DNA vaccination induces WT1-specific T-cell responses with potential clinical relevance

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The Wilms tumor antigen, WT1, is associated with several human cancers, including leukemia. We evaluated WT1 as an immunotherapeutic target using our proven DNA fusion vaccine design, p.DOM-peptide, encoding a minimal tumor-derived major histocompatibility complex (MHC) class I–binding epitope downstream of a foreign sequence of tetanus toxin. Three p.DOM-peptide vaccines, each encoding a different WT1-derived, HLA-A2–restricted epitope, induced cytotoxic T lymphocytes (CTLs) in humanized transgenic mice expressing chimeric HLA-A2, without affecting hematopoietic stem cells. Mouse CTLs killed human leukemia cells in vitro, indicating peptide processing/presentation. Low numbers of T cells specific for these epitopes have been described in cancer patients. Expanded human T cells specific for each epitope were lytic in vitro. Focusing on human WT1$_{37-45}$–specific cells, the most avid of the murine responses, we demonstrated lysis of primary leukemias, underscoring their clinical relevance. Finally, we showed that these human CTL kill target cells transfected with the relevant p.DOM-peptide DNA vaccine, confirming that WT1-derived epitopes are presented to T cells similarly by tumors and following DNA vaccination. Together, these data link mouse and human studies to suggest that rationally designed DNA vaccines encoding WT1-derived epitopes, particularly WT1$_{37-45}$, have the potential to induce/expand functional tumor-specific cytotoxic responses in cancer patients. (Blood. 2008;112:2956-2964)

Introduction

Despite major advances in chemotherapy and hematopoietic stem cell transplantation, many patients with leukemia will relapse because of reemergence of tumor. However, complete molecular remissions have been achieved by infusion of donor lymphocytes into patients, demonstrating that reactive T cells are able to eradicate leukemic cells in vivo.[1] Recently, various leukemia-associated antigens that are recognized by cytotoxic T lymphocytes (CTLs) in the context of major histocompatibility complex (MHC) class I have been identified.[2] One promising candidate is the Wilms tumor antigen, WT1, which is expressed by the majority of acute leukemias (both acute lymphoid leukemia (ALL) and acute myeloid leukemia (AML)), blast crisis of chronic myeloid leukemia (CML), and many solid tumors, including those of lung, breast, and colon.[3-6] The finding that blockade of WT1 function in primary leukemic cells significantly slows cell growth in vitro[7] additionally suggests that WT1 is of critical importance to the tumor phenotype and that tumor escape by simple WT1 down-modulation is unlikely to occur. In healthy adults, despite ubiquitous expression during embryogenesis, WT1 expression is limited to renal podocytes, gonadal cells, and a low frequency of hematopoietic precursor CD34$^+$ cells,[4,8-11] where it is expressed at significantly lower

levels than those described in tumors (10- to 100-fold).[4] These features indicate that WT1 could be a useful target for therapeutic vaccination.

Antibodies to WT1[12-14] and WT1-peptide specific CD8$^+$ T cells[15-17] have been detected in AML and CML patients, confirming that tolerance is incomplete. Furthermore, the presence of WT1-specific T cells correlates with graft-versus-leukemia (GvL) effects after allogeneic stem cell transplantation in ALL patients,[18] implying that expansion of the WT1-responsive repertoire in leukemic patients may help to mediate tumor clearance.

Facilitating the study of WT1-specific immunotherapeutic approaches, several MHC class I-restricted peptides have been identified, including the HLA-A*0201-restricted WT1.126 (WT1$_{126-134}$; RMFPNAPYL) and the HLA-A*0201 and A*24-restricted WT1.235 (WT1$_{235-243}$; CMTWNQMNL) epitopes.[19-21] T cells with specificity for WT1.126 or WT1.235 have been isolated from the peripheral blood of HLA-A2$^+$ leukemic patients,[15,22,23] although evaluation of lytic ability has been limited to HLA-A2$^+$WT1.126-specific cells, which were shown to lyse tumor cell lines.[22] Recently, a novel HLA-A2-binding epitope, WT1.37 (WT1$_{37-45}$; VLDFAPPGA), was identified.[24] High-avidity T cells with WT1.37-specificity were expanded

from patients with CML and AML, although the cytotoxic capacity of these cells was untested.[15]

Surprisingly, few groups have investigated vaccine strategies to increase the frequency of clinically relevant WT1-specific T cells in vivo. Nevertheless, clinical trials are underway using the WT1.126 or WT1.235 peptides mixed with adjuvant, and some responses in recipients have been reported.[23,25,26] An early study reported that vaccination using WT1.126 peptide with keyhole limpet hemocyanin plus granulocyte-macrophage colony-stimulating factor induced complete remission in the first tested patient with AML.[23] However, both the functionality of the induced WT1-reactive T cells and the means to achieve significant levels of response in patients require further investigation.

We have previously described a novel DNA fusion vaccine[27,28] that encodes a tumor-derived antigen fused to the fragment C (FrC) of tetanus toxin to provide CD4^{+} T-cell help, critical for the induction of long-term antitumor immunity.[29-31] Minimizing the FrC sequence to a single domain (DOM) incorporating the MHC class II–restricted peptide, p30,[32,33] reduces the potential for peptide competition. Fusing this single domain to a target MHC class I–restricted epitope then enhances induction of epitope-specific CTL capable of eliminating tumor cells.[34,35] These optimized DNA fusion vaccines, termed p.DOM-peptide, are capable of inducing high levels of epitope-specific CTL from a tolerized repertoire, probably because of provision of high levels of T-cell help via the antimicrobial repertoire.[36]

In the present study, we used humanized HLA-A2 transgenic mice[37] to model a WT1 vaccination strategy based on our optimized p.DOM-peptide design. This model has value for testing the ability of vaccines to induce CD8^{+} T-cell responses restricted by the human HLA-A2 haplotype and for demonstrating that the target peptide is naturally processed and presented.[38] Murine and human WT1 share a similar expression pattern and 96% identity at the amino acid level,[39] with all 3 HLA-A2–restricted epitopes identical in the 2 species. Thus, WT1-specific immune tolerance is expected to be similar in HLA-A2–transgenic mice and humans, making humanized mice an ideal test-bed for clinical vaccination approaches aimed at breaking tolerance to WT1. We show that T cells with specificity for all 3 WT1-derived epitopes can be expanded by vaccination and can kill human leukemic cells. To make a clinical connection and demonstrate a potential T-cell repertoire, we additionally showed that expanded WT1.37-specific human HLA-A2^{+} T cells from healthy donors were able to lyse primary leukemia cells. The ability of our vaccine design to present peptide to these T cells and therefore expand the repertoire was shown by transfecting human cells with the relevant p.DOM-peptide DNA vaccine. Thus, we suggest that vaccinating patients with p.DOM-peptide DNA vaccines to expand functional WT1-specific T cells, including those with specificity for WT1.37, may be an efficient means to induce cancer-specific cytotoxicity in patients with leukemia.

