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Detection and preliminary characterization of CD8⁺ T lymphocytes specific for Wilms' tumor antigen in patients with non-Hodgkin lymphoma

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Abstract

Wilms' tumor antigen (WT1) is overexpressed in many different solid tumors and hematologic malignancies. However, little is known about WT1 expression or WT1-specific immune responses in patients with non-Hodgkin lymphoma (NHL). In a cross-sectional survey study, we investigated the immune recognition of WT1 by NHL patients. Utilizing a WT1 overlapping peptide library, we discovered that a large percentage of NHL patients of all grades maintain WT1-specific T cells. *Ex vivo* frequencies of these T cells measured from unfractionated samples by the CD137 activation marker assay were high in many patients (some >1% CD8⁺). Using standard *in vitro* techniques we discovered they were cytotoxic to WT1 peptide library-loaded T2 cells and WT1 antigen-primed autologous EBV-transformed B cell lines (EBV-LCL) and expressed interferon gamma (IFN- γ). In addition, we detected WT1 mRNA transcripts in diseased lymph node tissues of NHL patients utilizing real-time quantitative polymerase chain reaction (RT-qPCR) technology. These results are the first example of strong T cell reactivity against WT1 in NHL patients which also demonstrate strong cytotoxicity against peptide-loaded tumor cells. The potential for developing WT1 as a target for immunotherapy in NHL deserves further exploration.

Keywords

Wilms' tumor antigen; non-Hodgkin lymphoma; CD8⁺ T cell response

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DISCLOSURE OF CONFLICTS OF INTEREST

None to disclose

INTRODUCTION

Non-Hodgkin lymphoma (NHL) is a progressive disease of the lymphatic system with multiple etiologies, high morbidity and mortality and rising incidence [1]. Though advances in clinical research have changed the outcome for some patients, many subtypes of NHL remain incurable. Active immunotherapy approaches hold promise for becoming important therapeutic options. The idiotype is the most commonly used tumor antigen in NHL cancer vaccination studies, which has therapeutic potential for patients with B-cell-derived NHL [2,3]. One of the major limitations is the need to generate a custom-made vaccine for each patient, because idiotype is considered a patient-specific tumor antigen, and not universal. Another approach involves immunotherapy targeting the T cell receptor (TCR) for T cell-derived lymphoproliferative disorders [4]. However, the same limitations exist for TCR vaccines.

A distinguished NCI panel prioritized 75 cancer antigens in the search for an “ideal” cancer vaccine target and ranked Wilms’ tumor antigen (WT1) as the top choice because of its favorable properties [5]. WT1 expression is tightly linked with hematological malignancies such as acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), chronic myelogenous leukemia (CML) and myelodysplastic syndrome (MDS), and positive correlations have been shown between WT1 mRNA expression levels and disease progression [6-9]. Moreover, a WT1 real-time quantitative polymerase chain reaction (RT-qPCR) assay is considered an important test for the detection of minimal residual disease in acute leukemia and MDS patients [6,8,10-12]. Despite being well-studied in leukemia, there are few published studies providing evidence for WT1 expression and none detecting WT1-specific T cells in NHL patients. An IHC approach is the most comprehensive study to date of WT1 expression in NHL subtypes [13]. WT1 antigen was detected in a subset of high-grade NHL samples as well as in lymphoma cell lines. Others found variability in the expression of WT1 transcripts when they examined a small number of Burkitt's lymphoma tissues by reverse transcriptase PCR [14]. However, there are contradictory reports by other investigators who failed to detect WT1 antigen in NHL patients or NHL-derived cell lines by IHC or PCR [7,15,16].

