Specificity for the tumor-associated self-antigen WT1 drives the development of fully functional memory T cells in the absence of vaccination

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Recently, vaccines against the Wilms Tumor antigen 1 (WT1) have been tested in cancer patients. However, it is currently not known whether physiologic levels of WT1 expression in stem and progenitor cells of normal tissue result in the deletion or tolerance induction of WT1-specific T cells. Here, we used an human leukocyte antigen-transgenic murine model to study the fate of human leukocyte antigen class-I restricted, WT1-specific T cells in the thymus and in the periphery. Thymocytes expressing a WT1-specific T-cell receptor derived from high avidity human CD8 T cells were positively selected into the single-positive CD8 population. In the periphery, T cells specific for the WT1 antigen differentiated into CD44-high memory phenotype cells, whereas T cells specific for a non–self-viral antigen retained a CD44^{low} naive phenotype. Only the WT1-specific T cells, but not the virus-specific T cells, displayed rapid antigen-specific effector function without prior vaccination. Despite long-term persistence of WT1-specific memory T cells, the animals did not develop autoimmunity, and the function of hematopoietic stem and progenitor cells was unimpaired. This is the first demonstration that specificity for a tumor-associated self-antigen may drive differentiation of functionally competent memory T cells. (*Blood*. 2011;117(25): 6813-6824)

Introduction

The Wilms Tumor antigen 1 (WT1) is an attractive target for immunotherapy of leukemia and solid tumors as it is expressed at high levels in many malignancies, whereas progenitor and stem cell populations express only low levels of the WT1 transcription factor.[1-5] In acute myeloid leukemia (AML), high WT1 levels are associated with poor prognosis, and the quantitative measurement of WT1 RNA transcripts is now widely accepted as a sensitive molecular marker for monitoring minimal residual disease in patients undergoing chemotherapy or transplantation.[6]

In the past few years, vaccination with WT1 peptides has been tested as a treatment option for various malignancies, including myelodysplasia and leukemia.[7,8] In these studies clinical responses were observed in 60% to 74% of evaluable patients (including stable disease and reduced expression of tumor markers), but correlation with the detection of immunologic responses in peripheral blood was variable. Studies in breast cancer patients showed that WT1-specific T cells were undetectable in peripheral blood, although present in tumor-draining lymph nodes (LNs).[9] Therefore, failure to detect WT1-sepcific T cells in the blood of cancer patients might be the result of selective migration to the site of tumor growth.

It is possible that low-level WT1 expression in normal progenitor cells may result in the central deletion of high avidity WT1-specific T cells or induce unresponsiveness by peripheral tolerance mechanisms. However, there is good evidence indicating that central and peripheral tolerance to WT1 is incomplete. First, vaccination in humans can induce self-restricted WT1-specific T-cell responses in some patients, although their frequency is generally low.[10] In addition, WT1-specific T cells were detectable in leukemia patients after allogeneic stem cell transplantation and their detection correlated with low risk of leukemia relapse.[11] Finally, WT1-specific T cells can be detected in the peripheral blood of healthy persons.[12-17]

It is currently unclear to what extent self-antigen expression in normal tissue shapes the phenotype and functional activity of WT1-specific T cells. For example, recent studies examining the in vivo generation of "natural" memory phenotype T cells have suggested that self-antigen might trigger naive T cells to differentiate into memory phenotype cells.[18-20] In this study, we designed murine model experiments to analyze the thymic development of HLA-A0201–restricted, WT1-specific T cells and to determine the phenotype and function of these cells in the periphery.

Previously, we have used the allo-restricted strategy to isolate HLA-A0201–restricted, pWT126-specific T cells from healthy persons.[21,22] High avidity T-cell lines were established and used to isolate the genes encoding the α- and β-chains of a pWT126-specific TCR. Retroviral TCR gene transfer readily converted human peripheral blood T cells into high avidity, pWT126-specific CTL,[23] which were able to recognize and kill CD34$^+$ cells of leukemia patients but not normal CD34$^+$ hematopoietic progenitor/stem cells. Furthermore, adoptive therapy with TCR-gene transduced T cells resulted in the elimination of autologous leukemia cells in the xenogenic NOD/SCID model.[24]

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In this study, we transferred the WT1-TCR gene into purified murine lineage-negative hematopoietic stem cell (HSCs). Transplantation of the gene-modified stem cells into HLA-A0201 transgenic recipients allowed us to study the phenotype and function of TCR-expressing cells in the thymus and periphery. Surprisingly, WT1-specific T cells were not impaired by central or peripheral tolerance but differentiated instead into memory phenotype T cells able to display antigen-specific effector function. Thus, self-specificity for a tumor-associated antigen can contribute to the establishment of natural memory phenotype T cells.[18,19] The implications of these findings for WT1-based vaccination strategies are discussed.

Methods

Mice

C57BL/6 mice were purchased from Charles River Laboratories or the Comparative Biology Unit of University College London, Royal Free Campus. HLA-A2Kb transgenic (A2Kb Tg) mice on a C57Bl/6 background were a kind gift from Theobald M (University Medical Center, Utrecht, The Netherlands). C57Bl/6 CD45.1 and A2KbxCD45.1 Tg mice were bred and maintained in the Comparative Biology Unit of University College London. All animals were housed in pathogen-free conditions in individually ventilated cages and were kept in accordance with the University and United Kingdom Home Office regulations. All procedures were performed aseptically, and irradiated animals received 2.5% enrofloxacin (Baytril, Bayer) in their water 1 week before irradiation and for 2 weeks after irradiation. Donor mice were 7 to 10 weeks old at the time of bone marrow (BM) harvest. Recipient mice were 12 weeks old at transplantation and received 9.4 Gy irradiation in 2 divided doses at day −2 and day 0. Secondary adoptive transfer recipients were sublethally irradiated (5 Gy) 4 hours before T-cell transfer.

Lentiviral vector constructs

A pSIN second-generation lentiviral vector, containing a spleen focus forming virus LTR promoter and the HIV-1 central polypurine tract cis-active element, was modified for this study. Both the codon-optimized hybrid HLA-A*0201-restricted WT1-specific TCR and the latent membrane protein 2 (LMP2)–specific TCR genes have been described before.[24,25] The WT1-TCR uses the Vβ2.1 and Vα1.5 TCR chains and is specific for the WT126 peptide, RMFPNAPYL. The LMP2-TCR uses the Vβ13.1 and Vα3.1 TCR chains and is specific for the CLG peptide, CLGGLLTMV. The hybrid TCR sequences contain murine constant and human variable region sequences. An additional disulphide bond between the α- and β-chains was created by the introduction of 2 additional cysteine residues in the α- and β-chain constant regions. The genes for the TCR α- and β-chains cloned into the lentiviral vector were separated by a porcine tsechovirus self-cleaving 2A sequence to optimize expression of both TCR α- and β-chain genes. The leader sequence was derived from the pMP71 retroviral vector, and the full-length woodchuck hepatitis virus post-transcriptional regulatory element was truncated to prevent encoding of the oncogenic protein X.[26]

Lentivirus preparation

Human embryonic kidney 293T cells were used to produce WT1-TCR and LMP2-TCR lentiviral particles. A total of 2×10^6 293T packaging cells were cultured in T150cm tissue-culture flasks in Iscove modified Dulbecco medium supplemented with 10% heat-inactivated FCS (Sigma-Aldrich), 1% penicillin and streptomycin (Pen/Strep), and 1% L-glutamine, for 24 hours at 37°C, 5% CO_2. To produce the appropriate lentiviral particles, the 293T cells were cotransfected with a plasmid encoding either WT1-TCR or LMP2-TCR genes, the pMD.G plasmid encoding the vesicular stomatitis virus glycoprotein envelope, and the pCMVΔ8.91 plasmid that encodes genes necessary for the production of functional viral particles. The Fugene6 transfection kit (Roche Diagnostics) was used for packaging cell transfection as per the manufacturer's protocol. Twenty-four hours later, the transfection medium was replaced with fresh IMDM. The lentiviral supernatant was harvested 48 hours later, concentrated 100 times by ultracentrifugation, before resuspension in StemSpam medium (StemCell Technologies) and stored at −80°C until required for transduction. Before storage, serial dilutions of each lentiviral supernatant were added to 1 mL of 5×10^5 $58^{-/-}$ cells, which were cultured in RPMI medium supplemented with 10% FCS, 1% Pen/Strep and 1% L-glutamine, to titrate the multiplicity of infection (MOI). An MOI 10 was used to transduce Lin^- BM cells in all experiments.

