Detection of Wilms' Tumor Antigen – Specific CTL in Tumor-Draining Lymph Nodes of Patients with Early Breast Cancer

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Abstract **Purpose:** The Wilms' tumor antigen (WT1) is overexpressed in ~90% of breast tumors and, thus, is a potential target antigen for the immunotherapy of breast cancer. We have tested the working hypotheses that WT1 can be immunogenic in patients with breast cancer and can stimulate CTL of sufficient avidity to kill tumor cells.

Experimental Design: Paired tumor-draining lymph node and peripheral blood samples were analyzed from five HLA-A2-positive patients with stage I/II breast cancer. Fluorescent HLA-A*0201/WT1 tetramers were used to quantify WT1-specific CTL and the functional capacity of the CTL was assessed using cytotoxicity assays and intracellular cytokine staining.

Results: WT1 tetramer – binding T cells expanded from all lymph node samples but none of the corresponding peripheral blood samples. Functional assays were carried out on T cells from the patient who had yielded the highest frequency of HLA-A*0201/WT1 tetramer – positive cells. The cytotoxicity assays showed WT1 peptide – specific killing activity of the CTL, whereas intracellular cytokine staining confirmed that the tetramer – positive T cells produced IFN-γ after stimulation with WT1 peptide. These WT1-specific T cells killed HLA-A2-positive breast cancer cell lines treated with IFN-γ but no killing was observed with untreated tumor cells.

Conclusions: These results show that WT1-specific CTL can be expanded from the tumor-draining lymph nodes of breast cancer patients and that they can display peptide-specific effector function. However, the CTL only killed IFN-γ-treated tumor targets expressing high levels of HLA-A2 and not tumor cells with low HLA expression. This suggests that induction of autologous WT1-specific CTL may offer only limited tumor protection and that strategies that allow a high level of peptide/MHC complex presentation and/or improve CTL avidity may be required.

Breast cancer is the most frequently diagnosed cancer among women in the United States and the second most common cause of cancer-related deaths (1). Standard therapies for early breast cancer include surgery, radiotherapy, chemotherapy, and hormonal manipulation. Although these therapies reduce the risk of recurrence and improve overall survival, the 10-year survival for women under the age of 50 years discovered to have lymph node involvement is still only 53% (2). Furthermore, standard therapy is not without its toxicities to normal tissues. More recently, immunotherapeutic approaches to treating breast cancer have been investigated, the advantage being potential tumor specificity and thus fewer side effects.

One of the most important immune effector cells for tumor protection is the $CD8^+$ CTL (3). To date, the vast majority of described CTL responses to tumors have been directed against tumor-associated antigens. One such tumor-associated antigen currently under investigation as a potential target for the treatment of breast cancer is the Wilms' tumor protein (WT1). Most of the early work published on the oncogenic role of WT1 focused on patients with leukemia. WT1 has been shown to be expressed in the majority of acute leukemias and levels of WT1 mRNA expression at diagnosis have been found to correlate inversely with prognosis (4–10). Phase I trials are currently under way, investigating the therapeutic potential of vaccinating patients with WT1-derived peptides (11, 12).

WT1 is an attractive target antigen for breast cancer immunotherapy for several reasons. First, WT1 is overexpressed in >90% of breast cancers (13, 14)[8] contrasting, for example,

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the Her-2/neu oncogene that is overexpressed in only 25% to 30% of cases (15, 16). Second, WT1 may contribute to maintenance of the malignant phenotype because treatment of a panel of breast cancer cell lines with WT1 antisense oligodeoxynucleotides, directed against the translational site, resulted in a reduced rate of growth in 8 of 10 breast cancer cell lines tested (17). This finding suggests that tumor cells would be unlikely to escape WT1-directed immunotherapeutic approaches by down-regulating WT1. Third, there is evidence that WT1 is overexpressed in a wide variety of other solid tumors, including ovarian and colon cancer, and thus strategies targeting WT1 may be of use not only in breast cancer patients but potentially in a much larger group of patients (18, 19).