In this study, we demonstrate for the first time strong levels of T cell responsiveness to WT1 antigen in patients with NHL. Using the efficient and comprehensive *ex vivo* CD137 (4-1BB) assay [17,18], we were able to detect strong WT1-specific CD8⁺ T cell responses in a large percentage of NHL patients studied in contrast to sporadic positives among healthy donors (HD). Our *in vitro* assays confirmed that WT1-specific T cells were functional as they were cytotoxic and could express interferon gamma (IFN- γ). Utilizing sensitive TaqMan RT-qPCR techniques, we were not able to detect high levels of WT1 mRNA transcripts in the peripheral blood of NHL patients. However, we detected significantly higher levels of WT1 mRNA transcripts in diseased lymph node (LN) tissues of a different cohort of NHL patients whereas minimal levels were found in the control lymph nodes from non-cancer patients. The demonstration of functional T cell responses to the WT1 antigen in NHL patients as well as the detection of the possible antigenic reservoir for the observed responses may offer a novel translational direction for NHL immunotherapy.

MATERIALS AND METHODS

Study Subjects

Patients gave written informed consent in accordance with the Declaration of Helsinki for laboratory-based studies on peripheral blood samples obtained before and after hematopoietic cell transplantation (HCT) or chemotherapy at various time points. Patient characteristics are summarized in **Table I**. Samples from anonymous healthy donors were

obtained from platelet donation aphereses. All the data used for the analyses are cross-sectional with each sample/point representing a single patient.

Cells

Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll-Hypaque density gradient centrifugation (Organon Teknica, Durham, NC) and subsequently frozen in 90% heat-inactivated fetal calf serum (FBS) and 10% dimethyl sulfoxide according to standard protocols. Before use, frozen cells were thawed, washed, and suspended in RPMI medium supplemented with 5% FBS.

Peptide Synthesis

The WT1 peptide library was prepared in-house according to our recently published methods [19]. We synthesized a peptide library of 15mers with 11 residues overlapping from full-length human WT1 (**Table SI**; supplemental data). Exceptions were based on minimal coding potential or synthetic feasibility as follows: aa19-21 and aa55-66 regions were not covered; peptides in bold were not synthesized; peptides 43, 80 and 95 were made as 17mers (**Table SI**). The WT1 library contained a total of 103 peptides. The survivin library was a modification of a published overlapping peptide library [19] to reflect human sequences (sequences available upon request). The Preferentially expressed antigen of melanoma (PRAME) library was acquired from JPT Innovative Peptide Solutions (Berlin, Germany; catalog ID: P78395).

Ex Vivo Stimulation of PBMCs and CD137 Surface Staining

Adopting previously published methods [17,18,20], PBMCs were stimulated with 1 µg/mL of WT1, Survivin, or PRAME peptide library for 24 h, stained with antibodies anti-CD8, CD4, and CD137, and analyzed by FACSCanto flow cytometry system using FACSDiva software (all from BD Biosciences, San Jose, California). Medium with 0.25% DMSO (dimethyl sulfoxide) was used as negative control (medium/DMSO) for cell stimulations. The positive control was the mix of minimal viral peptides (CMV, EBV, Flu), CEF [21]. Lymphocytes were initially gated using forward versus side scatter, then CD8⁺ and CD4⁺ lymphocytes were gated separately. The number of CD137 expressing cells was shown as a percentage of the CD8⁺ lymphocyte population. The CD137 baseline expression on PBMCs was studied prior to the 24-hour stimulation utilizing the same staining and analyses procedures.

In Vitro Stimulation of PBMCs

For the generation and expansion of antigen-specific CD8⁺ T cells, approximately 3.5×10^6 PBMCs were placed in 100 µl of serum free RPMI medium and stimulated with WT1 peptide library at 50 µg/mL for 2 h in 37°C, 5% CO₂ incubator. Cells were then resuspended and distributed into 5 wells (200 µl/well) of a 96 well u-bottom plate by increasing volume to 1 mL using expansion media [RPMI, 20% human serum (Valley Biomedical, Inc., Winchester, Virginia), IL-2 at 100 IU/mL] for 24 hrs. Cells were then pooled and placed into 1 well of a 48 well plate with addition of 1 mL of expansion media for expansion. Growth was observed by confluency and media color change, and expansion was done by increasing well size with addition of equal volume of expansion media for a minimum of 14 days.