HSC isolation, infection, and transfer

Whole BM was harvested from 7- to 10-week-old donor mice (C57BL/6 or A2Kb Tg as specified in each experiment). Briefly, femurs, tibias, and pelvic bones were removed and the BM was harvested by flushing it with cold RPMI medium containing 1% Pen/Strep with a syringe equipped with a 25-gauge needle. Uncommitted BM progenitors, highly enriched in HSCs, were negatively selected using either the lineage cell depletion kit for mouse (Miltenyi Biotec) or the Hematopoietic Progenitor Enrichment kit (StemCell Technologies) following the manufacturer's protocols. The $lineage^-$ selected cells (Lin^- BM cells) were placed in culture at 1×10^6 cells/mL in StemSpan medium (StemCell Technologies) containing 1% Pen/Strep, 100 ng/mL murine stem cell factor, 100 ng/mL human Fms-like tyrosine kinase 3, 100 ng/mL human IL-11, and 20 ng/mL murine IL-3. All cytokines were purchased from PeproTech. The Lin^- BM cells were transduced with the appropriate lentiviral particles at an MOI of 10. At 18 to 24 hours after transduction, the transduced Lin^- BM cells were harvested, washed, and resuspended in RPMI medium containing 1% Pen/Strep at 2.5×10^6 cells/mL. Lethally irradiated recipients received 5×10^5 Lin^- BM cells/each by tail vein injections. Tail bleeds were performed at weeks 5, 7, and 9 after transplantation. Recipient mice were killed 11 weeks after transplantation, and thymus, spleen, LNs, and BM were harvested for phenotypic analysis and functional characterization of T cells.

Adoptive T-cell transfer into secondary recipients

Lentiviral TCR-transduced C57BL/6 Lin^- BM cells were transferred into lethally irradiated A2KbxCD45.1 Tg recipients as described in "HSC isolation, infection and transfer." Seventeen weeks after transplantation the mice were killed, and their spleens were harvested. Untouched CD3 T-cell selection was performed on splenocytes pooled from 5 primary recipients, using a mouse pan-T cell isolation kit (Miltenyi Biotec). A total of 3×10^6 T cells were transferred to secondary A2KbxCD45.1 recipients that were sublethally irradiated (5 Gy) 1 day before the transfer. Tail bleeds were performed at day 9 and day 28 after adoptive transfer. On days 69 and 70, in vivo cytotoxicity assays (See "In vivo cytotoxicity assays") were performed. Splenocytes from the secondary recipients were used in ex-vivo proliferation assays (See "Ex vivo proliferation assays").

Serial HSC transplants

BM cells from primary WT1-TCR HSC transplant recipients (A2KbxCD45.1 to A2Kb transplant) were pooled together and then transferred into lethally irradiated (9.4 Gy) C57BL/6 mice (5×10^6 cells/recipient). Tail bleeds were performed at 3, 5, 8, and 12 weeks after transplantation. Peripheral blood was stained with anti-CD45.1 allophycocyanin-Cy7 (APC-Cy7), CD3 fluorescein isothiocyanate (FITC), B220 phycoerythrin-Cy5 (PE-Cy5), and CD11b APC antibodies to monitor the reconstitution kinetics of all hematopoietic lineages. BM cells from untreated A2KbxCD45.1 Tg mice were used to reconstitute lethally irradiated C57BL/6 mice as a control for donor-derived hematopoietic reconstitution.

Antibodies and FACS analysis

The following fluorescently labeled anti–murine antibodies (BD Bioscience) were used in the study: CD45.1 APC Cy7, CD3 APC, CD3 FITC,

CD4 FITC, CD8a PE Cy5, CD44 APC, CD62L FITC, B220 PE Cy5, CD11b APC, and streptavidin APC. Anti–human Vβ2.1 PE, Vβ2.1 biotin, and Vβ13 were purchased from Immunotech. An LSR II cytometer (BD Bioscience) was used for flow cytometric analysis, and data were analyzed using FlowJo Version 7 software (TreeStar).

In vivo cytotoxicity assays

Splenocytes from female A2Kb Tg mice were peptide loaded with 100μM of either relevant peptide (WT1-TCR WT126p, LMP2-TCR CLGp) or an irrelevant HLA-A0201 presented peptide before labeling with 1.5μM carboxyfluorescein diacetate succinimidyl ester (CFSE; $CFSE^{high}$) or 0.15μM CFSE ($CFSE^{low}$), respectively, for 5 minutes at 37°C. The cells were then washed once in ice-cold RPMI with 8% FCS and twice with ice-cold PBS. Labeled cells were mixed at a 1:1 ratio, relevant: irrelevant targets and a total of 10×10^6 mixed cells were injected per mouse. Eighteen hours later, splenocytes of injected animals were analyzed by flow cytometry to identify CFSE-labeled cells. Control untreated A2Kb Tg mice were injected with labeled target cells, and in vivo cytotoxicity was calculated as previously described. Percentage antigen-specific cytotoxicity was determined using the following formula: $[1 - ((A \div B)/(C \div D))] \times 100$, where A = mean number of relevant peptide-loaded splenocytes at 18 hours in experimental mice; B = mean number of irrelevant peptide-loaded splenocytes at 18 hours in experimental mice; C = mean number of relevant peptide-loaded splenocytes at 18 hours in control mice; and D = mean number of irrelevant peptide-loaded splenocytes at 18 hours in control mice.

Ex vivo proliferation assays

Splenocytes from mice that had been transplanted with WT1 or LMP2-TCR-transduced HSCs, or mice that had received an adoptive transfer of T cells from primary transplant recipients, were labeled with 1.5μM CFSE as in "In vivo cytotoxicity assays" and placed in culture at 1×10^6 cells/mL. CFSE-labeled splenocytes were stimulated with 100μM of relevant (WT126p/CLGp) or irrelevant peptide for 5 days before FACS analysis for CFSE dilution after anti–human Vβ2 or Vβ13 antibody staining. In the case of A2Kb Tg mice that received C57BL/6 TCR-transduced HSC cells, professional antigen-presenting cells were almost completely replaced with donor C57BL/6 BM-derived antigen-presenting cells. Therefore, to ensure adequate antigen presentation, splenocytes were harvested from A2Kb Tg mice, peptide loaded with relevant/irrelevant peptides (100μM), and added to the cell cultures at a 1:1 ratio to the splenocytes from the experimental mice. The final cell concentration was 1×10^6 cells/mL.

ELISA

Supernatants from the ex vivo proliferation assays were harvested on day 5 and stored at −20°C. IL-2 and IFN-γ production after peptide stimulation was determined by performing ELISA on these supernatants using the BD OptEIA mouse IL-2 and IFN-γ ELISA sets, as per the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software Version 5.0, and Microsoft Excel.

Results

TCR gene transfer into stem cells to study the development of HLA-restricted, WT1-specific T cells

Previous studies have shown that retroviral TCR gene transfer into HSCs can serve to analyze the development and function of antigen-specific T cells in the thymus and periphery.[27,28] In these studies, retroviral vectors were used to infect bulk BM cells followed by transfer into myeloablated recipients. We found that lentiviral vectors efficiently transduced purified, lineage-negative stem cells that efficiently reconstituted conditioned recipients and developed into mature T cells. Hence, we assembled a lentiviral vector containing the HLA-A0201-restricted TCR specific for the WT1-derived peptide pWT126 (Figure 1A). The human constant regions of the TCR-α and -β genes were replaced with murine sequences to facilitate expression in murine cells.[29] The constant regions were further modified to encode a cysteine residue to improve the pairing of the introduced TCR-α and -β chains (Figure 1B).[30] HSCs were transduced with the WT1-TCR lentiviral construct before transplantation into myeloablated transgenic A2Kb mice (Figure 1D). The transgenic mice expressed hybrid HLA-A0201 molecules consisting of human α1 and α2 domains, which together form the peptide binding groove required for antigen presentation (Figure 1C). The α3 domain was of murine origin to facilitate appropriate interaction between murine CD8 coreceptors and the hybrid HLA molecule. Importantly, both human and murine WT1 proteins are highly conserved, and the HLA-A0201 presented pWT126 peptide is identical between the 2 species. It has been demonstrated that murine cells expressing murine WT1 can generate the pWT126 for major histocompatibility complex (MHC) presentation and T-cell recognition.[31] Further, the endogenous WT1 expression pattern in normal tissues is very similar in humans and mice.[32] Thus, the HLA transgenic host provides an in vivo model to investigate how physiologic WT1 expression affects the development and function of HLA-restricted, WT1-specific T cells.

