer has made significant progress in recent years, with impressive results being achieved in patients with melanoma. The adoptive transfer of *in vitro*-expanded tumor infiltrating lymphocytes into lymphodepleted recipients results in clinical responses in approximately 50% of patients.[36,37] In contrast, the adoptive transfer of TCR gene-modified T cells has been less successful with clinical responses seen in only two out of 15 treated patients.[38] Several factors might contribute to the impaired functional activity of the TCR gene-modified T cells, including the inferior expression of the introduced TCR in gene-modified T cells compared to high expression of endogenous TCR in tumor infiltrating lymphocytes.

In preparation for clinical trials, a major goal of this study was to improve the expression and function of the WT1-TCR in gene-modified T cells. We took advantage of two modifications that were recently shown to improve TCR gene expression.[28,39] Codon optimization leads to more efficient synthesis of TCR chains, and cysteine modification in the TCR constant domains enhances the correct pairing between the introduced TCR chains and reduces mis-pairing between the introduced and endogenous TCR chains. In addition, we linked the TCR α and β genes with a viral 2A element[31] to favor equimolar expression of both TCR chains. Collectively, these modifications produced a new WT1-SS-TCR construct that was at least 5-fold more active than the other retroviral TCR constructs tested in this study.

In the planned clinical trial, we aim to use freshly transduced T cells to avoid the possible loss of functional plasticity that is associated with prolonged *in vitro* culture of T cells.[30] The production of a high frequency of antigen-responsive T cells is a major advantage of the new SS-TCR construct. In this study, up to 25% of freshly transduced CD8 T cells were able to respond to WT1 antigen stimulation, which represents a high frequency that is desirable for our planned clinical trials.

It is possible that improving WT1-TCR function could increase the risk of T-cell attack of normal stem/progenitor cells expressing low levels of WT1. Using purified $CD34^+$ cell populations we found that the T cells transduced with the new WT1-SS-TCR construct showed selective killing of $CD34^+$ cells from leukemia patients but not of $CD34^+$ cells from healthy donors. This suggests that the functionally improved T cells retain the ability to attack leukemic cells selectively.

We used the new WT1-SS-TCR construct to demonstrate that T cells from a patient with chronic myeloid leukemia can be redirected to attack autologous blast cells. To our knowledge, this is the first demonstration that TCR gene-modified patient's T cells can eliminate autologous primary leukemia cells in an *in vivo* model of adoptive T-cell therapy.

Overall, this study demonstrates how combinations of vector and TCR modifications can improve the safety features of TCR gene transfer, while at the same time enhance the expression and function of the introduced TCR chains. This work provides the basis for the use of the WT1-specific SS-TCR in the planned clinical trials in patients with chronic and acute myeloid leukemias.

Authorship and Disclosures

SAX and HJS designed the experiments, SAX, LG, ST, DPH, JZX, and RG performed experiments, RHV, EM and HJS analyzed the data, SAX and HJS wrote the paper.

EM and HJS are consultants for Cell Medica. EM received honoraria from Pfizer and Gilead. The other authors reported no potential conflicts of interest.

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