IFN-γ Intracellular Staining

Same as *ex vivo* stimulation of PBMCs but with the addition of GolgiPlug™ (Cytotfix/Cytoperm Plus with GolgiPlug; BD Biosciences) after 1.5 h. Medium with 0.25% DMSO and unrelated HIV gag peptide were used as negative controls for cell re-stimulations. After 12 h of incubation, PBMCs were harvested, washed, labeled with FITC-conjugated anti-

CD8 antibody, fixed, and permeabilized (Cytofix/Cytoperm; BD Biosciences) before they were labeled with APC-conjugated antibody to IFN- γ . The stained cells were analyzed as for CD137.

Generation of EBV-immortalized B Cell Lines

EBV-transformed B cell lines (EBV-LCL) were generated according to previously published methods [22].

Cytotoxicity Assay

A standard 4 h ^{51}Cr release cytotoxicity assay at E:T ratios of 100, 20 and 4 was used to measure the presence of specific functional WT1 cytotoxic T lymphocytes (CTL) as previously described [23] following 1 or 2 rounds of *in vitro* stimulation (IVS).

Quantitative RT-qPCR Analysis

WT1 antigen in NHL-involved and normal control LN specimens, as well as in peripheral blood of NHL patients and HD was measured by TaqMan RT-qPCR similar to previously published techniques [6,10,11] utilizing commercially available primer and probe sets for WT1 and cABL genes and the ABI7300 instrument (Applied Biosystems, Carlsbad, CA). Total RNA was isolated from frozen LN tissues using RNeasy^{TM} (Qiagen, Valencia, CA). cDNA was made from total RNA using the RevertAid first strand cDNA synthesis kit (Fermentas Inc, Glen Burnie, Maryland). Absolute quantification of the transcript copy number was achieved for WT1 and c-ABL genes from the corresponding standard curves enabled by WT1 and c-ABL control genes cloned into plasmids. Plasmid dilutions were generated to span the anticipated transcript copy range (10^1 - 10^6 copies). Results were expressed as a ratio: (WT1 copy number/c-ABL copy number) $\times 10^4$. RNA from control positive cell line K562 as well as WT1 overexpressing mesothelioma and Wilms' tumor served as positive control. Results showing a >1 cycle deviation from threshold cycle number (Ct) between replicate wells were repeated. Samples containing less than 1000 copies of c-ABL were considered degraded and new cDNA was generated.

Statistical Analysis

Statistical significance for comparisons between two groups was calculated with the Graphpad Prism Software v4.03 using the Mann-Whitney test. A p value <0.05 was considered significant. Univariate logistic regression model was used to evaluate the associations between WT1-specific T cell responses and the following factors: treatment (alloHCT, chemotherapy/autoHCT), age at blood draw ($<$ median 54, $\geq$ median 54), NHL grade (low, high/intermediate), time from treatment to the lab test ($<$ or $\geq$ median 174 days), disease status at HCT or last chemo (CR, non-CR), donor type (related, matched-unrelated), prior acute GVHD maximum grade (none or grade 1, grade 2-4), and conditioning regimen (full-intensity, reduced-intensity conditioning). SAS® version 9.2 (SAS Institute, Cary, NC) was used to calculate p values. The statistical significance was set at the $p < 0.05$ level.

RESULTS

Ex Vivo Detection of CD8⁺ T Cells Specific for WT1 in NHL Patients

We developed a detection system for WT1-specific T cells that is HLA independent by constructing an overlapping peptide library of full length WT1 antigen using peptide synthesis algorithms to generate peptides with permissible residues at both termini (Table SI; supplemental data). This reagent was used for all *ex vivo* studies in clinical specimens. We analyzed PBMC specimens from NHL patients and healthy donors (HD) for the presence of immune responses to the WT1 peptide library by flow cytometry based on the