One factor, however, which needs to be considered when targeting tumor-associated antigens is the existence of immunologic tolerance. In postnatal life, the expression of WT1 is limited to specialized cells within the kidney, testis, ovary, as well as hematopoietic and myoepithelial progenitor cells (20, 21). Although studies in patients with leukemia suggest that tolerance to WT1 is incomplete (22 – 24), there is currently little information concerning the capability of WT1-specific CTL to kill tumor cells.

In the present study, we have investigated if WT1-specific CTL can be detected in patients with early breast cancer and if they are capable of killing breast cancer cells. Paired tumor-draining lymph node and peripheral blood samples were taken from five HLA-A2-positive patients and fluorescent HLA-A*0201/WT1 tetramers were used to quantify the numbers of WT1-specific T cells. T cells capable of binding the HLA-A*0201/WT1 tetramer were expanded from five of five lymph node samples but none of the peripheral blood samples. These WT1-specific T cells displayed peptide-specific cytotoxicity and IFN-γ production. Further analyses showed that the CTL killed IFN-γ-treated breast cancer cell lines, expressing WT1 endogenously, in an HLA-A2-restricted fashion. However, in the absence of IFN-γ, tumor cells expressed reduced levels of HLA-A2 and were not killed by the CTL. These data suggest that autologous WT1-specific T cells may be of limited clinical benefit and that strategies aimed at enhancing target cell peptide/MHC presentation and/or enhancing T-cell avidity may be required to optimize tumor rejection.

Materials and Methods

Tumor samples. Approval was obtained from the Institutional Ethics Committee for the collection of samples from patients with stage I/II breast cancer undergoing surgery. After patient consent was given, peripheral blood samples and one draining lymph node were taken at the time of surgery. Peripheral blood mononuclear cells (PBMC) were separated from whole blood using Ficoll density gradient centrifugation (Lymphoprep-Apogent Discoveries, Wilmslow, United Kingdom) and fluorescence-activated cell sorting (FACS) staining was carried out to determine the HLA-A2 status of the patient. Samples from the five patients that were HLA-A2 positive were used for molecular subtyping. Three patients were HLA-A*0201, one patient was A*0202, and one patient was A*0211. HLA-A*0202, which differs from A*0201 by three amino acid residues, is a relatively common HLA-A2 subtype found in Black Africans. Elution studies have shown that A*0202 binds a similar spectrum of peptides as HLA-A*0201. In addition, some antigen-specific CTL can cross-recognize relevant peptides presented by A*0201 and A*0202 (25). A*0211 is a rare subtype that differs from A*0201 by two amino acid residues. These changes result in A*0211 being the most acidic HLA-A2 subtype (26). Lymph node samples were blunt dissected and separated to single cell suspensions. Cell numbers permitting, tetramer staining was done *ex vivo* before setting up the T-cell cultures.

Cell lines. The breast cancer cell lines used in this study were BT-474, CAL-51, MDA-MB-231, and ZR-75-1 (all kindly donated by Prof. Charles Coombes, Imperial College, London, United Kingdom). All cell lines were maintained in RPMI 1640 (Invitrogen, Paisley, United Kingdom) supplemented with 10% heat inactivated FCS (BioWest, Ringmer, United Kingdom), 1% penicillin, 1% streptomycin, and 2 mmol/L glutamine. For IFN-γ treatment, cells were incubated for 3 days with growth medium containing 300 units/mL IFN-γ (Peprotech, London, United Kingdom). The T2 cell line is deficient in the genes encoding the transporter associated with antigen processing, which results in inefficient loading of HLA class I molecules with endogenous peptides. Thus, the class I molecules (HLA-A*0201) can be efficiently loaded with exogenous peptides (27). T2 cells pulsed with the pWT126 peptide derived from WT1 have previously been shown to be capable of inducing autologous WT1-specific CTL (28). The K-562 cell line, used as a positive control for WT1 mRNA expression, was established from the pleural effusion of a patient with chronic myeloid leukemia in blast crisis (29). The C1R-A*0201 cell line, used as a positive control for HLA-A2 expression, is an EBV-transformed lymphoblastoid cell line that has been transfected with the *HLA-A*0201* gene (30).