WT1-specific T cells are selected in the thymus

Purified Lin^- BM stem cells from A2Kb transgenic mice were transduced with the lentiviral WT1-TCR vector. Using CD4/CD8 phenotyping and staining with antihuman Vβ antibodies, we found expression of the WT1-TCR in immature double-negative thymocytes and also in the more mature double-positive population (Figure 2A) in A2Kb transplant recipients. As expected, the HLA class I-restricted TCR was selected into the CD8 single-positive thymocyte population, with little TCR expression in CD4 single-positive cells. We observed similar frequencies of TCR-expressing cells in the $CD4^+CD8^+$ population and $CD8^+$ cells (Figure 2B), suggesting no preferential loss of WT1-specific T cells at the double-positive to single-positive stage of thymic selection. The relative high frequency of WT1-TCR-expressing thymocytes in the double-negative population is most probably the result of nonphysiologic early expression of the lentiviral TCR construct, before the expression of endogenous TCR-α/β heterodimers. Together, the thymus data were compatible with positive selection of WT1-specific T cells at the double-positive to single-positive stage.

WT1-specific T cells persist in the periphery and differentiate into memory phenotype cells without vaccination

Next, we explored whether TCR-expressing cells were detectable in the periphery of transplanted mice (A2Kb → A2Kb). Mature T cells expressing the WT1-TCR were readily detectable in the spleen, LNs, and BM (Figure 3A). As expected, the WT1-TCR was expressed in peripheral $CD8^+$ T cells and not in $CD4^+$ cells (Figure 3B). Anti-CD3 antibodies were used to measure the level of TCR/CD3 complex expression in all T cells derived from the transplanted stem cell population. There was a substantial reduction in the level of CD3 expression in the WT1-TCR^+ T cells compared with the WT1-TCR^- T cells (Figure 3C). Low expression of the WT1-TCR could be the result of the self-specificity of this TCR, or alternatively it could be the result of low promoter

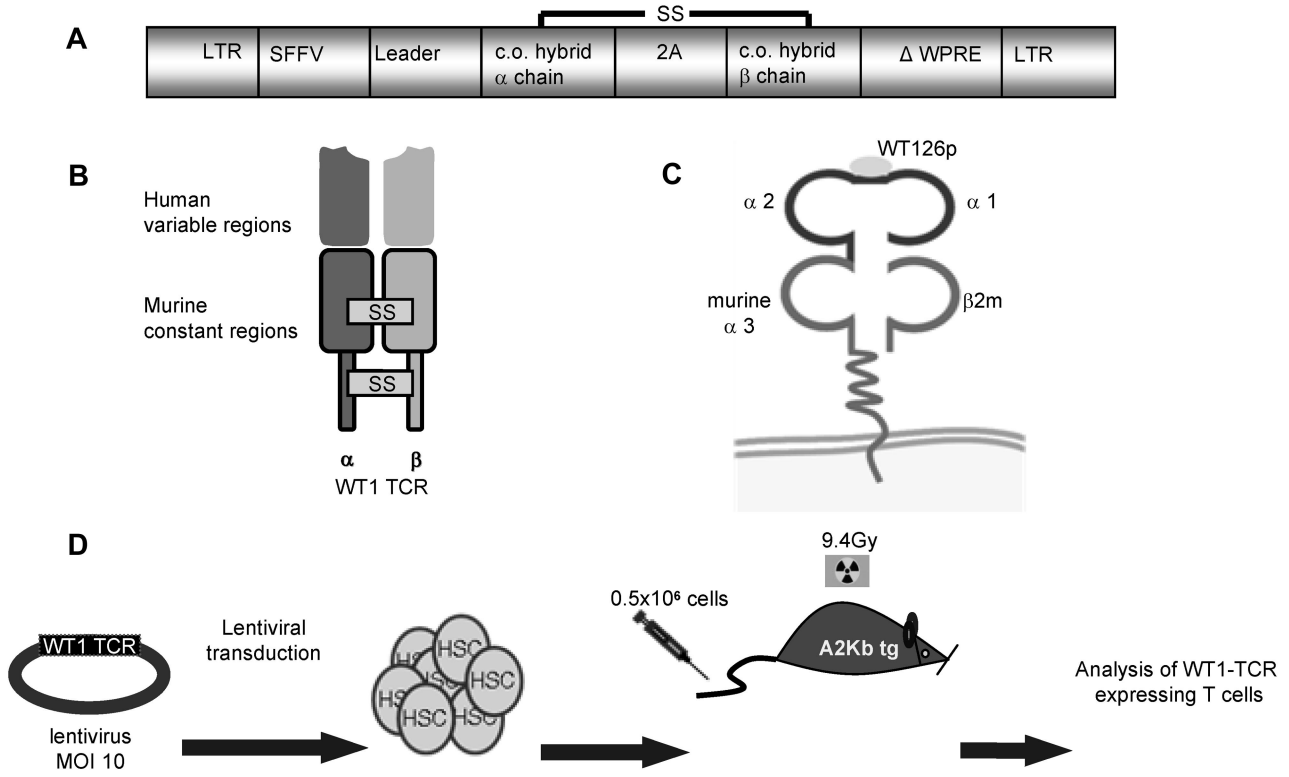

Figure 1. Schematic representations. (A) Schematic representation of the pSIN second-generation lentiviral vector encoding the codon-optimized, murinized hybrid HLA-A0201–restricted pWT126-specific Cys-1 modified TCR α- and β-chain genes (or the HLA-A0201–restricted LMP2-specific Cys1 modified TCR chains) separated by a self-cleaving porcine tsechovirus 2A sequence. SFFV indicates spleen-forming focus virus; LTR, long terminal repeat; and WPRE, woodchuck hepatitis virus posttranscriptional regulatory element. (B) Schematic representation of the Cys-1 modified, codon-optimized hybrid TCR containing murine constant regions (black outline) and human variable regions. (C) Schematic representation of the hybrid HLA-A2Kb molecule expressed in HLA-A2Kb (A2Kb) transgenic mice. The hybrid MHC class I molecules in A2Kb mice contain human alpha1 and alpha2 domains that interact with the human HLA-A2–restricted TCR, fused to a murine alpha3 domain, which facilitates interaction with the murine CD8 coreceptor. (D) Schematic representation of TCR-transduced HSC transplantation. Lineage$^-$ BM stem cells (HSCs) were transduced with lentivirus at an MOI of 10. Each lethally irradiated recipient received 5×10^5 bulk transduced HSCs.

activity of the lentiviral vector driving TCR expression. We therefore replaced the WT1-TCR variable gene segments in the lentiviral vector with the variable region gene segments of an HLA-A0201–restricted TCR specific for a non–self-peptide derived from the LMP2 of Epstein Barr virus.[25] Transplantation experiments with transduced stem cells indicated that mature T cells expressing the LMP2-TCR were readily detectable in the spleen, LNs, and BM of transplanted mice (Figure 3A). As expected, LMP2-TCR expression was limited to CD8$^+$ T cells and not seen in CD4$^+$ T cells (Figure 3B). Importantly, the level of CD3 expression in the LMP2-TCR$^+$ T cells was similar to that seen in control T cells expressing endogenous TCR (Figure 3C). This indicated that the reduced level of TCR/CD3 expression was not caused by poor expression from the lentiviral vector but was a feature of the self-reactive WT1-TCR and not of the non–self-reactive LMP2-TCR.

In previous transgenic models, the down-modulation of TCR and CD8 was described as an important mechanism of peripheral tolerance induction.[33] Unexpectedly, TCR down-modulation in WT1-specific T cells was associated with an up-regulation of CD8 coreceptor expression (Figure 3D). The down-modulation of TCR and up-regulation of CD8 coreceptors were only seen in WT1-specific T cells and not in T cells specific for the non–self-LMP2 antigen (Figure 3D).

Differences in TCR and CD8 expression between WT1 and LMP2-specific T cells correlated with differences in their activation status. The expression of the CD44 activation marker in LMP2-specific (TCR$^+$) T cells was similar to that seen in endogenous control (TCR$^-$) T cells (Figure 4A). In contrast, a large proportion of the WT1-specific T cells expressed high levels of CD44 and CD62L, a phenotype normally associated with central memory T cells (Figure 4A).