CD137 activation marker and focusing on CD8⁺/CD137⁺ T cell response [17,18,20]. The results from the CD137 assay can be interpreted equivalently to those generated by *ex vivo* detection of antigen-specific T cells [18]. The cohort included patients who received HCT or were treated with biological or chemotherapy. The data are summarized in **Figure 1** and in **Table SIIa** (supplemental data). Shown in **Figure 1A** are measurements of CD137 response levels in PBMCs of 63 NHL patients (with medium/DMSO values subtracted). The WT1-specific CD8⁺ T cell responses were significantly greater in NHL patients than a randomly chosen group of HD. Remarkably, some patients have *ex vivo* levels that rival CMV-specific T cell levels in HCT recipients [24]. In contrast, when we examined 30 HD, we confirmed that most individuals have low levels, but there were isolated cases in which higher magnitudes were found similar to NHL and leukemia patients (**Figure 1A**) [25]. The median of CD8⁺CD137⁺ cells (percentage) for the patient group was 0.019 and it was 0 for the HD group. The *p* value equaled 0.0007 when the results of the two groups were analyzed by the Mann Whitney test. No significant differences were found when levels of CD8⁺ T cells in patients specific to other antigens associated with hematologic malignancies and solid tumors, such as Survivin and PRAME [26-29], were compared to those in HD (Survivin: median for patient group 0.005; median for HD 0; Mann Whitney test, *p*=0.15; PRAME: median for patient group 0.002; median for HD 0.0005; Mann Whitney test, *p*=0.68). The levels of CD8⁺/CD137⁺ T cells in NHL patients (N=63) in response to Survivin and PRAME peptide libraries are shown in Table SIIa (supplemental data). Representative CD137 flow plots showing the levels of WT1-specific T cells detected in NHL patients are shown in **Figure 1B**. The WT1-specific CD4⁺ T cell responses were significantly lower and did not correlate with the WT1-specific CD8⁺ T cell responses (data not shown). The CD137 baseline expression (prior to the 24-hour stimulation) studied in a subset of our cohort of NHL patients was undetectable or minimal on all samples analyzed with levels equal to or lower than that of the medium/DMSO negative control sample (**Table SIIb**, supplemental data).

WT1 IVS Cells are Able to Expand and Produce IFN- γ

To evaluate the level of expansion and IFN- γ production of antigen-stimulated cells, available samples with high levels of CD137⁺/CD8⁺ T cells based on the CD137 *ex vivo* screening of NHL patient PBMCs (**Figure 1A**) were identified. PBMCs from one HD (data not shown) and 12 patients were expanded using IVS and the WT1 peptide library. All the PBMC samples were able to expand in culture after 10-14 days by single IVS (7 fold median expansion). Specifically, the HD sample (data not shown) as well as 11 out of 12 patients tested were able to produce high levels of IFN- γ assessed by intracellular cytokine staining (ICS) and detailed in **Table SIII** (supplemental data). As illustrated in **Figure 2**, significantly higher IFN- γ responses in CD8⁺ T cells were induced when restimulating IVS cells with the WT1 library (median=2.985, range 0.077-5.54) as opposed to the medium/DMSO control (median=0.39, range 0.045-1.7). The *p* value of the difference between control and WT1 groups was significant (0.002). This was also true when an HIV gag peptide was used as an unrelated control for restimulations in a subset of our NHL patients with available specimens (median=2.46 for the WT1 group, range 0.389-5.54; median=0.049 for the HIV gag group, range 0.03-0.09; Mann Whitney test, *p*=0.0079; **Table SIII**, supplemental data). We demonstrated that antigen-specific cells could be amplified, an important characteristic of a successful T cell response to a WT1 vaccine candidate.