Methods

Construction of DNA vaccines

DNA vaccines are shown schematically in Figure 1. Construction of the p.DOM plasmid containing the gene encoding the first domain of FrC of tetanus toxin (DOM, $TT_{865-1120}$) with a leader sequence derived from the V_H of the IgM of the BCL_1 tumor at the N-terminus has been previously described.[35] Three additional DNA vaccines were constructed encoding $WT1_{37-45}$ (WT1.37; p.DOM-WT1.37), $WT1_{126-134}$ (WT1.126; p.DOM-WT1.126), or $WT1_{235-243}$ (WT1.235; p.DOM-WT1.235) peptides fused
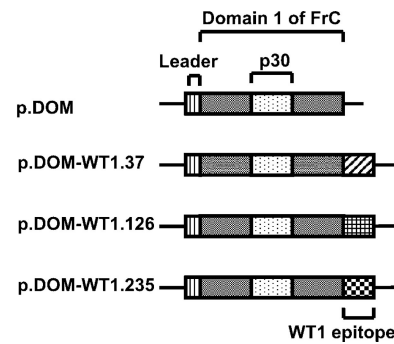

Figure 1. Schematic representation of DNA vaccines. All DNA vaccines encode the leader sequence of the V_H heavy chain gene from the BCL_1 lymphoma upstream of sequence encoding the first domain of fragment C (FrC) of tetanus toxin ($TT_{865-1120}$), which contains a promiscuous MHC II-binding sequence, p30. The control vaccine p.DOM contains no additional sequence, whereas p.DOM-WT1.37 additionally encodes VLDFAPPGA ($WT1_{37-45}$), p.DOM-WT1.126 additionally encodes RMFPNAPYL ($WT1_{126-134}$), and p.DOM-WT1.235 encodes CMTWNQMNL ($WT1_{235-243}$) downstream of the FrC sequence.

directly 3′ to DOM. p.DOM-WT1.126 was constructed by polymerase chain reaction (PCR) amplification using p.DOM as template with the forward primer 5′-TTTT*AAGCTT*GCCGCCACCATGGGTTGGAGC-3′ and the reverse primer 5′-TTTT*GCGGCCGC*TTACAGGTAGGGCG-CATTGGGGAACATCCTGTTACCCCAGAAGTCACGCAGGAA-3′. The PCR product was gel purified, digested using *Hin*dIII and *Not*I restriction sites, and cloned into the expression vector pcDNA3 (Invitrogen, Paisley, United Kingdom). The vaccines p.DOM-WT1.235 and p.DOM-WT1.37 were constructed similarly using the same forward primer and reverse primers 5′-TTT*GCGGCCGC*TTATAAGTTCATCTGATTCCAGGTC-ATGCAGTTACCCCAGAAGTCACGCAGGAA-3′ and 5′-TTT*GCGGCC-GC*TTAAGCGCCCGGAGGCGCAAAGTCCAGCACGTTACCCCAGA-AGTCACGCAGGAA-3′, respectively. Restriction sites within primers are italicized. Integrity of the inserted sequence was confirmed by DNA sequencing and translated product size was checked in vitro using the TNT T7 coupled reticulocyte lysate system (Promega, Southampton, United Kingdom).

Peptides

The HLA-A*0201-restricted WT1.37 (VLDFAPPGA), WT1.126 (RMFP-NAPYL), WT1.235 (CMTWNQMNL) peptides[19-21] and the HLA class II-restricted p30 (FrC-derived: TTFNNFTVSFWLRVPKVSASHLE)[33] and PADRE (KSSAKXVAAWTLKAAA: $\times$ = cyclohexylalanine)[40] peptides were synthesized commercially and supplied at more than 95% purity (PPR, Southampton, United Kingdom).

HHD transgenic mice

HHD mice express a transgenic chimeric monochain MHC class I molecule in which the COOH-terminus of human β_2-microglobulin is covalently linked to the NH$_2$-terminus of chimeric HLA-A2 α1 and α2 domains fused with the murine H-2D[b] α3 domain. These mice lack cell-surface expression of mouse endogenous H-2[b] class I molecules because of targeted disruption of the H-2D[b] and mouse β_2-microglobulin genes.[37]

Vaccination protocol

HHD mice at 6 to 10 weeks of age were injected intramuscularly into both quadriceps with a total of 50 μg DNA in saline solution on day 0. Unless stated otherwise, mice were boosted with the same DNA vaccine delivered with in vivo electroporation on day 28 as previously described.[41] For comparing DNA and peptide vaccination, mice were immunized intramuscularly with p.DOM-WT1.126 or subcutaneously into 2 sites on the flanks with a total of 50 μg WT1.126 and 100 μg PADRE peptides mixed together. Peptides were administered with an equal volume of Incomplete Freunds Adjuvant (IFA). Splenocytes were harvested 10 days later for analysis. Animal experimentation was conducted within local Ethical Committee and

UK Coordinating Committee for Cancer Research (London, United Kingdom) guidelines under Home Office License.

Mouse interferon-γ ELISpot

Vaccine-specific interferon-γ (IFN-γ) secretion by lymphocytes from individual mice was assessed ex vivo (BD ELISpot Set; BD PharMingen, San Diego, CA) on day 10 or 36, as described previously.[36] Briefly, viable lymphocytes were selected from splenocyte preparations by density centrifugation. Cells (2-4 × 10^{5} cells/well) were incubated in complete medium (RPMI 1640, 1 mM sodium pyruvate, 2 mM L-glutamine, nonessential amino acids [1% of 100× stock], 50 μM 2-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin [all Invitrogen] with 10% heat-inactivated fetal calf serum) with WT1.37, WT1.126, or WT1.235 peptides to assess CD8^{+} T-cell responses, or with p30 peptide or PADRE peptide to assess CD4^{+} T cells. Samples were plated in triplicate; control samples were incubated without peptide or with an irrelevant HLA-A2-binding peptide. Data are expressed as the frequency of spot-forming cells (SFCs) per million lymphocytes. For analysis of peptide-specific T-cell sensitivity, splenic lymphocytes from immunized mice were incubated with a range of WT1 peptide concentrations and the frequency of specific cells assessed by ELISpot analysis as described. The number of SFCs/million cells at the peptide concentration inducing the greatest response was assigned a value of 100%. For each peptide concentration tested, the percentage maximal response was then calculated by the formula: (experimental SFCs per million cells/maximal SFCs per million cells) × 100% for each individual animal.