Peptides and tetramers. The peptides and tetramers used in this study were obtained from Proimmune (Oxford, United Kingdom). The synthetic peptide pWT126 corresponds to amino acids 126 to 134 (RMFPNAPYL) of the Wilms' tumor protein. The synthetic peptide pMelan-A corresponds to amino acids 26 to 35 (ELAGIGILTV) of the Melan-A protein but with a leucine substituted for the alanine at position 27. This substitution results in increased binding of the peptide to the HLA-A*0201 molecule and increased antigenicity of the peptide (31). All peptides were dissolved in PBS and stored at a concentration of 2 mmol/L at −20°C. Phycoerythrin-labeled tetramers consisting of the same peptides bound to HLA-A*0201 molecules were used for the detection of antigen-specific T cells. These were stored in the dark at 4°C.

T-cell culture. Mononuclear cells from lymph node samples or PBMCs were placed in culture and stimulated in a peptide-specific manner. Mononuclear cells/PBMCs were resuspended in T-cell medium [RPMI 1640 supplemented with 10% FCS, 1% penicillin/streptomycin, 2 mmol/L L-glutamine, and 50 μmol/L 2-mercaptoethanol (Sigma, Dorset, United Kingdom)] at a density of 3×10^6 cells per 2 mL medium in a 24-well plate. The cytokines interleukin-2 and interleukin-7 were added to the medium on day 0 at concentrations of 20 units/mL (Roche, Lewes, United Kingdom) and 2.5 ng/mL (Roche), respectively. Peptide-specific stimulation was done by adding a 10 μmol/L concentration of either the pWT126 or the pMelan-A peptide directly to the medium. After a culture period of 10 to 11 days, tetramer staining was done before the cultures were restimulated. Restimulation involved plating the cells at a density of 5×10^5 per 2 mL of T-cell medium containing interleukin-2 at a concentration of 20 units/mL. Cultures were stimulated using irradiated (80 Gy) T2 cells loaded with 100 μmol/L of the relevant peptide. Because sufficient samples were not obtained to use autologous PBMCs as feeders, irradiated (30 Gy) PBMCs from healthy HLA-A2-positive individuals were used. These were also loaded with the relevant peptide before addition to cultures.

Flow cytometry. Unless stated otherwise, all flow cytometric agents were purchased from BD Biosciences (Oxford, United Kingdom). All data acquisition was done on FACSCalibur and analyzed using CellQuest Software (BD Biosciences).

To determine HLA-A2 status, cells were stained by indirect immunofluorescence for surface HLA-A2 with the monoclonal antibodies HB-54 (anti-HLA-A2, B17; American Type Culture Collection)

[8] Unpublished data.

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and HB-117 (anti-HLA-A2, A28; American Type Culture Collection) followed by staining with a FITC-conjugated sheep anti-mouse IgG (Sigma). To determine the level of surface MHC class I expression, the same protocol was carried out but the monoclonal antibody HB-95 (anti-HLA-A, B, and C; American Type Culture Collection) was used followed by staining with the FITC-conjugated sheep anti-mouse IgG.

Tetramer staining was done using phycoerythrin-labeled tetramers on 7×10^5 cells. Ten microliters of a 1:50 dilution of the tetramer were added for 20 to 30 minutes at room temperature. Cells were washed once in PBS/1% FCS and then stained with directly conjugated antibodies to CD3 (FITC) and CD8 (allophycocyanin). Samples were incubated for 15 to 20 minutes at 4°C and then washed twice with PBS/1% FCS. Before data acquisition, cells were resuspended in 500 μL PBS/1% FCS. Propidium iodide was added to discriminate dead cells from viable cells.

To determine the level of nonspecific HLA-A*0201/pWT126 tetramer staining, samples from five healthy HLA-A2-negative and five healthy HLA-A2-positive individuals were analyzed. This allowed a lower detection limit to be defined for tetramer staining for the breast cancer samples. This lower detection limit was ~0.017% of viable $CD3^+$ cells (cutoff = mean + 3 SD = 0.01749%).