Cytotoxic Activity of WT1-specific T Cells Against WT1 Peptide Library-loaded Targets

Positive results in an IFN- γ assay are not always associated with functional killing. Therefore, to evaluate the cytotoxic effector function of WT1 library-expanded T cells, IVS cultures were tested by *in vitro* chromium release assay (CRA) using autologous WT1

peptide library-loaded EBV-LCL or HLA-A2 derivative T2 cells [30] as targets. IVS cells from 7 out of 10 HLA*A0201 patient samples showed high levels of cytotoxicity against WT1-loaded T2 targets in contrast to the HIV gag-loaded controls ($p < 0.005$ between control and WT1 groups in all cases) demonstrating WT1-specific killing. From these patients, two were chosen and tested in an additional CRA utilizing EBV-LCL as target cells. We observed potent lysis of WT1-loaded EBV-LCL targets, though irrelevant HIV gag-loaded controls were negative in both cases (data shown for UPN222 in **Figure 3**; $p < 0.005$). Similarly, a patient sample having HLA*A0101 evaluated using EBV-LCL cells as targets after one round of IVS, showed high levels of cytotoxicity against WT1-loaded target compared to the control (**Figure 3**, UPN010; $p = 0.0004$). A patient sample with HLA*A0206 was also able to kill WT1-loaded T2 cells (**Figure 3**, UPN016; $p = 0.0019$). The representative CRA results are shown in **Figure 3**. These results demonstrate the functional killing of WT1 antigen-loaded T2 and EBV-LCL target cells by WT1-specific T cells from NHL patients, while the same cells loaded with irrelevant control were not recognized. We confirmed that WT1-specific T cells expanded by IVS methods had high enough avidity to kill peptide-loaded tumor targets.

WT1 T Cell Responses in PBMC by NHL Subtype Category and Grade

We classified 63 patients enrolled in our study based on their diagnosis and lymphoma grade (**Tables I and II**). Based on prior studies of WT1-specific T cell responses [31], patients were considered positive responders if they had a minimum of 0.05% CD137⁺CD8⁺ WT1-specific T cells by the *ex vivo* CD137 assay (after subtracting the medium/DMSO values). We observed a greater number of responders among patients with high/intermediate grade NHL, such as DLBCL/transformed FL, MCL, and PTCL/AILD. In contrast, low-grade lymphomas (FL, MZL, SLL/CLL, lymphoplasmacytic lymphoma) had lower proportions of patients with WT1-positive T cells. 24 of 43 patients with high/intermediate-grade NHL (55.8%) had a positive CD8 T cell response to WT1 compared with 4 of 20 patients with low-grade NHL (20%) ($p = 0.011$; odds ratio 5.053 (95% CI: 1.447 - 17.636) (**Table II**). None of the other clinical variables such as treatment modality (alloHCT, chemotherapy/autoHCT), disease status (CR, non-CR), prior acute GVHD (in alloHCT, none or grade 1, grade 2-4) were significantly associated with the WT1-specific CD8 T cell response (see Materials and Methods section for the complete list of variables analyzed). The correspondence of WT1-specific T cells and NHL grade is consistent with IHC results [13].

WT1 Expression in LN from NHL Patients

In evaluating possible antigen reservoirs to account for the expansion of WT1-specific T cells, we used the RT-qPCR TaqMan approach to measure WT1 transcript levels in 31 frozen diseased LN specimens from NHL patients. Since biopsies of the LN were not available for the corresponding patients who were immunologically analyzed, the LN specimens in the form of cDNA were obtained from a separate cohort of NHL patients through collaboration with University of Wurzburg (Wurzburg, Germany). Frozen specimens of reactive LN from individuals without a cancer diagnosis were also tested for the detection of WT1 transcripts. As shown in **Figure 4** (detailed data in **Table SIV**, supplemental data), we were able to detect higher levels of WT1 mRNA transcripts in the NHL LN with a median value of 6.06 WT1 copies/cABL copies $\times 10^4$ (range 0 to 2524) as opposed to controls where 0 or minimal levels were found (median=0 WT1 copies/cABL copies $\times 10^4$; range 0 to 30; $p = 0.0034$). K562 erythroleukemia cells, Mesothelioma and Wilms' tumor samples served as positive controls and were included for each qPCR run. These results provide a scenario in which WT1-specific T cells may persist because of WT1 antigen expressed in LN of NHL patients.