Murine cytotoxic T-cell expansion and detection

For the generation and maintenance of CTL lines, mice were killed at the indicated time points and cell suspensions made from each spleen. Splenocytes were washed and resuspended in 10 to 15 mL complete media per spleen in upright 25-cm[2] flasks together with WT1.37 (10^{-9} M), WT1.126 (10^{-6} M), or WT1.235 (10^{-6} M) peptides. After 7 to 10 days of stimulation in vitro, cytolytic activity of the T-cell cultures was assessed. For further cycles of in vitro restimulation, CTLs were washed, resuspended at 3 × 10^{5}/mL with 2.5 × 10^{6}/mL syngeneic splenocytes preincubated for 1 hour with the relevant peptide at 10^{-9} M (WT1.37) or 10^{-6} M (WT1.126 and WT1.235), washed 4 times in unsupplemented RPMI 1640 (Invitrogen), and irradiated at 25 Gy. Recombinant human interleukin-2 was added to cultures at 20 IU/mL (interleukin-2 (IL-2); Perkin-Elmer, Foster City, CA) and cells were incubated at 2 mL/well of a 24-well plate. Subsequent cycles of in vitro restimulation were carried out similarly every 10 to 12 days. Specific cytotoxic activity was assessed by standard 4- to 5-hour ^{51}Cr release assay as previously described.[42]

Assessment of autoimmune damage

For the detection of autoimmune damage to colony-forming cells in bone marrow, HHD mice were vaccinated as indicated on day 0 and day 28 (+ electroporation). Between days 36 and 42, mice were culled and bone marrow cells collected under aseptic conditions before incubation in Methocult media containing IL-3, SCF, IL-6, and erythropoietin according to the manufacturer's instructions (StemCell Technologies, London, United Kingdom). Colonies of hematopoietic cells were enumerated on days 9 to 11 of in vitro incubation.

Patients and healthy donors

Peripheral blood mononuclear cells (PBMCs) from 10 HLA-A*0201–positive healthy subjects were isolated from leukapheresis using Ficoll-Hypaque density-gradient centrifugation and subsequently cryopreserved in complete medium supplemented with 20% heat-inactivated AB serum (SAB) and 10% dimethyl sulfoxide. Tumor cells from 6 patients with WT1^{+} acute leukemias were isolated from one HLA-A*0201–positive patient with ALL (patient I), 3 from HLA-A*0201–positive patients with AML (patients II, III, and IV), and 2 from HLA-A*0201–negative patients with AML (patients V and VI). WT1 expression was confirmed by WT1-specific RT-PCR (data not shown). Informed consent was obtained

from all healthy subjects and patients before blood sampling. Informed consent was obtained in accordance with the Declaration of Helsinki.

Cell lines and autologous PHA blasts

Cells used as targets in murine CTL assays were the human leukemia lines C1RA2 (WT1^{−}HLA-A*0201[+]), KYO-1 (WT1^{+}HLA-A*0201[−]), or 697 (WT1^{+}HLA-A*0201[+]) either alone or retrovirally transduced with HHD DNA using standard methods. For human in vitro studies, the pro-B cell line RS 4,11 (WT1^{+}HLA-A*0201[+]) was purchased from ATCC (Manassas, VA), the pre-B leukemia cell line 697 and follicular lymphoma cell line VAL (WT1^{−}HLA-A*0201[+]) were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Phytohemagglutinin-blasts were differentiated from nonadherent PBMCs (10[6] cells/mL) by culture in complete medium supplemented with 10% SAB (RPMI 10% SAB), 50 IU/mL of IL-2 (R&D systems, Oxford, United Kingdom), and 2 μg/mL of phytohemagglutinin (Sigma-Aldrich, Saint Quentin Fallavier, France). IL-2 (50 IU/mL) was added every 2 to 3 days.

Human cytotoxic T-cell expansion

Dendritic cells (DCs) were generated from donor monocytes as previously described.[43] Briefly, adherent PBMCs were cultured at 10 × 10^{6} cells/well in 6-well plates with 100 ng/mL of IL-4 and 80 ng/mL of granulocyte-macrophage colony-stimulating factor in AIM V serum free medium (Invitrogen) for 1 day. Maturation was induced by additional 24-hour incubation with 10 ng/mL tumor necrosis factor-α, 20 ng/mL IL-6, 1 μg/mL PGE-2, and 10 ng/mL IL-1β (all R&D Systems, Abingdon, United Kingdom). Mature DCs pulsed for 2 hours at 37°C with 10 μg/mL of WT1-derived peptide in AIM V were then irradiated (50 Gy), washed in RPMI 10% SAB and used as stimulator cells. Autologous CD8^{+} T cells were purified from healthy HLA-A*0201–positive donor PBMCs by positive selection using CD8 magnetic microbeads (Miltenyi Biotech, Paris, France). The binding fraction was adjusted to 5 × 10^{6} cells/mL, whereas the CD8-negative fraction was irradiated (30 Gy) and adjusted to 5 × 10^{6} cells/mL to be used as helpers. CD8^{+} T cells were then plated together with stimulator DC at a 10:1 or 5:1 ratio and with helpers at a 2:1 ratio in RPMI 10% SAB. IL-2 (25 IU/mL) and IL-7 (5 ng/mL; R&D Systems) were added every 2 to 3 days from day 3. One million CTLs were restimulated every 10 days in 24-well plates with autologous adherent monocytes from 4 × 10^{6} PBMCs pulsed with 5 μg/mL relevant peptide and 100 ng/mL β₂-microglobulin (Sigma-Aldrich) in serum-free RPMI 1640. IL-2 (25 IU/mL) and IL-7 (5 ng/mL) were added every 2 to 3 days.