Previous studies indicated that survival of naive T cells required tonic TCR stimulation by MHC molecules expressed on hematopoietic cells, whereas memory T-cell survival did not require tonic triggering by hematopoietic cells.[34] However, the role of hematopoietic cells in the generation of memory phenotype T cells in the absence of vaccination has not yet been explored. Hence, we analyzed whether the generation and maintenance of WT1-specific memory phenotype T cells was dependent on antigen-presentation by hematopoietic cells. Stem cells from normal B6 mice were transduced with the WT1-TCR lentiviral vector and transplanted into HLA-A2Kb transgenic B6 recipients. In the transplanted mice, hematopoietic cells, including professional antigen-presenting cells, were unable to present antigen to the HLA-A0201–restricted WT1-TCR. Analysis of WT1-specific T cells in these mice showed that the development of CD44^{high}CD62L^{high} memory phenotype cells was not dependent on antigen presentation by hematopoietic cells (Figure 4A bottom row).

A more detailed analysis of the naive (CD44^{low}CD62L^{high}), central memory (CD44^{high}CD62L^{high}), and effector memory (CD44^{high}CD62L^{ow}) T-cell frequencies in mice expressing WT1-TCR or LMP2-TCR showed that the WT1-TCR–expressing population contained significantly less naive phenotype T cells

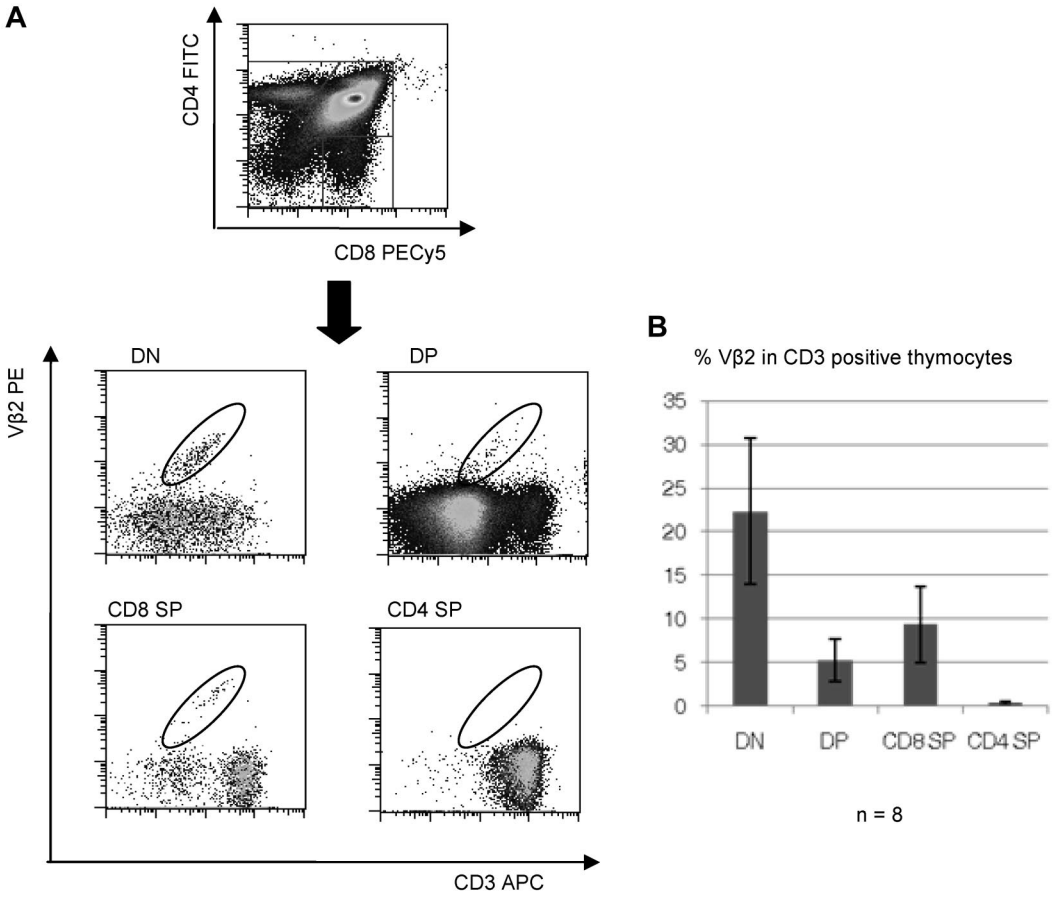

Figure 2. WT1 specific T cells are selected in the thymus. (A) FACS analysis of thymocytes isolated from A2Kb mice transplanted with lin$^-$ A2Kb BM stem cells transduced with the lentiviral WT1-TCR vector. Thymocytes were stained with antimurine CD3, CD4, and CD8 antibodies together with anti–human Vβ2.1 to detect the WT1-TCR β chain. SP indicates single positive; DP, double positive; and DN, double negative. Representative plots from a total of 8 mice are shown. (B) Percentage of murine thymocytes expressing the WT1-TCR as detected by anti–human Vβ2.1 antibody at each stage of thymic selection was determined by FACS analysis after gating on DN, DP, or SP populations. A total of 8 transplanted A2Kb → A2Kb mice were analyzed.

compared with LMP2-TCR–expressing population ($P < .001$; Figure 4B). In contrast, central memory cells were significantly more frequent in the WT1-TCR–expressing population ($P < .001$) in both recipients of TCR-Td A2Kb and TCR-Td B6 stem cells. An increased frequency of effector memory T cells in the WT1-TCR–expressing population (compared with the frequency of LMP2-TCR–expressing effector memory T cells) was only observed in mice reconstituted with A2Kb stem cells ($P < .05$) but not those reconstituted with B6 stem cells (P = not significant; Figure 4B). Therefore, antigen presentation by hematopoietic cells promoted the differentiation of WT1-specific T cells into effector memory phenotype cells. At the time of the phenotypic analysis, the percentage donor chimerism of the CD3$^-$ cells (including antigen-presenting cells) in the B6 → A2Kb transplant recipients showed between 92% and 97% donor cells (data not shown). It is therefore possible that the small number of residual A2Kb$^+$ antigen-presenting cells contributed to, or was sufficient for, the development of central memory and effector memory phenotype cells in the mice transplanted with B6 stem cells.

Within the WT1-specific T cells, the TCR-low population contained primarily memory phenotype cells, whereas the TCR-high population contained both naive and memory phenotype T cells (Figure 4C). Together, these observations suggested that naive phenotype T cells differentiated into memory phenotype cells by a mechanism that involved TCR triggering, which resulted in TCR down-modulation and up-regulation of the CD44 activation marker. It is possible that the lymphopenic environment of transplanted mice facilitates the generation of WT1-specific memory T cells, although this environment does not promote LMP2-specific memory T-cell development.

WT1-specific T cells display antigen-specific effector function without vaccination

We explored whether WT1-specific T cells, and more specifically the memory phenotype T cells, were able to display antigen-specific effector function. In vivo cytotoxicity assays revealed strong antigen-specific killing activity in A2Kb mice transplanted with WT1-TCR–transduced A2Kb stem cells or B6 stem cells (Figure 5A). In contrast, no antigen-specific killing was observed in mice transplanted with LMP2-TCR–transduced stem cells, although the number of peripheral T cells expressing the LMP2-TCR or WT1-TCR was similar (Figure 5A). Ex vivo, the WT1-specific T cells isolated from both A2Kb → A2Kb and B6 → A2Kb transplanted mice displayed antigen-specific proliferation (Figure 5B), IL-2 and IFN-γ production (Figure 5C, B6 → A2Kb not shown), whereas LMP2-specific T cells displayed no antigen-specific functions (Figure 5B-C). These data indicated that the phenotypic differences between WT1-specific and LMP2-specific T cells correlated with the ability of WT1-specific T cells to display antigen-specific effector function without prior vaccination.