WT1 Expression Analysis of Peripheral Blood in NHL Patients

We detected high levels of WT1 mRNA transcripts in LN from NHL patients which suggests a possible antigenic stimulus for circulating WT1-specific T cells. Since we had access to peripheral blood samples from NHL patients, it was important to study if there was also high levels of WT1 transcripts which could provide an alternative antigen source to stimulate T cell proliferation. We performed RT-qPCR measurements of WT1 mRNA transcript levels on patient specimens, similar to a previous study detecting circulating NHL cells using MAGE-A3 gene expression [26]. WT1 mRNA expression was measured in 15 available PBMC specimens of NHL patients that were studied immunologically (8 specimens from positive responders and 7 specimens from patients that had lower than 0.05% CD8⁺CD137⁺ WT1-specific T cells) and compared to a control group of 18 HD. Interestingly, our molecular analyses did not detect significant levels of WT1 mRNA transcripts in the 15 patient peripheral blood specimens studied compared to 18 HD (median for patient group 1.45; median for HD 1.78; Mann Whitney test, $p=0.58$). The WT1 mRNA transcript levels were not significantly different when positive responders were compared to non-responders (Mann Whitney test, $p=0.5$). We conclude that the analyzed patient specimens likely did not have circulating hematopoietic or NHL cells that had significant enough WT1 expression to account for providing an antigenic stimulus to stimulate WT1-specific T cell proliferation.

DISCUSSION

Our data demonstrate for the first time a major population of WT1-specific CD8⁺ T cells with a proportion having cytotoxic function in PBMCs of NHL patients. In this cross-sectional study, we discovered that a large percentage of NHL patients of all disease grades and HLA haplotypes maintain WT1-specific T cells as assessed by the expression of the CD137 (4-1BB) activation marker [18,20]. Using the novel CD137 assay allowed us to detect a greater number of activated CD8⁺ T cells, 24 hours after stimulation than could be detected using individual cytokine expression probes. CD137 is upregulated on the surface of antigen-specific CD8⁺ T cells of all phenotypes regardless of their individual cytokine profiles or differentiation stage [17,18]. In addition, we were able to induce high levels of IFN- γ producing cells after expanding them through IVS, in contrast to a previous study analyzing the IFN- γ production of WT1-specific CD8⁺ T cells *ex vivo* after a short stimulation [31]. Our molecular analysis did not detect higher levels of WT1 mRNA transcripts in the peripheral blood of NHL patients compared to HD, strongly suggesting the absence of circulating malignant cells capable of expressing WT1. While having paired LN specimens from the corresponding patients would be ideal for the analysis of WT1 mRNA transcript levels, we did not have access to the LN tissue samples of the patients that were evaluated immunologically. However, when we analyzed the diseased LN of a different cohort of NHL patients, we found examples of strong WT1 antigen expression, which may act as an antigenic stimulus for WT1-specific CD8⁺ T cells. While detecting WT1 in diseased LN may be an explanation for having large numbers of antigen-specific T cells in the periphery, it is not known if the levels of WT1 detected by RT-qPCR are enough to evoke such strong T cell responses. Our analysis did not reveal a correlation between WT1 transcript levels in the NHL LN specimens and disease subtype/grade (not shown) possibly due to the small sample size. A larger study is needed to further investigate the associations between WT1 transcript quantities and NHL disease grade or histologic subtypes.

Our preliminary investigations of associations between WT1-specific CD8⁺ T cell responses and NHL disease grade showed that patients with high/intermediate grade NHL were more likely to have WT1-specific CD8⁺ T cell responses. However, because this was a cross-sectional study, it is unknown whether these T cells have any long-term beneficial effects for the patients. Previous vaccination studies targeting WT1 in hematologic malignancies have

resulted in clinical responses that were not consistently correlated with the simultaneous increase in WT1-specific T lymphocytes [31,32]. Such observations can likely be explained by the quality of the responses generated such that a majority of the WT1-specific T lymphocytes are of low avidity or somehow suppressed. Since WT1 is also expressed in normal adult cells [33], it may function as a self-antigen, and the majority of high avidity CD8⁺ T lymphocytes may have been deleted during thymic maturation [34]. Alternatively, high avidity T cells that could be elicited by vaccination may be suppressed innately or by cancer-induced immune suppression. The inability to generate effective WT1-specific T lymphocyte responses using current therapies re-emphasizes the need for novel WT1-targeting strategies [35].