Human cytotoxic T-cell detection

CD8^{+} T cells specific for WT1 peptides were quantified by double-staining using biotinylated HLA-A*0201/WT1.126 or HLA-A*0201/WT1.235 pentamers (Proimmune, Oxford, United Kingdom) and phycoerythrin (PE)-Cy5-coupled anti-CD8 antibody (clone 3B5; Caltag, Burlingame, CA). Briefly, 5 × 10^{5} T cells were incubated for 30 minutes on ice with 0.5 μg pentamer, washed and incubated for a further 20 minutes at 4°C with 5 μL anti-CD8 antibody and 200 ng streptavidin-PE (Invitrogen) concurrently. Fluorescence was determined using a FACS FC500 (Beckman Coulter, Villepinte, France). For determining cytotoxic activity, target cells were either peptide-pulsed (10 μg/mL peptide plus 100 ng/mL β₂-microglobulin 2 hours at 37°C) or unpulsed and labeled with ^{51}Cr sodium chromate for 1 hour at 37°C before plating at 2.5 × 10^{3} cells/well in a 96-well plate. Cytolytic activity at graded effector-to-target ratios was determined in a standard 4-hour ^{51}Cr-release assay. In some experiments, target cells were incubated with the antihuman HLA-class I monoclonal antibody W6.32 (Diaclone, Besançon, France) or with IgG2a isotype control (5 μg/well) for 15 minutes at 4°C before the addition of T cells and for the duration of the assay. Means of triplicate cultures were expressed as (experimental release − spontaneous release)/(total ^{51}Cr incorporated − spontaneous release) × 100%. The average spontaneous release never exceeded 15% of the total incorporated [51]Cr.
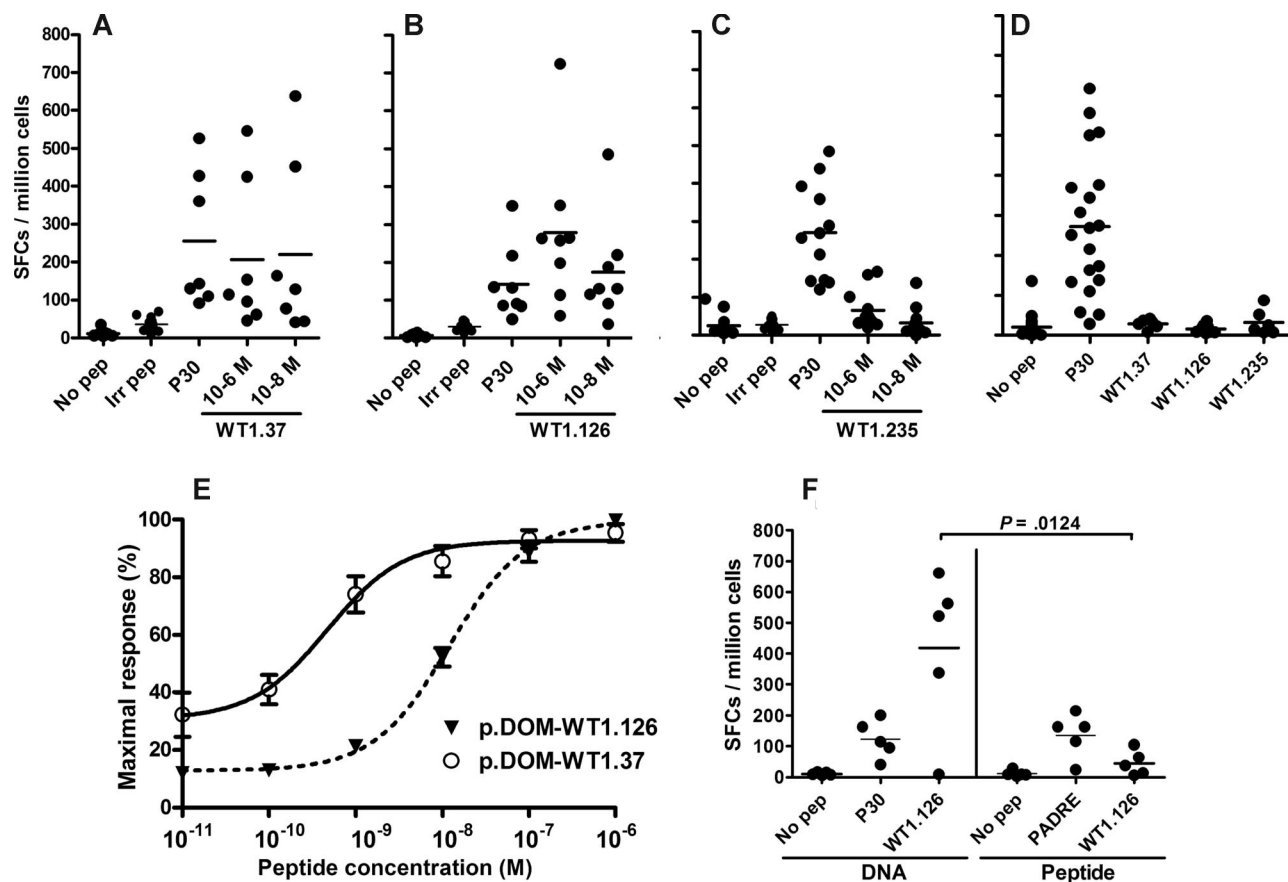
Figure 2. DNA vaccination induces WT1-specific IFN-γ–secreting T cells in HHD mice. HHD mice were vaccinated with (A) p.DOM-WT1.37 (n = 7), (B) p.DOM-WT1.126 (n = 8), (C) p.DOM-WT1.235 (n = 12), or (D) p.DOM (n = 20) and were boosted 28 days later with the same vaccine delivered using in vivo electroporation. On day 36, splenic lymphocytes were harvested by density centrifugation of spleen cells and the frequency of specific T cells assessed by IFN-γ ELISpot following a brief incubation alone (no pep), with an irrelevant peptide (irr pep; 10^{-6} M) with p30 peptide (10^{-6} M) or with the relevant WT1 peptide (10^{-6} M or 10^{-8} M). Data are expressed as the number of spot-forming cells (SFCs) per million lymphocytes and are a combination of 2 experiments with similar results; group means are represented by a horizontal bar. Responses were considered significant if the frequency of IFN-γ–secreting cells was more than double the frequency detected in wells without peptide. (E) Lymphocytes from mice vaccinated with p.DOM-WT1.37 (n = 4) or p.DOM-WT1.126 (n = 9) were incubated with a range of peptide concentrations and the frequency of specific cells assessed by ELISpot analysis as before. Data are shown as the mean percentage of the maximum obtained for each mouse with the SEM indicated. A nonlinear line of best fit was plotted using GraphPad Prism 4 software. (F) HHD mice were injected with DNA vaccine (p.DOM-WT1.126; DNA) or with peptide vaccine (WT1.126 peptide in IFA mixed with PADRE peptide: peptide). Lymphocytes were harvested on day 10 and the frequency of vaccine-specific T cells assessed by IFN-γ ELISpot following a brief incubation alone (no pep), with the Th peptides p30 or PADRE (10^{-6} M) or with the WT1.126 peptide (10^{-6} M). Group means are represented by a horizontal bar.

VAL cell line transfection

VAL cells (5×10^6 cells) were transfected with 5 µg of plasmid (p.DOM, p.DOM-WT1.37, or p.EGFPmax) using the Amaxa Nucleofector apparatus (Amaxa, Cologne, Germany) according to the manufacturer's protocol for human B cells. Flow cytometric analysis of p.EGFPmax-transfected cells (Amaxa) showed a transfection efficiency of between 60% and 70% at 24 hours. Cells were used as targets in a ^{51}Cr-release assay at this time point.