Intracellular IFN-γ detection was done using 5×10^5 T cells stimulated with 5×10^5 T2 cells loaded with 10 μmol/L of either the pWT126 or pMelan-A peptide. After 2 hours, Brefeldin A (Sigma) was added at a concentration of 10 μg/mL to block cytokine secretion. After another 4 hours, staining was done. Cells were initially stained with either the HLA-A*0201/pWT126 or HLA-A*0201/pMelan-A tetramer and incubated at room temperature for 30 minutes followed by staining with a directly conjugated antibody to CD8-allophycocyanin and incubated at 4°C for 20 minutes. Following this, cells were fixed/permeabilized with FACS permeabilizing solution and after two washes with PBS/1% FCS 20 μL of a 1:10 dilution of mouse serum was added and incubated for 15 minutes at room temperature. After another two washes with PBS/1% FCS, cells were stained with IFN-γ FITC for 30 minutes at room temperature. Finally, samples were resuspended in 300 μL of PBS/1% FCS before data acquisition.

CTL assay. Cytotoxic activity was determined in a 4-hour ^{51}Cr release assay. Target cells (T2 and in some assays breast cancer cell lines) were incubated for 1 to 2 hours in assay medium (RPMI 1640 with 5% heat-inactivated FCS) containing 100 μmol/L synthetic peptide at 37°C. Peptide coated or uncoated targets cells were labeled with ^{51}Cr for 1 hour washed and added to serial 2-fold dilutions of T-cell cultures as effectors in round-bottomed 96-well plates to obtain a total volume of 200 μL/well. Assay plates were incubated for 4 hours at 37°C, 5% CO_2, and 100 μL supernatant was harvested and counted using a Wallac Gamma Counter (Milton Keynes, United Kingdom). The specific lysis was calculated by the equation (experimental release − spontaneous release) / (maximum release − spontaneous release) × 100%.

RNA extraction and reverse transcription. Total RNA was isolated from 3×10^6 cells using the RNeasy Minikit according to the instructions of the manufacturer (Qiagen, Crawley, United Kingdom). RNA (5 μg) was reverse transcribed to cDNA. The reconstituted RNA was incubated at 65°C for 10 minutes followed by a 2-hour incubation at 37°C with a mixture of 200 units murine leukemia virus reverse transcriptase (Life Technologies), 1 μL of 0.1 mol/L DTT (Life Technologies), 40 units RNase inhibitor (Promega, Southampton, United Kingdom), 3 μL of 5 mmol/L deoxynucleotide triphosphates (Promega), 1 μL oligo-dT primer (Invitrogen). Following reverse transcription, the reaction was terminated by incubating the mixture for 10 minutes at 65°C. The synthesized cDNA was diluted in 10 mmol/L Tris-HCl/0.1 mmol/L EDTA to a final volume of 100 μL.

Real-time PCR. The synthesized cDNA was subjected to quantitative real-time PCR using the ABI 7700 system (Applied Biosystems, Foster City, CA). The WT1 mRNA expression levels were measured using a dual labeled probe; FAM-CAGGATGTGCGACGTGTGCCTGGAG-TAMRA (exons 6 to 7) and primers, WT-1F 5′-AGAATACACACG-CACGGTGTCT-3′ (sense, nucleotides 1,259-1,280; exon 6) and WT-1R 5′-GATGCCGACCGTACAAGAGTC-3′ (antisense, nucleotides 1,324-1,344; exon 7). The PCR conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute with a final reaction volume of 25 μL containing 1× Universal Master Mix (Applied Biosystems) and 5 μL cDNA. The WT1 expression levels were normalized to ABL control gene using the following probe (exon 4) and primers (exons 3 and 4) set: FAM-TGCTTCTGATGGCAAGCTCTACGTCTCCT-TAMRA; ABL-146F: 5′-GATACGAAGGGAGGGTGTACCA-3′; ABL-240R: 5′-CTCGGCCAGGGT-GTTGAA-3′.

The WT1 and ABL copy number were measured off from the standard curves generated for each quantitative real-time PCR assay using serially diluted plasmids. The WT1 plasmid was created by cloning full-length WT1 DNA from the K-562 leukemia cell line and inserting it into a TOPO vector (Invitrogen). The plasmid was linerized before performing quantitative real-time PCR by digesting with *Hin*dIII restriction enzyme (Life Technologies), as per instructions of the manufacturer. The reaction was terminated by incubating the mixture at 70°C for 16 minutes. Serial dilutions were prepared, giving copy numbers ranging from 500 to 5×10^6 copy numbers in 1 log increments.