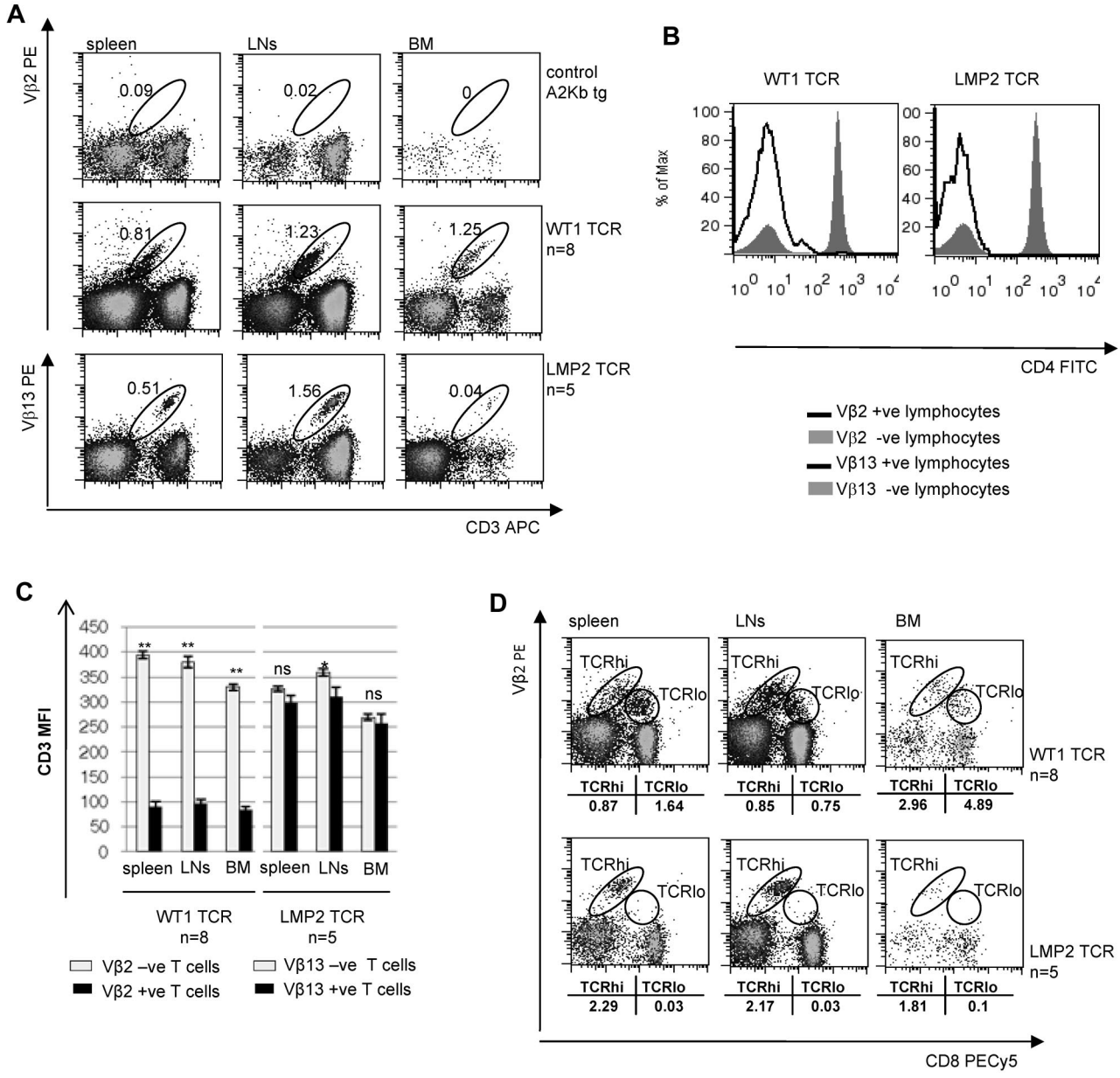

Figure 3. WT1 specific T cells persist in the periphery. (A) FACS analysis of peripheral T cells in spleen, LNs, and BM of A2Kb Tg mice killed 11 weeks after transplantation with TCR-transduced Lin$^-$ A2Kb BM stem cells. Mice received untransduced stem cells (top row), WT1-TCR-transduced stem cells (middle row, n = 8), or LMP2-TCR–transduced stem cells (bottom row, n = 5). Viable lymphocytes were stained with anti-CD3, and anti–human Vβ2.1 (WT1-TCR) and anti–human Vβ13 (LMP2-TCR) antibodies before FACS analysis. Percentages of Vβ2.1$^+$ and Vβ13$^+$ cells in total CD3$^+$ cells are indicated. (B) FACS analysis of splenocytes isolated from A2Kb Tg mice transplanted with Lin$^-$ A2Kb BM stem cells transduced with the lentiviral WT1-TCR or LMP2-TCR vector. Splenocytes were stained with anti–murine CD3, CD4, and CD8 antibodies together with anti–human Vβ2.1 and anti–human Vβ13. (C) Cell surface CD3/TCR complex expression levels in peripheral T cells were determined by FACS analysis after staining with anti-CD3, and anti–human Vβ2.1 and anti–human Vβ13 antibodies. CD3/TCR expression levels of the WT1-TCR (self-reactive) and the LMP2-TCR (non–self-reactive) were compared with endogenous polyclonal T cells derived from transplanted stem cells (Vβ2.1$^-$ and Vβ13$^-$, respectively). Data are mean ± SD of CD3 mean fluorescence intensity (MFI; n = 8 mice for WT1-TCR and n = 5 mice for LMP2-TCR). *$P < 0.05$, 1-way ANOVA. **$P < .01$, 1-way ANOVA. (D) Modulation of TCR and/or CD8 expression was determined by FACS analysis of peripheral T cells of spleen, LNs, and BM stained with anti-CD8, and anti–human Vβ2.1 (WT1-TCR, n = 8) and anti–human Vβ13 (LMP2-TCR, n = 5) antibodies. After gating on viable CD3$^+$ cells, percentages of TCR^{hi} and TCR^{lo} populations are indicated. All recipient mice were A2Kb and received TCR-Td A2Kb stem cells.

To test the in vivo function of WT1-specific memory phenotype T cells, cell transfer experiments were performed to generate mice lacking naive phenotype cells. T cells were purified from A2Kb mice transplanted with WT1-TCR-transduced B6 stem cells and then adoptively transferred into irradiated secondary A2Kb-transgenic recipients. The secondary recipients were unable to generate "new" WT1-specific T cells in the thymus, which was expected to result in the loss of naive phenotype cells and in the persistence of memory phenotype T cells. The analysis of LNs, spleen, and BM of the secondary recipients 10 weeks after transfer demonstrated a loss of the CD44^{low}CD62L^{high} naive phenotype T cells with high TCR expression level and persistence of CD44^{high}CD62L^{high} memory phenotype T cells expressing reduced TCR and elevated CD8 (Figure 6A-B). In transiently lymphopenic hosts, the WT1-specific T cells converted more efficiently into memory phenotype cells than the WT1-TCR negative control