Results

p.DOM-WT1 vaccines induce WT1-specific T-cell responses in HHD mice

Three DNA vaccines were constructed, each encoding the first domain of FrC of tetanus toxin (DOM) fused to a minimal WT1-derived peptide sequence, and conforming to our previously described vaccine design.[34-36] Specifically, vaccines were p.DOM-WT1.37, p.DOM-WT1.126, and p.DOM-WT1.235, each incorporating sequence encoding the promiscuous helper T-cell epitope, p30, and one of the 3 MHC class I–binding peptides, WT1.37, WT1.126, or WT1.235, respectively (Figure 1). To test vaccine

efficacy, humanized HHD mice were immunized on days 0 and 28 with one of the p.DOM-WT1-peptide vaccines or with the control vaccine p.DOM. The booster injection on day 28 was immediately followed by in vivo electroporation at the site of injection, a technique we have previously shown to be of benefit for the activation of T-cell responses, particularly at boosting.[41] On day 36, mice were killed and splenic responses assessed by IFN-γ ELISpot analysis. All vaccines, including the p.DOM control, induced a significant response to the p30 peptide confirming the integrity, in vivo expression, and immunogenicity of the DNA vaccine protein products (Figure 2A-D). Furthermore, 100% of HHD mice immunized with p.DOM-WT1.37 or p.DOM-WT1.126, and 75% of mice immunized with p.DOM-WT1.235, induced significant numbers of WT1-peptide specific IFN-γ–secreting T cells detectable directly ex vivo (Figure 2A-C). CD8+ T-cell responses to WT1.235 were notably lower than responses to either of the other 2 WT1-peptide vaccines, although these were significantly above background levels ($P = .005$, 2-tailed Mann-Whitney U test). As expected, the control p.DOM vaccine failed to induce any significant WT1-specific responses (Figure 2D). Encouragingly, the frequency of T cells specific for the WT1.37 peptide, which has not before been
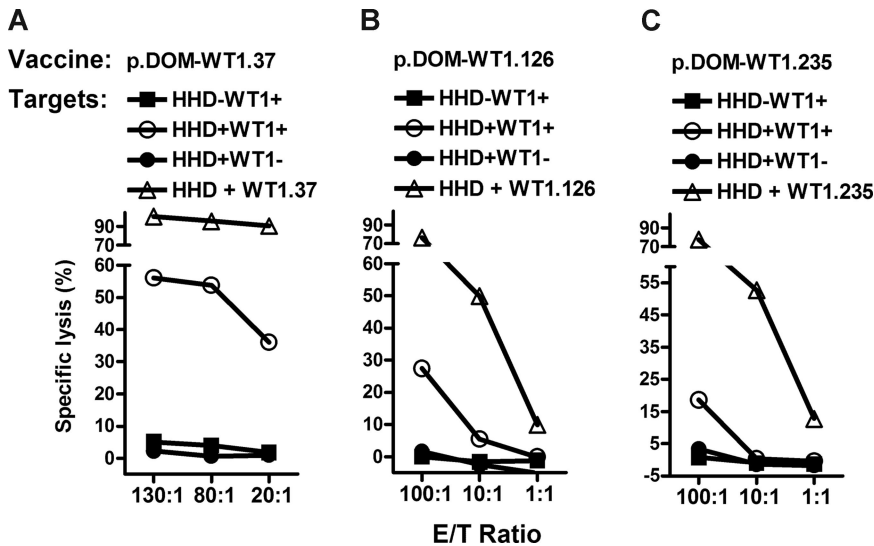
Figure 3. WT1-specific T cells induced by DNA vaccination are peptide specific and kill target cells presenting WT1. Splenocyte cultures from individual mice previously immunized with (A) p.DOM-WT1.37, (B) p.DOM-WT1.126, or (C) p.DOM-WT1.235 were established by stimulation in vitro for 2 weeks before assessing cytotoxicity by ^{51}Cr-release assay. Cytotoxicity was assessed at the effector:target ratios shown, against human tumor cells endogenously expressing WT1 and stably transduced with the chimeric humanized MHC class I molecule, HHD, denoted as HHD$^+$WT1$^+$ (specifically, KYO-HHD (A and C) or 697-HHD (B) cells). Cell lines expressing either WT1 (HHD$^-$WT1$^+$) or HHD (HHD$^+$WT1$^-$) were included as negative controls (KYO-1 and C1RA2-HHD, respectively), HHD-transduced cells pulsed with the relevant peptide were used as positive controls.

tested in any vaccination strategy, was comparable with that elicited to the WT1.126 epitope after vaccination (Figure 2A,B). We also noted that T cells responsive to the WT1.37 epitope exhibited no decrease in the frequency of responding cells when incubated with 10 nM rather than 1 μM peptide (Figure 2A). Further analysis of the WT1.37-specific response over a range of peptide concentrations confirmed that these T cells continue to respond to peptide at concentrations approximately 50-fold lower than WT1.126-specific cells (Figure 2E).

One question concerning vaccine design was the comparative performance of the peptide immunogen when delivered via DNA or as an exogenous peptide plus IFA. This was tested using peptide WT1.126, with a source of T-cell help for the exogenous peptide provided by coinjection of the PADRE peptide. Priming of CD8$^+$ T-cell responses was clearly superior when using DNA delivery with approximately 9-fold higher levels of IFN-γ–producing T cells detectable at day 10 ($P = .012$, 2-tailed t test; Figure 2F). Levels of CD4$^+$ T-cell help against either p30 (DNA vaccine) or PADRE (peptide vaccine) appeared adequate in both cases (Figure 2F).

p.DOM-WT1 vaccines induce WT1-specific cytotoxic T lymphocytes

To evaluate the cytotoxic activity and peptide specificity of WT1-specific T cells induced by DNA vaccination, splenocytes from immunized mice were restimulated in vitro and their lytic ability tested in a ^{51}Cr-release assay. CTLs from mice immunized with each of the p.DOM-WT1 vaccines demonstrated significant lysis of peptide-pulsed target cells, demonstrating the functionality of WT1-specific T cells induced by pDOM-WT1 vaccination (Figure 3). Lysis was not observed against target cells pulsed with an irrelevant peptide (data not shown).

To ensure that all 3 epitopes are naturally processed and presented by tumor cells, vaccine-induced CD8$^+$ T cells were tested for their ability to lyse human leukemia cells endogenously expressing WT1 and transfected with the chimeric HLA-A2 (HHD). T cells specific for each individual peptide lysed human target cells expressing both WT1 and HHD, but not those expressing only HHD or WT1, demonstrating their ability to kill human tumors (Figure 3). In addition, WT1.126-specific T cells induced lysis of target cells expressing HLA-A2 and WT1 in the

absence of the HHD molecule (data not shown), indicating lytic activity independent of CD8.