Results

T cells capable of binding the HLA-A*0201/pWT126 tetramer can be expanded from the tumor-draining lymph nodes of patients with early breast cancer but not from the peripheral blood. Using the HLA-A*0201/pWT126 tetramer, the numbers of tetramer–positive T cells in paired tumor-draining lymph node and peripheral blood samples were quantified in patients with stage I/II breast cancer. Samples were obtained from 14 patients of whom eight were HLA-A2 positive. Unfortunately, the lymph node samples of two of these patients contained a large proportion of dead cells and attempts to recover viable cells after 1 week of *in vitro* culture were unsuccessful. A lymph node sample from another patient was too small and contained insufficient numbers of cells for tetramer staining and *in vitro* culture. Thus, samples of five patients contained sufficient numbers of viable cells for tetramer analysis and *in vitro* expansion (see Table 1). Cells were placed in culture and stimulated with peptide-loaded T2 stimulator cells every 10 to 11 days. Tetramer analysis was done *ex vivo* and before each stimulation. In all five patients, HLA-A*0201/pWT126 tetramer–positive T cells expanded from the tumor-draining lymph node samples (Fig. 1A and B). These pWT126-tetramer binding T cells were not detectable immediately *ex vivo* but were seen after only one (patients 1 and 3) or two (patients 2, 4, and 5) rounds of peptide-specific stimulation. Figure 1A shows the typical kinetics of expansion of HLA-A*0201/pWT126 tetramer–positive cells from the lymph nodes, as exemplified by patient 3. By contrast, despite the same culture conditions, pWT126-tetramer-binding T cells were not detected from the corresponding peripheral blood samples even after undergoing three rounds of peptide-specific stimulation (Fig. 1B). To explore if the lack of T-cell responses in the peripheral blood was due to poor stimulation conditions, we analyzed T-cell responses against the Melan-A antigen, a melanoma differentiation antigen with a high CTL precursor frequency in the T-cell repertoire (32). T cells specific for this peptide have been characterized as phenotypically and functionally naïve (33). Hence, Melan-A-specific CTL should be detectable in lymph nodes and in peripheral blood. Stimulation of the patients' samples with the pMelan-A peptide resulted in expansion of

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Table 1. Characteristics of the five HLA-A2-positive patients from whom peripheral blood and tumor-draining lymph node samples were obtained

Patient no.	Age	Histology	Vascular invasion	Lymph node involvement	ER status	PR status	HLA-A2 subtype
1	65	Grade 2 lobular carcinoma with widespread LCIS	No	0 of 12 lymph nodes involved	ND	ND	A*0202
2	60	Grade 1 invasive ductal carcinoma	No	0 of 5 lymph nodes involved	+3	+3	A*0211
3	66	Grade 2 invasive ductal carcinoma with extensive high grade DCIS	Yes	18 of 21 lymph nodes involved	Negative	Negative	A*0201
4	65	Multifocal grade 3 invasive ductal carcinoma with extensive high-grade DCIS	No	0 of 15 lymph nodes involved	Negative	Negative	A*0201
5	85	Grade 2 invasive ductal carcinoma with high grade DCIS	Yes	3 of 16 lymph nodes involved	+2	+2	A*0201

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; ND, not done; LCIS, lobular carcinoma *in situ*; DCIS, ductal carcinoma *in situ*.

pMelan-A-specific T cells from all four lymph node and five peripheral blood samples tested (Fig. 2) showing that the experimental conditions were sufficient to expand antigen-specific T cells from both the peripheral blood and tumor-draining lymph node.

Compared with the other patients, the $CD8^+$ CTL of patient 2, who was A*0211, gave consistently dull staining with the A*0201 tetramers (Figs. 1 and 2). This suggested a low-affinity TCR-tetramer interaction, which might be due to poor recognition of the presented peptide or binding to the A*0201 molecule irrespective of peptide. Further tetramer and functional analyses revealed that these CTL were not peptide-specific but recognized A*0201 in a peptide nonspecific fashion. Thus, using the T2-based stimulation protocol employed in this study, we were not able to show pWT126-specific CTL responses in this patient. By comparison, the CTL from patient 1, who was A*0202, gave bright staining with the A*0201 tetramers. Further tetramer analyses revealed that the T cells bound to the A*0201 molecule in a pWT126 peptide–dependent manner, suggesting that these CTL were indeed WT1-specific.