Our data provide strong evidence that the high frequencies of WT1-specific CD8⁺ T lymphocytes found in NHL patients are functional because they kill WT1 peptide-loaded targets *in vitro* and may represent a high avidity subset. This further suggests that mechanisms of suppression may exist in patients to prevent proliferation and activation of WT1-specific T cells. In leukemia patients Rezvani *et al* detected high avidity T lymphocyte clones within the WT1-specific CD8⁺ T lymphocyte repertoire in patients and concluded that T lymphocytes reactive to WT1 are polyclonal [36]. The natural occurrence of such high-avidity WT1-specific T lymphocytes in the periphery can potentially be boosted through a combination of vaccination and therapy to reduce immune suppression, generating an enhanced response to eliminate tumors [37]. Alternatively, WT1-specific T lymphocytes for adoptive immunotherapy strategies can be generated utilizing molecular technologies [35,38-40]. These approaches could be adopted for NHL patients, since we have documented functional WT1-specific T cells in the immune repertoire.

Exploring strategies for the development of an immunotherapeutic for NHL should be of major importance since currently, alloHCT is the only known curative treatment available for patients with low or high/intermediate-grade NHL who have failed standard chemotherapy/autoHCT. The T cell-mediated graft-versus-lymphoma (GVL) effect frequently generated following alloHCT supports the hypothesis that generating lymphoma-specific T cell responses may contribute to disease control of NHL [41]. This is further supported by work linking the presence of tumor infiltrating T lymphocytes to extended survival of NHL patients [42]. WT1-specific T cells have already been shown to contribute to the graft-versus-leukemia effect in acute and chronic leukemia patients after alloHCT [25,43]. Moreover, some studies of WT1 vaccines for immunotherapy of solid tumors, MDS, and AML have reported correlations between clinical responses and increased frequencies of WT1-specific CD8⁺ T lymphocytes with complete remission in some cases [44-47]. Thus, it is conceivable that boosting such WT1-specific responses after HCT may enhance GVL effects, and therefore, WT1-targeting immunotherapy following HCT could be envisioned as beneficial for alloHCT patients with high risk for relapse. In fact, a recent study utilizing DCs electroporated with full-length WT1 mRNA showed promise in a Phase I/II vaccination trial of AML patients [47]. In our study, the cross-sectional design limits analyses of associations between WT1-specific CD8⁺ T cell levels and subsequent death or relapse. A limited subset analysis in our cohort did not reveal significant correlations with either death or relapse (data not shown). It will be critical to conduct larger longitudinal studies with multivariate analyses to address the questions of the associations between these important clinical outcomes and WT1-specific T cell responses of patients with NHL.

In summary, using the *ex vivo* CD137 comprehensive screening approach we were able to detect strong CD8⁺ T cell responses to WT1 antigen in NHL patients. Our *in vitro* findings demonstrating the proliferative potential and functional killing of WT1 T cells opens a possibility for the development of a WT1-targeted immunotherapy for NHL patients. that

could substitute for toxic chemotherapy, minimally effective autoHCT, or higher risk alloHCT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. CD137 *ex vivo* responses in PBMCs of NHL patients and healthy donors

(A) WT1-specific CD8⁺ T cell response levels to WT1 peptide library were measured by the CD137 assay in NHL patients (N=63) and healthy donors (N=30). Medians are shown for each group illustrated by grey horizontal lines (median for patient group 0.019; median for HD 0; Mann Whitney test, $p=0.0007$). (B) Representative plots of CD137 assay in an NHL patient (top plot: response to medium/DMSO diluent control; middle plot: response to WT1 peptide library; bottom plot: response to CEF peptides).