WT1-specific CD8$^+$ T cells do not inhibit hematopoietic colony formation

In light of the ability of our DNA vaccine approach to induce WT1-specific T cells, we sought to investigate whether vaccination causes damage to hematopoietic precursor cells known to express WT1.[11] HHD mice were vaccinated twice with one of the 3 p.DOM-WT1 vaccines, with control p.DOM or were left unimmunized before analysis of hematopoietic stem cell frequency in the bone marrow. Ex vivo ELISpot analyses of splenic lymphocytes were performed concurrently and only mice in which a WT1-specific T-cell response was evident were evaluated (data not shown). There was no difference between the frequencies of hematopoietic stem cells (either of the granulocyte-monocyte [CFU-GM; Figure 4A] or erythroid [BFU-E; Figure 4B] lineages) present in mice vaccinated with any of the p.DOM-WT1 DNA vaccines, compared with either p.DOM recipients or unimmunized
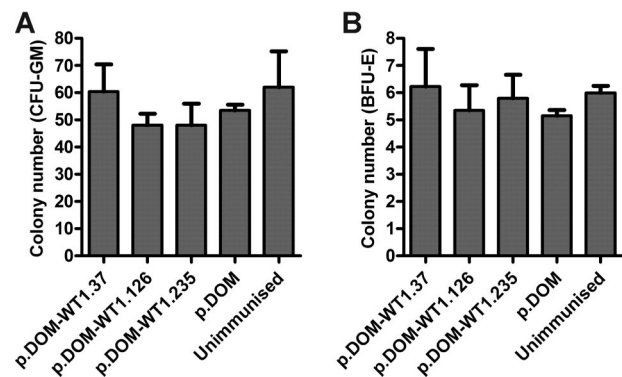

Figure 4. Vaccination with p.DOM-WT1 DNA vaccines does not lead to destruction of hematopoietic stem cells. Groups of mice were injected with the vaccines shown on days 0 and 28 (+ electroporation), or were left unimmunized. Mice were culled between days 36 and 42 and spleen and bone marrow collected. Splenocytes were used to confirm the presence of WT1-specific T cells by ELISpot; nonresponding mice were excluded from analysis (data not shown). Bone marrow cells were assessed for the frequency of (A) colony-forming units granulocyte-macrophage (CFU-GM) and (B) burst-forming units-erythroid (BFU-E). Data are a pool of 3 of 3 experiments with group means and standard deviations shown.

Figure 5. Human WT1-peptide–specific CTLs can be expanded in vitro and specifically kill peptide-pulsed cells. (A) Independently in vitro expanded WT1.126 (top row) and WT1.235-specific (bottom row) CD8+ T cells were identified on day 31 and day 45 of culture, respectively, by double-labeling using specific–HLA-A*0201/peptide-biotin labeled pentamers with streptavidin-PE and anti–CD8-PE-Cy5 labeled antibody. Negative controls were similarly stained with the opposite and irrelevant HLA-A*0201/peptide pentamer. Numbers indicate the percentage of CD8+ cells of whole culture capable of binding pentamer. On day 54 of culture, (B) WT1.37, (C) WT1.126, or (D) WT1.235-peptide–specific CD8+ T cells were tested for their ability to lyse HLA-A*0201 autologous PHA-Blasts (B) or the VAL cell line (C and D) pulsed with the indicated peptides at the effector/target (E/T) ratios shown. For each CTL line generated (3 WT1.37-specific, 1 WT1.126-specific, and 2 WT1.235-specific), the assay was performed twice. Data were similar in each case and representative results are shown.
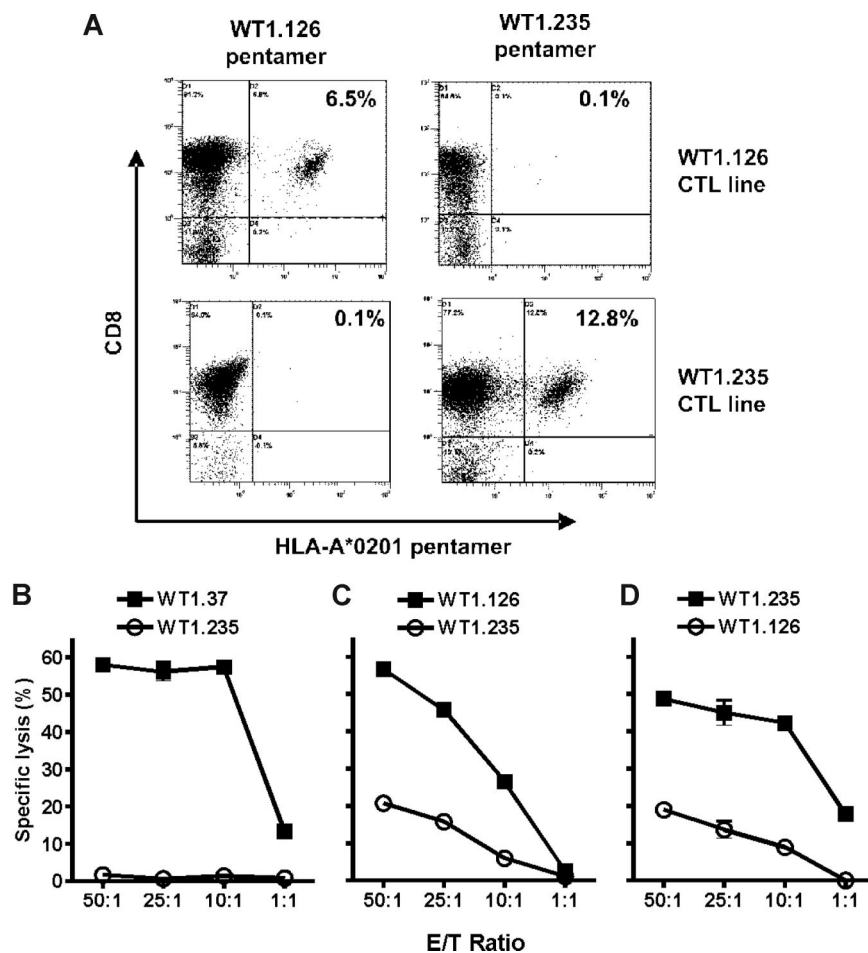
mice, showing that induction of CD8+ T cells against WT1 does not lead to detectable loss of hematopoietic stem cells.

Human WT1-specific CTL can be expanded from healthy donors

Confirming that a T-cell repertoire specific for these same peptides exists in humans,[15,16,44] WT1-specific CD8+ T cells were successfully expanded from a total of 5 of 10 healthy HLA-A2+ donor PBMCs. Briefly, cells were exposed to autologous DCs pulsed with one of the WT1-derived peptides and were then restimulated at 10-day intervals with autologous monocytes pulsed with the corresponding peptide. Rezvani et al previously reported, using IFN-γ mRNA production in response to peptide stimulation, that T cells specific for WT1.126 or WT1.235 could be identified in 3 of 12 healthy persons.[15] We have used pentamer staining and functional assays to show that 1 of 5, and 2 of 6, healthy donors had a repertoire of T cells specific for WT1.126 and WT1.235, respectively (Figure 5; and data not shown), broadly reflecting the frequency of responders identified previously.[15] WT1.126-expanded cells did not bind to the WT1.235 pentamer and vice versa, confirming the specificity of the multimers used (Figure 5A). CTLs specific for the WT1.37 epitope could not be quantified in this way as HLA-A*0201/WT1.37 pentamers could not be made.