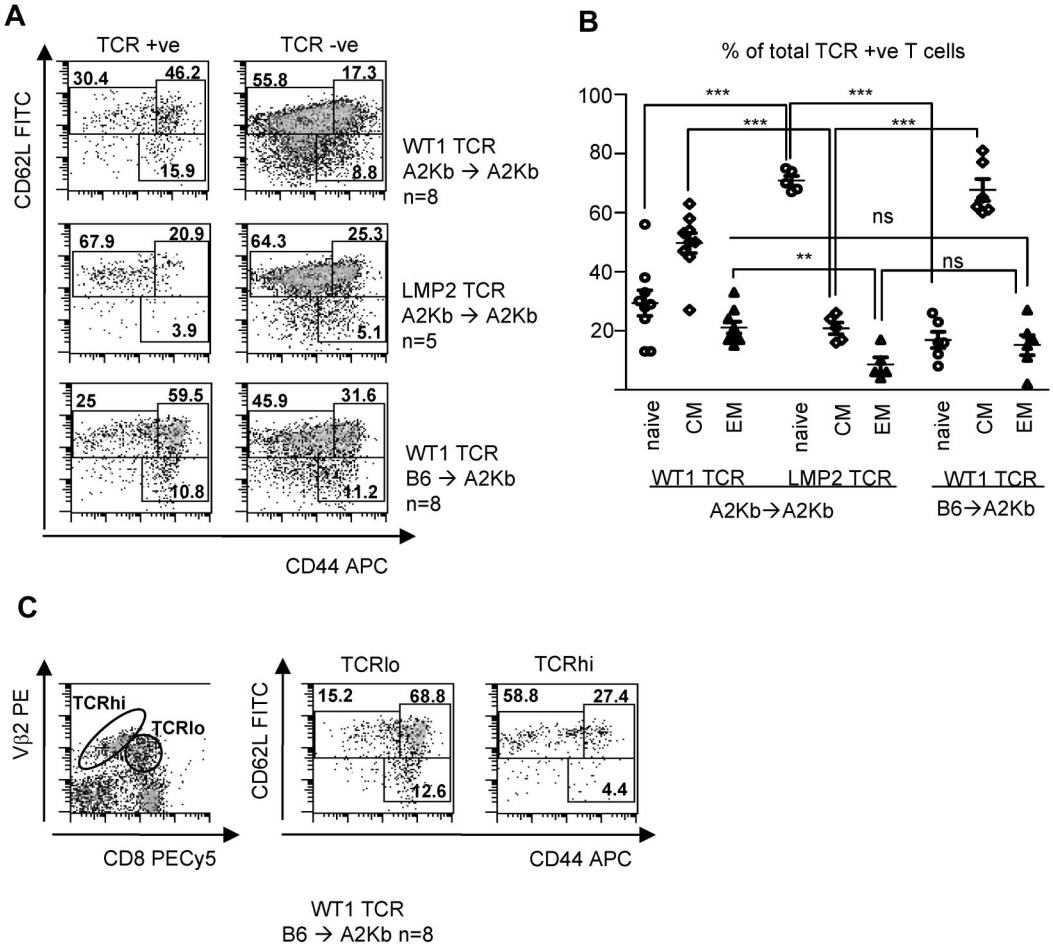

Figure 4. Peripheral WT1-specific T cells differentiate into memory T cells without vaccination. (A) CD44 and CD62L expression on gated TCR-expressing (TCR^+) and gated TCR^- peripheral T cells as determined by FACS analysis after staining with anti–murine CD44 and CD62L antibodies. Similar staining patterns were observed for both splenocytes and LN (shown here). (Top and middle panels) Resentative plots from A2Kb Tg mice transplanted with A2Kb stem cells transduced with the WT1-TCR (top panel, n = 8) or the LMP2-TCR (middle panel, n = 5). (Bottom panel) Representative plots from A2Kb Tg mice transplanted with B6-derived stem cells transduced with the WT1-TCR (n = 8). (B) Summary data of naive ($CD44^{low}$, $CD62L^{high}$), CM ($CD44^{high}$, $CD62L^{high}$), and EM ($CD44^{high}$, $CD62L^{low}$) T-cell frequencies in transplanted mice expressing WT1-TCR or LMP2-TCR. ***$P < .001$. **$P < .01$. A2Kb → A2Kb or B6 → A2Kb as indicated in the figure. (C) Phenotypic analysis of gated WT1-TCR^{hi} and WT1-TCR^{lo} peripheral T-cell populations in A2Kb recipients after transplantation with TCR-Td B6 stem cells, after staining with anti–murine CD44, CD62L, and CD8 antibodies. ns indicates not significant.

T cells expressing endogenous TCRs (Figure 6B left panel). Cytotoxicity experiments in the secondary hosts demonstrated efficient WT1-specific effector function, which correlated with robust WT1-specific proliferation ex vivo (Figure 6C). This clearly demonstrated that the $CD44^{high}/CD62L^{high}$ memory phenotype T cells were capable of displaying antigen-specific effector function without prior vaccination.

WT1-specific T cells accumulate in the BM without impairing stem cell function

Considering that BM resident stem/progenitor cells express low levels of WT1, we explored whether WT1-specific T cells accumulated in the BM of mice transplanted with A2Kb stem cells. Analysis of the donor T-cell compartment showed an increased percentage of WT1-specific T cells in the BM compared with LNs and spleen (Figure 7A). In contrast, analysis of LMP2-TCR transplanted mice demonstrated no enrichment of LMP2-specific T cells in the BM compared with LNs and spleen of these mice (Figure 7A). It was possible that the accumulation of WT1-specific T cells was the result of selective recruitment and proliferation of memory phenotype T cells to the BM, as shown in other studies.[35] However, analysis of WT1-specific T cells showed that the relative frequency of naive and memory phenotype cells was similar in BM compared with spleen and LNs (data not shown). This suggests that the observed accumulation in BM of WT1-specific T cells, but not LMP2-specific T cells, was primarily driven by the specificity and not the memory phenotype of WT1-T cells.

Finally, we examined whether the presence of WT1-specific T cells resulted in damage to the stem/progenitor cells in the BM of transplanted mice. BM was harvested from WT1-TCR transplanted mice and from control mice, followed by transplantation into myeloablated secondary recipients to measure long-term reconstitution of hematopoiesis. Analysis revealed that both myeloid and lymphoid lineages were efficiently reconstituted by BM isolated from donors transplanted with WT1-TCR–transduced stem cells, demonstrating that WT1-specific T cells did not impair the functional competence of the hematopoietic progenitor/stem cells (Figure 7B). However, T-cell engraftment was slower in the experimental mice compared with control mice ($P < .01$). It is possible that lentiviral vector-driven TCR expression in developing thymocytes alters the kinetics of T-cell repertoire selection and T-cell reconstitution in the thymus of transplanted mice.