Figure 2. WT1-specific T cells expanded from patient PBMCs produce IFN- γ . The PBMCs were expanded for 10-14 days in IVS cultures using WT1 library as antigen. The cultures (N=12) were then evaluated by ICS for IFN- γ using WT1 library or medium/DMSO diluent control. Medians are shown for each condition represented by grey horizontal lines (2.985 for WT1 and 0.39 for control; Mann Whitney test, $p=0.002$).

Figure 3. Cytotoxic function of WT1-specific T cells expanded by WT1 15mer peptide library PBMCs from NHL patients were expanded in IVS cultures with WT1 library to generate effectors for use in chromium release assays against T2 and/or EBV-LCL targets. Effectors were then incubated in a 4-hour CRA with T2 and/or EBV-LCL targets loaded with WT1 library or unrelated control at E:T ratios of 100:1, 20:1, 4:1, in triplicate. All assays were repeated twice. *p* values are shown for each representative assay (Mann Whitney test). Error bars represent the standard error of the mean.

Figure 4. Detection and quantitative assessment of WT1 transcripts

WT1 transcript expression was measured in diseased LN from NHL patients (N=31) and in control LN from non-cancer individuals (N=8) utilizing the TaqMan qPCR technology. Medians are illustrated by grey horizontal lines for each group (6.06 for NHL LN and 0 for control LN; Mann Whitney test, $p=0.0034$).

Table I

Characteristics of NHL patients

	Chemotherapy/AutoHCT N=21	AlloHCT * N=42
Median age (range)	56 (36-83)	52.5 (25-65)
Gender (female/male)	9/12	13/29
Grade		
High/Intermediate	16	27
Low	5	15
Subtype		
DLBCL/Transformed FL	12	9
MCL	4	9
PTCL/AILD	0	5
LBL	0	4
FL	1	11
MZL	3	1
SLL/CLL	0	2
Lymphoplasmacytic	0	1
Low grade lymphoma, NOS	1	0
Median time (range) from most recent therapy (chemotherapy/autoHCT/alloHCT)	501 days (46-2329)	163 days (75-1187)
Disease status at HCT (auto/alloHCT) or at sample draw (chemotherapy)		
CR	14	11
Non-CR	7	31
Donor type (related/matched-unrelated)	NA	(25/17)
Conditioning		
Full-intensity	NA	9
Reduced-intensity	NA	33
Acute GVHD		
None or Grade 1	NA	19
Grade 2-4	NA	23

AutoHCT indicates autologous hematopoietic cell transplantation; AlloHCT, allogeneic hematopoietic cell transplantation; DLBCL, Diffuse Large B-Cell Lymphoma; FL, Follicular Lymphoma; MCL, Mantle Cell Lymphoma; PTCL, Peripheral T-Cell Lymphoma; AILD, Angioimmunoblastic Lymphoma; LBL, Lymphoblastic Lymphoma; MZL, Marginal Zone Lymphoma; SLL/CLL, Small Lymphocytic Lymphoma/Chronic Lymphocytic Leukemia; NOS, Not Otherwise Specified; CR, Complete Remission; and GVHD, Graft-Versus-Host Disease; NA, not applicable.

* The applied regimen successfully achieves 100% donor chimerism within one month after alloHCT.

Table II

Associations between WT1 T cell responses in PBMCs and NHL subtype and grade

	Lymphoma subtype	Number of patients	Number of positive WT1 T cells (%)
High/Intermediate Grade	PTCL/AILD	5	5 (100%)
	DLBCL/Transformed FL	21	13 (61.9%)
	MCL	13	6 (46.2%)
	LBL	4	0 (0%)
	High/Intermediate Grade (total)	43	24 (55.8%)*
Low Grade	FL	12	3 (25%)
	MZL	4	1 (25%)
	SLL/CLL, Lymphoplasmacytic, Low grade lymphoma, NOS	4	0 (0%)
	Low Grade (total)	20	4 (20%)*

Abbreviations are explained in Table I.

*
p=0.011