To determine whether WT1.37-specific T cells were expanded and to investigate the functionality of WT1-specific T cells, particularly those specific for WT1.235 and WT1.37 whose lytic ability has not been previously reported, CTL lines were established and used in ^{51}Cr-release assays. T cells of all 3 specificities

were able to mediate lysis against peptide-loaded target cells (Figure 5B-D). Peptide-specific cytotoxic responses were detected in 3 of 7 WT1.37 cultures; for WT1.126 and WT1.235, only cultures in which specific cells were detected by pentamer staining exhibited significant levels of lysis.

Human WT1.37-specific CTL kill WT1-expressing tumor cell lines and primary leukemia cells in an HLA-A*0201–restricted manner

Natural processing and presentation of WT1.126 and WT1.235 by HLA-A*0201 and WT1-expressing leukemic cells have been reported.[19,21,22,45] However, it is not known whether this is similarly the case for the WT1.37 epitope, to which particularly avid T cells exist in vaccinated transgenic mice (Figure 2E) and in healthy humans.[15] We therefore chose to focus on human WT1.37-specific T cells and evaluated their ability to kill human tumor cell lines (697 and RS 4,11) and primary tumor cells expressing both HLA-A*0201 and WT1. Our data show that WT1.37-specific CTL efficiently lyse both the 697 and the RS 4,11 cell lines and 4 of 4 primary tumor cell samples from HLA-A*0201+ patients with acute leukemia (either ALL [patient I] or AML [patients II-IV]) (Figure 6A). A significant proportion of the observed lysis against both cell lines and the 2 WT1+HLA-A*0201+ primary AML tumors tested, was HLA-class I–restricted, underscoring the specificity of the effector T cells (Figure 6B). Specificity was further confirmed by the absence of lysis against WT1-expressing, yet HLA-A*0201 negative, primary tumor cells (patients V and VI; Figure 6C). These data clearly show that the WT1.37 epitope is
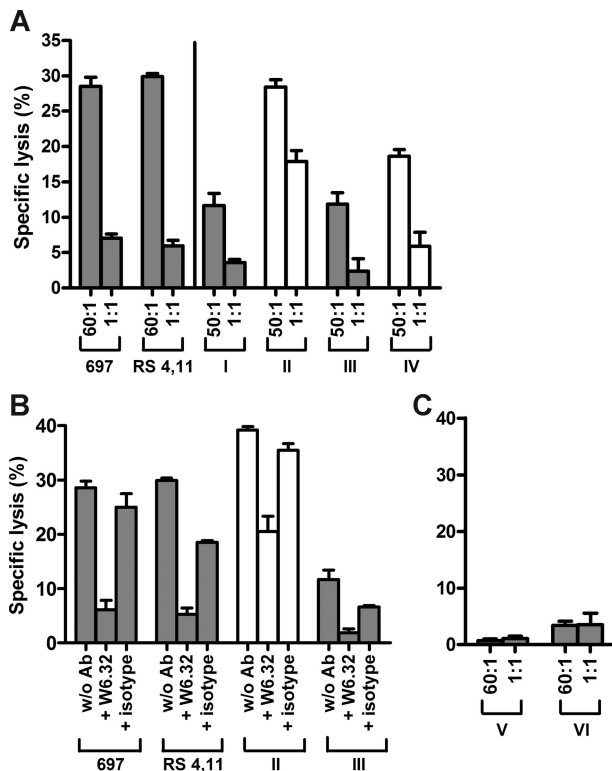
A

B

C

Figure 6. Human WT1.37-specific CTL kill WT1-expressing tumor cell lines and WT1-positive primary leukemia cells in a HLA-class I–dependent manner. WT1.37-specific CTLs were tested in a chromium release assay for lytic activity against (A) 697 and RS 4,11 cell lines (both HLA-A*0201+WT1+) and primary WT1+ leukemia cells from 4 different HLA-A*0201+ patients (I-IV) alone at the effector/target (E/T) ratios indicated or (B) in the presence of an antibody specific for HLA-class I (W6.32), alone (without Ab) or with an isotype control (+ isotype) at an effector/target cell ratio of 60:1 (targets 697, RS 4,11 and sample III) or 100:1 (sample II). (C) WT1.37-specific CTL were tested for lytic activity against target cells from 2 different WT1+ and HLA-A*0201− patients (V and VI). Data are a combination of 2 experiments with 2 different WT1.37-specific CTL lines, differentiated by □ or ▓.

processed and presented by HLA-A*0201–positive and WT1-expressing human tumor cell lines and primary tumor cells from patients with acute leukemia.

The WT1.37 peptide is processed and presented similarly by tumors and following DNA vaccination

Finally, human-derived WT1.37-specific CTLs were used to verify that WT1 peptides are processed and presented by human cells expressing the DNA fusion vaccine product. To this end, a human B-cell line, VAL (expressing HLA-A*0201 but not WT1), was transfected either with p.DOM-WT1.37 or with the control plasmids p.DOM or p.EGFP. WT1.37-specific CTL were able to specifically kill these antigen-presenting cells transfected with pDOM-WT1.37, at levels higher than those transfected with control plasmids, confirming that the protein product of pDOM-WT1.37 is expressed, and the WT1.37 epitope presented, by human cells (Figure 7).

Discussion

In this report, we made use of humanized mice to test our DNA vaccination approach against the leukemia-associated antigen WT1. The similar sequence and distribution pattern of WT1 in mice and humans strongly imply that the extent of WT1-specific
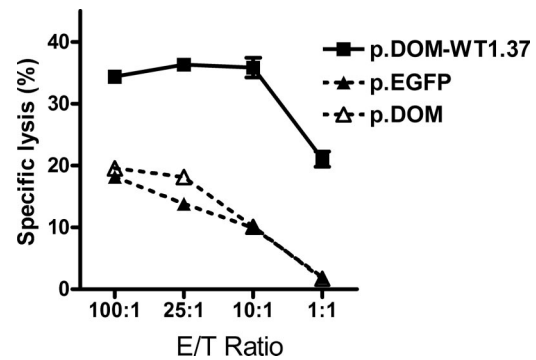

Figure 7. Human B cells transfected with p.DOM-WT1.37 present the WT1.37/HLA complex to, and are lysed by, WT1.37-specific T cells. The VAL follicular lymphoma (HLA-A*0201+WT1−) cell line was transfected with p.DOM-WT1.37 or with control plasmids p.DOM or p.EGFP. Twenty-four hours later, transfectants were tested for their susceptibility to lysis by human WT1.37-specific CTLs. Data shown are representative of 3 experiments performed.