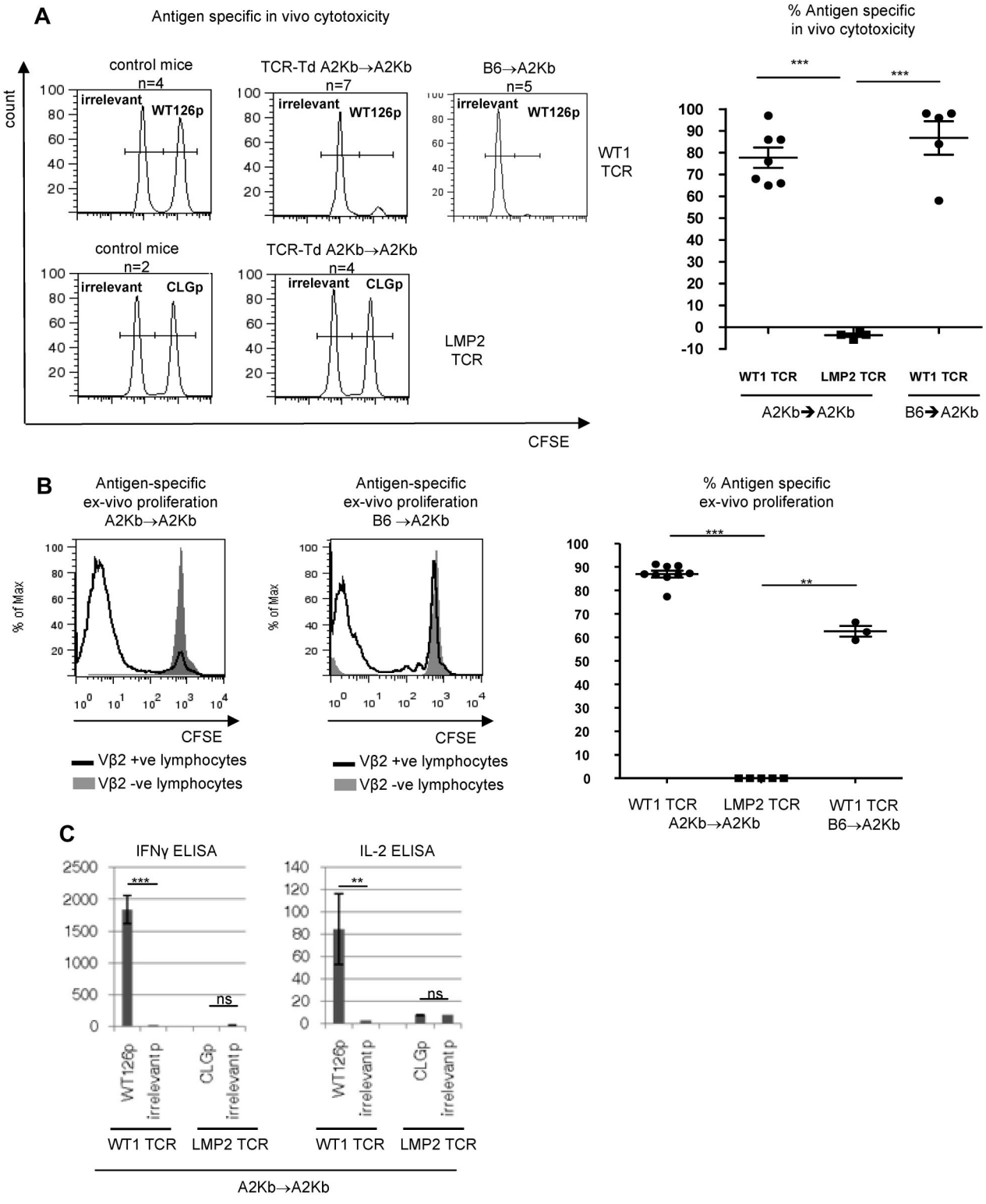

Figure 5. WT1 specific T cells display peptide-specific effector function. (A) In vivo cytotoxicity of CFSE-labeled peptide-loaded target cells. A2Kb Tg mice, 11 weeks after transfer of BM stem cells transduced with the WT1-TCR (top panel, n = 7 A2Kb → A2Kb, n = 5 B6 → A2Kb) or LMP2-TCR (bottom panel, n = 4 A2Kb → A2Kb) were intravenously injected with a 1:1 mix of relevant: irrelevant peptide-loaded A2Kb Tg splenocytes, differentially labeled with CFSE (WT1-TCR is specific for WT126 peptide, and LMP2-TCR is specific for CLG peptide). Eighteen hours later, splenocytes of injected animals were harvested and analyzed by FACS to identify CFSE-labeled cells. Representative plots are shown. Control untreated A2Kb Tg mice were injected with CFSE-labeled peptide-loaded target cells. Summary data of in vivo cytotoxicity assays are shown on the right. Percentage antigen-specific cytototoxicity was calculated as described in "In vivo cytotoxicity assays." ***$P < .001$. (B) Ex vivo proliferation of splenocytes from mice previously transplanted with WT1-TCR and LMP2-TCR-transduced A2Kb or B6 BM stem cells. Splenocytes were stimulated for 5 days with 100μM relevant or irrelevant peptide. CFSE-labeled splenocytes were analyzed by FACS for CFSE dilution after anti–human Vβ2.1 or Vβ13 antibody staining. A representative plot of pWT126-specific proliferation is shown on the left. Summary data of ex vivo proliferation assays using T cells harvested from mice transplanted with WT1-TCR (n = 8 A2Kb → A2Kb, n = 3 B6 → A2Kb) and LMP2-TCR (n = 5 A2Kb → A2Kb) transduced BM stem cells is shown on the right. **$P < .01$, ***$P < .001$. (C) Splenocytes harvested from A2Kb mice transplanted with WT1-TCR and LMP2-TCR-transduced A2Kb BM stem cells were stimulated ex vivo with 100μM relevant or irrelevant peptide for 5 days. ELISAs were performed to detect antigen-specific IFN-γ and IL-2 secretion. ns indicates not significant.

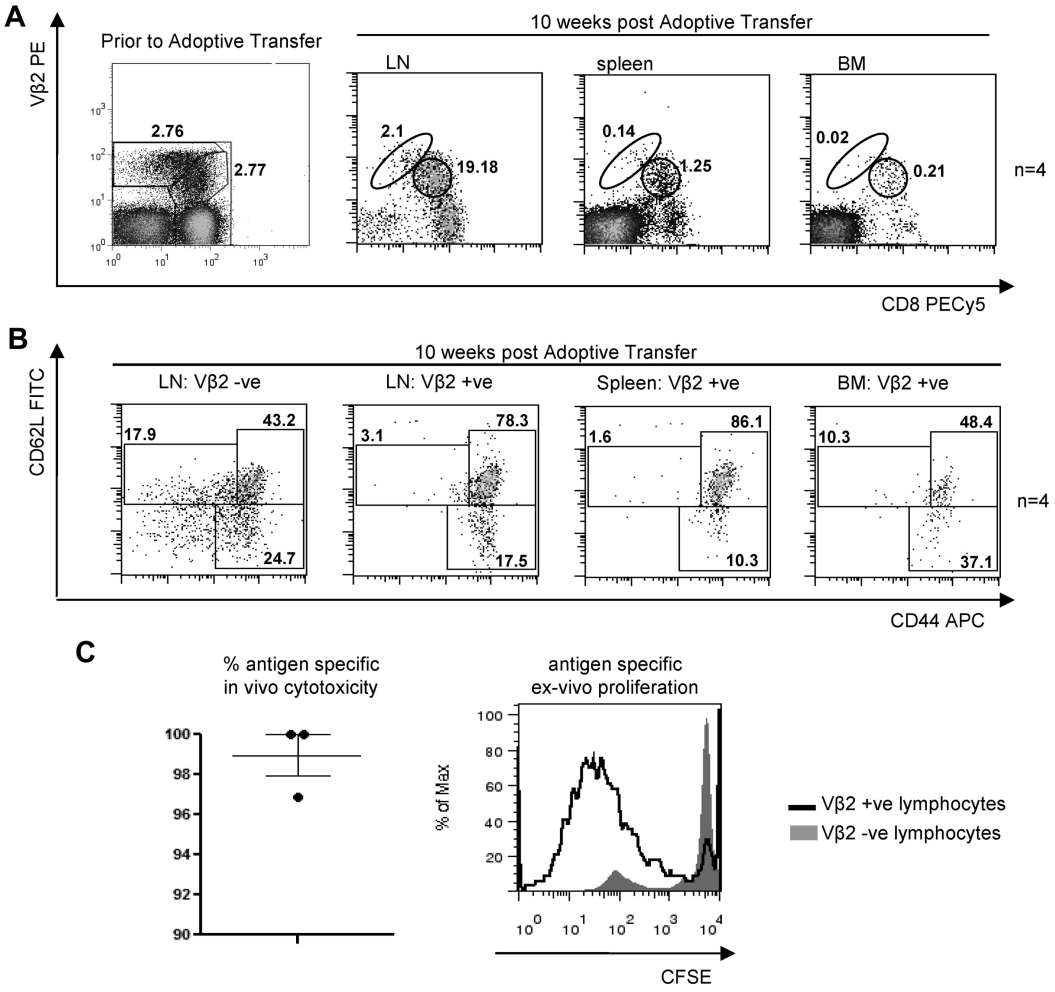

Figure 6. WT1-specific memory phenotype T cells display antigen specific effector function. (A) T cells were purified from A2Kb mice transplanted with WT1-TCR-transduced B6 BM stem cells and used for adoptive transfer into irradiated secondary A2Kb Tg recipient mice (n = 4). At 10 weeks after transfer, CD8/TCR expression of the transferred T cells was determined by FACS analysis of splenocytes, LNs, and BM after staining with antimurine CD8 and antihuman Vβ2.1 antibodies, respectively. (Left panel) The TCR and CD8 profile of the T cells before transfer. (Right panel) The profile 10 weeks after transfer. Percentage of TCR^{hi} and TCR^{lo} populations are shown after gating on total $CD3^+$ cells. (B) Analysis of the naive/memory phenotype of gated WT1-TCR^+ ($V\beta2.1^+$) T cells from spleen, LNs, and BM 10 weeks after adoptive transfer was determined by FACS analysis after staining with anti-CD44 and CD62L antibodies. (Left panel) The naive/memory phenotype of gated WT1-TCR^- T cells in LNs. (C) In vivo cytotoxicity experiments were performed in A2Kb Tg secondary recipients 10 weeks after adoptive transfer of T cells. Mean percentage killing of CFSE-labeled relevant peptide-loaded splenocytes from 3 mice is shown on the left. Ex vivo proliferation of WT1-TCR^+ and WT1-TCR^- T cells after stimulation with relevant peptide was determined by FACS analysis for CFSE dilution after staining with antihuman Vβ2.1 antibodies.

Discussion

We have used TCR gene transfer into HSCs to analyze the thymic development and peripheral function of T cells specific for a WT1 peptide presented by HLA-A0201. In this approach, populations of stem cells carrying distinct gene insertions give rise to mature T cells, thus avoiding possible founder effects of transgenic mice because of insertion of the transgene at a single genomic site. Further, in this model, the TCR-expressing cells represent only a small proportion of the whole T-cell repertoire providing an environment of polyclonal competition that contributes to the survival and maintenance of the peripheral T-cell pool.[36]

The WT1-specific TCR used was derived from high avidity human allorestricted $CD8^+$ T cells that efficiently recognize WT1-expressing human leukemia cells without impairing the function of human hematopoietic stem/progenitor cells.[23] In this study, the TCR was not deleted in the thymus but successfully selected into the single-positive CD8 lineage. Although some studies have demonstrated thymic WT1 expression, we found that the expression was high in the mesothelial lining but not in the epithelial stroma of the thymus, in agreement with previously described WT1 expression in cells with dual mesenchymal and epithelial properties, such as mesothelium, renal podocytes, and testicular Sertoli cells.[37] Our data indicate that physiologic WT1 expression does not prevent selection of a high-avidity WT1-specific human TCR.