T-cell tolerance in the 2 species is probably comparable. By using mice transgenic for a chimeric form of HLA-A2, we have been able to model the ability of 3 epitope-specific DNA vaccines to induce HLA-A2–restricted CD8+ T-cell responses against WT1.

Previous studies have revealed that vaccinating wild-type C57BL/6 mice with peptide-pulsed splenocytes,[46] or peptides in adjuvant,[47,48] can induce T-cell responses against WT1.126 in vivo. In this case, the response was restricted by mouse H-2Db class I molecules,[46,47] indicating that this peptide can bind to both human and mouse MHC class I molecules but diminishing the relevance to human responses. In contrast, we have made use of HHD transgenic mice expressing only human HLA-A2 MHC class I chimeric molecules. Clearly, DNA vaccination can induce functional T cells to 3 clinically relevant HLA-A*0201–restricted epitopes, WT1.126, WT1.235, and WT1.37, in vivo. The ability of DNA delivery to prime CD8+ T-cell responses against WT1.126 was found to be approximately 9-fold higher than that of exogenous peptide plus adjuvant and a source of T-cell help. Although further comparisons are required, this suggests an advantage for peptide delivery via DNA. Importantly, the CTLs generated also lysed human leukemia cells expressing WT1 from an endogenous source. To link preclinical findings to the clinic, we have studied the natural repertoire of human WT1-specific T cells with similar specificity, to ensure that there is a potential repertoire available for expansion by vaccination.

Using an assay for IFN-γ mRNA, Rezvani et al previously detected CD8+ T cells specific for each of the 3 epitopes, WT1.37, WT1.126, and WT1.235, in the blood of patients with leukemia,[15] showing that a repertoire of WT1-reactive cells persists in cancer patients. Notably, human T cells with specificity for WT1.37 were of approximately 2.5-fold greater avidity than WT1.126-reactive cells,[15] mirroring our findings in p.DOM-WT1.37–vaccinated HHD mice and underscoring the relevance of HHD mice for preclinical modeling. For WT1.37, we have taken this a step further in 2 ways to show that (1) a DNA vaccine can induce high levels of avid CTLs in a setting where there is natural expression of WT1 and therefore the potential for immune tolerance; and (2) WT1.37 is processed and presented naturally by primary tumor cells.

Although CD8+ T cells against all 3 peptides can kill WT1-positive targets, there was no evidence for loss of normal stem cells. Previous studies of WT1 responses in wild-type mice also failed to find any evidence of autoimmune disease,[46,48] and thus far only mild adverse effects have been reported in patients immunized against WT1.126 or WT1.235.[25,26] Our data additionally suggest

that this might similarly be the case after clinical vaccination against the WT1.37 epitope.

To date, WT1.126, WT1.235, and a modified version of WT1.235 (CYTWNQMNL), designed to improve binding to HLA-A*2402,[49] are being evaluated in clinical trials for patients with acute leukemia.[23,25,26,50] In each case, peptide is injected with adjuvant and some success has been reported, with a recent trial showing specific responses after just one injection.[50] However, one problem for peptide vaccines is the failure to provide CD4^{+} T-cell help, known to be essential for induction of effective CD8^{+} T-cell memory.[51-53]

Delivery via DCs is an alternative strategy to activate CD8^{+} T-cell responses and could be attractive for AML, especially because AML-derived DC can express leukemia-associated antigens.[54] However, WT1 expression appears to be down-regulated in AML DCs, arguing against the approach for this antigen.[54] We have chosen to use active vaccination with our p.DOM-peptide DNA vaccines specifically designed to provide CD4^{+} T-cell help. Indeed, our previous work has confirmed that CD4^{+} T-helper cells expanded from the nontolerized tetanus-specific repertoire are critical for effective priming of CD8^{+} T cells in vivo after p.DOM-peptide immunization.[31] Importantly, we have previously shown in an alternative model that our DNA vaccine design can overcome tolerance in mice.[36] This is probably an issue in patients exposed to high loads of leukemic cells. Other strategies exist to circumvent this toleragenic pressure on the repertoire, including the generation of allogeneic CTLs against mismatched MHC/WT1 peptide complexes. Thus, allogeneic and lytic HLA-A*0201[−] CTLs are generated against HLA-A*0201/WT1.126 or HLA-A*0201/WT1.235 complexes in vitro.[19,21] This adoptive therapy approach has to face destruction of the transferred cells; and to avoid this, genes encoding the TCR expressed by these allogeneic CTLs are being transduced into autologous T cells with the intention of subsequent adoptive transfer into patients.[55,56]

Although successful vaccination has the advantage of providing continuous surveillance of emergent tumor, a strong immune response in the clinic is required. It is now evident that performance of DNA vaccines in larger animals, including human subjects, is not optimal. The reason for this appears to be the delivery system which, for intramuscular injection, uses relatively large volumes for mice. Because these cannot be scaled up for human subjects, an alternative strategy to increase transfection rate and inflammation at the site has been sought. One approach is the technique of electroporation, which amplifies responses dramatically, especially at boosting.[41] This exciting technology is now undergoing clinical testing in our institution, and several prostate cancer patients have received p.DOM-peptide DNA vaccines delivered in this way with no serious adverse effects.

One criticism of using epitope-specific vaccines is that tumors may escape immune detection by losing epitope expression. To cover this possibility, DNA vaccines encoding the 3 promising HLA-A*0201–binding epitopes, ideally injected at different sites

to avoid competition, could be used to induce a wide range of CTLs. Although encoding a longer sequence from WT1 may achieve this, our previous work has shown that encoding long tumor antigen sequences may compromise epitope presentation.[35] In addition, immunodominance predicts that immune responses will focus on one epitope after subsequent booster injections.[57]

In conclusion, our data reveal that a repertoire of T cells with specificity for the tumor-associated antigen, WT1, exists in humans and humanized transgenic mice, and the preclinical model has revealed a DNA vaccine strategy to induce or expand specific CTLs. This sets the scene for clinical testing of these safe, specific DNA vaccines in cancer patients with HLA-A*0201[+] and WT1^{+} tumor cells, in settings where there is a need to suppress emergence of residual tumor.

Authorship

Contribution: C.C. and S.L.B. conceived, designed, and performed the research, analyzed data, and wrote the report; J.M. assisted with experiments and processed samples; H.R. and M.K. supplied clinical samples; G.E.V. provided essential novel reagents; V.M.-F., J.-P.F., and H.J.S. provided intellectual support; J.R. conceived and supervised the study; and M.-H.D.-L. and F.K.S conceived and supervised the study and wrote the report.

Conflict-of-interest disclosure: J.R. and F.K.S. hold a patent relating to the DNA vaccine design described in the article and have declared a financial interest in a company that holds an exclusive license relating to this design. All other authors declare no competing financial interests.

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