Unexpectedly, in our study, WT1-specific T cells did not display signs of self-antigen–induced tolerance but instead differentiated spontaneously into fully functional memory phenotype T cells. Previous studies have clearly demonstrated that lymphopenia-induced proliferation can result in the differentiation of naive T cells into memory phenotype T cells,[38,39] and such cells can display antigen-specific effector function. However, this effect was transient, as antigen-specific effector function was only detectable during a time period of 12 to 31 days after T-cell transfer into

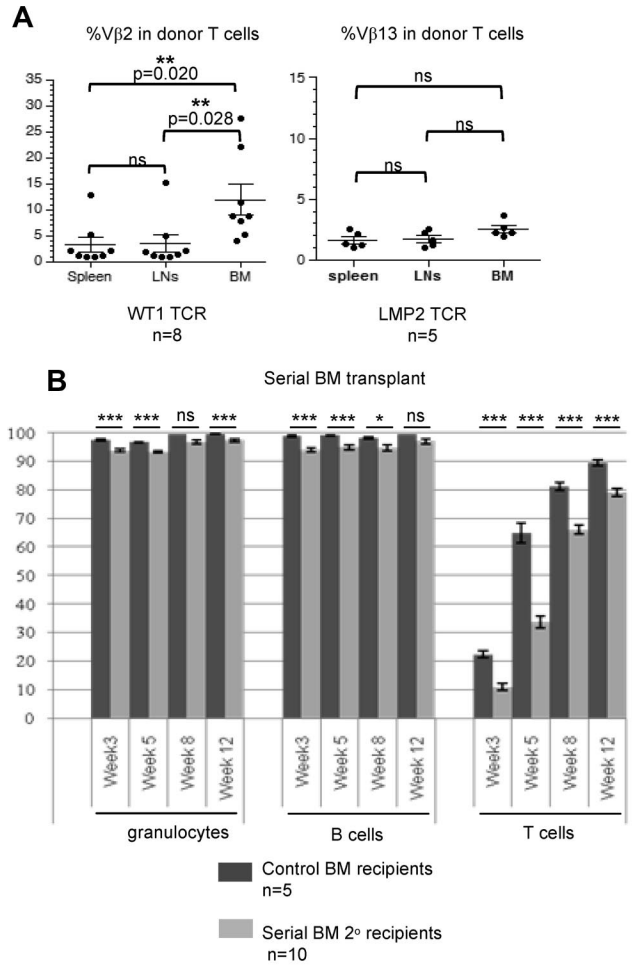

Figure 7. WT1 specific T cells accumulate in the BM without impairing stem cell function. (A) Donor T cells (CD45.1$^-$) were analyzed in the spleen, LNs, and BM of A2Kb Tg mice transplanted with BM stem cells transduced with the WT1-TCR (n = 8) or the LMP2-TCR (n = 5) at 12 weeks after transplantation. The percentage of CD45.1$^-$ donor T cells expressing Vβ2.1 (WT1-TCR) or Vβ13 (LMP2-TCR) was determined by FACS analysis. (B) Hematopoietic engraftment in secondary C57Bl/6 recipients (CD45.2) after transplantation of BM cells from A2Kb Tg mice that were previously transplanted with A2Kb BM stem cells (CD45.1) transduced with the WT1-TCR (n = 10). Peripheral blood of secondary recipients was stained with antimurine CD45.1, CD3, B220, and CD11b to identify donor hematopoietic cells, donor T cells, donor B cells and donor granulocytes, respectively. The peripheral blood analysis was done at weeks 3, 4, 8, and 12 after second transplantation. Control C57Bl/6 recipients received BM stem cells from untreated A2Kb (CD45.1) Tg mice (n = 5). Percentage of donor-derived cells are shown after gating on total granulocytes, B cells, and T cells, respectively. *$P < .05$, **$P < .01$, ***$P < .001$. ns indicates not significant.

lymphopenic hosts.[39] Although there are some similarities between our model and lymphopenia-induced memory phenotype induction, there are also important differences. In our stem cell transplantation model, thymic development generates a polyclonal T-cell population that reconstitutes the peripheral compartment. In this situation, the majority of non–WT1-specific T cells do not develop a memory phenotype, whereas a large proportion of the WT1-specifc T cells differentiate into memory T cells that maintain the ability to display antigen-specific effector function for several months after the initial stem cell transplantation. The reconstitution of a lymphopenic periphery after stem cell transplantation is similar to the generation of a peripheral T-cell repertoire in newborn mice, where adoptive T-cell transfer has previously demonstrated that lymophopenia-induced proliferation promoted memory phenotype development in approximately 20% of a transferred polyclonal T-cell population.[40] It was also shown that 2 monospecific TCR transgenic T-cell populations did not proliferate in the newborn hosts, suggesting that these TCRs, such as the TCRs of 80% of the polyclonal T cells, were not stimulated sufficiently by endogenous MHC/peptide ligands to trigger proliferation and memory development. These findings in neonates are very similar to our findings of T-cell reconstitution after stem cell transplantation. However, we found that the self-specific WT1-TCR was more effective than the polyclonal endogenous TCRs or the non–self-specific LMP2-TCR in differentiating into memory phenotype cells.

Our data are compatible with the proposal that chronic stimulation by self-antigen triggered the spontaneous differentiation of WT1-specific T cells into persisting memory cells. Unfortunately, both WT1 knockout mice as well as inducible tissue-specific knockout animals are not viable.[41] Thus, it is not possible to perform stem cell transplantation experiments in a WT1-deficient host to unequivocally prove that WT1 is the self-antigen driving the generation of central memory T cells.

Although specificity for self-antigen has been suggested to play a role in the generation of natural central memory phenotype cells, experimental evidence to support this has not yet been described.[18] A population of memory phenotype T cells was found in germ-free mice, suggesting that exposure to microbial antigens is not essential for their development.[19,20] Typically, the number of natural memory phenotype T cells increase with age,[18] which is similar to our observation that over time the frequency of WT1-specific T cells increased relative to the frequency of WT1-negative T cells (data not shown). A recent report demonstrated that, although natural memory phenotype T cells rapidly up-regulated CD69 on encountering peptide antigen in vivo, this did not correlate with in vivo cytokine production.[19] The lack of detectable effector function led the authors to propose the term "virtual memory cells." In contrast, the WT1-specific T cells were not "virtual memory cells" as they readily displayed in vivo cytotoxicity. A possible explanation for this difference is that the cytotoxic effector function is more readily triggered than in vivo cytokine production. Alternatively, it is possible that the natural memory phenotype population contains less differentiated virtual memory cells as well as more differentiated T cells capable of full antigen-specific effector function. It is tempting to speculate that the generation of virtual memory T cells is primarily driven by homeostatic proliferation, as suggested by Haluszczak et al,[19] whereas the generation of more differentiated memory T cells involves additionally TCR stimulation, as suggested by the observed TCR down-modulation in WT1-specific memory T cells.

The analysis of TCR-β usage showed that naive and natural memory phenotype T cells in young mice used similar TCR-β variable gene segments.[40] Our study extends this observation by demonstrating that T cells expressing identical TCR sequences can be present in both compartments. The data presented here are supported by a recent study in healthy human volunteers showing that both the naive and memory phenotype compartments contained WT1-specific T cells.[15] In patients with WT1-expressing malignancies, the frequency of naive phenotype cells was reduced, whereas memory phenotype T cells were increased. Whether this shift was the result of priming and differentiation of naive T cells or to the stimulation and expansion of memory phenotype T cells is currently not clear. The HLA transgenic model described here provides an opportunity to dissect which of the WT1-specific T-cell populations responds more effectively when encountering WT1-expressing malignancies or WT1-containing vaccines. It is conceivable that the antigen dose and the costimulatory signals for

the productive vaccination of naive T cells are different from the conditions required for optimal triggering and expansion of the memory phenotype T cells. Thus far, immune responses triggered by WT1 peptide vaccination were often transient and weaned soon after the vaccination.[10]

The transgenic murine model will help to define optimal conditions for WT1-directed T cell immunotherapies including vaccination with the aim of efficiently priming naive T cells while at the same time supporting the expansion and persistence memory phenotype T cells.

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Authorship

Contribution: E.C.M. and H.J.S. designed the experiments, interpreted results, and wrote the manuscript; C.P. designed and performed experiments and contributed to writing the manuscript; S.-A.X., A.H., C.V., M.P., F.F.-A., and B.F. assisted with and performed selected experiments; J.K. provided experimental data for the revision manuscript; and R.C. contributed to experimental design and writing the manuscript.

Conflict-of-interest disclosure: H.J.S. is on the scientific advisory board of CellMedica Ltd. E.C.M. has attended advisory boards for Gilead Sciences. The remaining authors declare no competing financial interests.

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