T cells from a breast cancer patient can kill and produce IFN-γ in a pWT126-specific manner. Although the HLA-A*0201/pWT126 tetramer permitted quantification of antigen-specific T cells, it did not provide any information on the functional ability of these cells. Therefore, functional assays were done on T cells from patient 3 because this patient had yielded the highest frequency of HLA-A*0201/pWT126 tetramer–positive cells. Tetramer staining was done before killing assays to determine the number of antigen-specific CTL present in the bulk cultures used for these experiments. For example, Fig. 3A shows a frequency of 9.5% of tetramer–positive, $CD8^+$ T cells

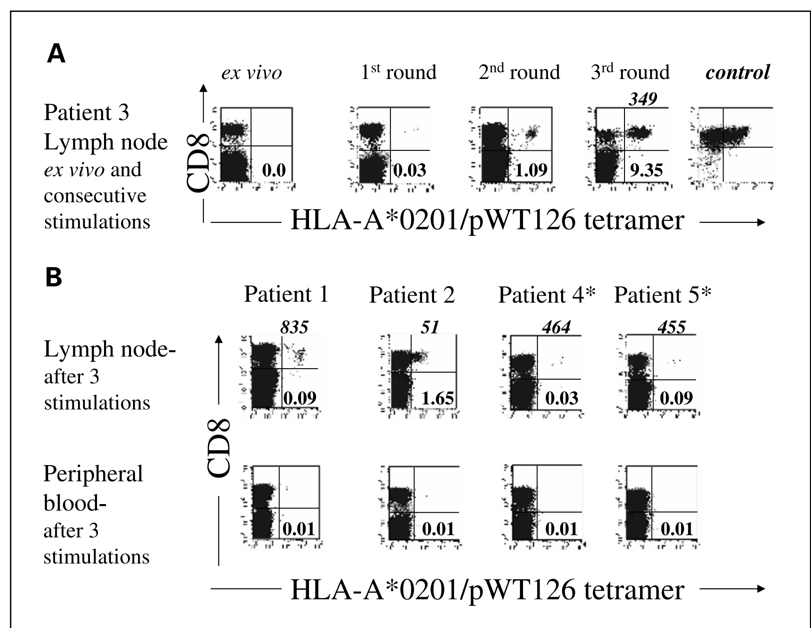

Fig. 1. HLA-A*0201/pWT126 tetramer–binding T cells can be expanded from the tumor-draining lymph nodes of patients with early breast cancer but not from the peripheral blood. Paired draining lymph node and peripheral blood samples were obtained from five HLA-A2-positive patients. Cells were stained with phycoerythrin-labeled HLA-A*0201/pWT126 tetramers, anti-CD8-allophycocyanin, anti-CD3-FITC antibodies, and propidium iodide and analyzed by flow cytometry. FACS plots, gated viable $CD3^+$ lymphocytes. *A*, typical kinetics of pWT126 tetramer–positive cell expansion seen best in the lymph node of patient 3. Staining was done immediately *ex vivo* and after one, two, and three rounds of *in vitro* stimulation. Numbers in plot, percentage of viable $CD3^+$ cells that are CD8-positive and HLA-A*0201/pWT126 tetramer–positive; numbers in italics above the dot plots, mean fluorescence intensity of staining with the tetramer. As a comparison, staining of WT1-specific control cells with the HLA-A*0201/pWT126 tetramer is shown. These control cells are $CD8^+$ T cells that have been transduced with a high-avidity HLA-A*0201/pWT126–specific T-cell receptor. The background level of staining with the HLA-A*0201/pWT126 tetramer was determined to be 0.017% of viable $CD3^+$ cells (see M + M). *B*, results obtained from the lymph node and peripheral blood from patients 1, 2, 4*, and 5* after three rounds of peptide-specific stimulation (for patients 4* and 5* staining is shown from the lymph node after two rounds of stimulation, when the pWT126-specific T cells were first detected). pWT126-specific T cells were detected in all lymph node samples after only one to two rounds of *in vitro* stimulation but were not found in the corresponding peripheral blood samples despite carrying out three rounds of stimulation.

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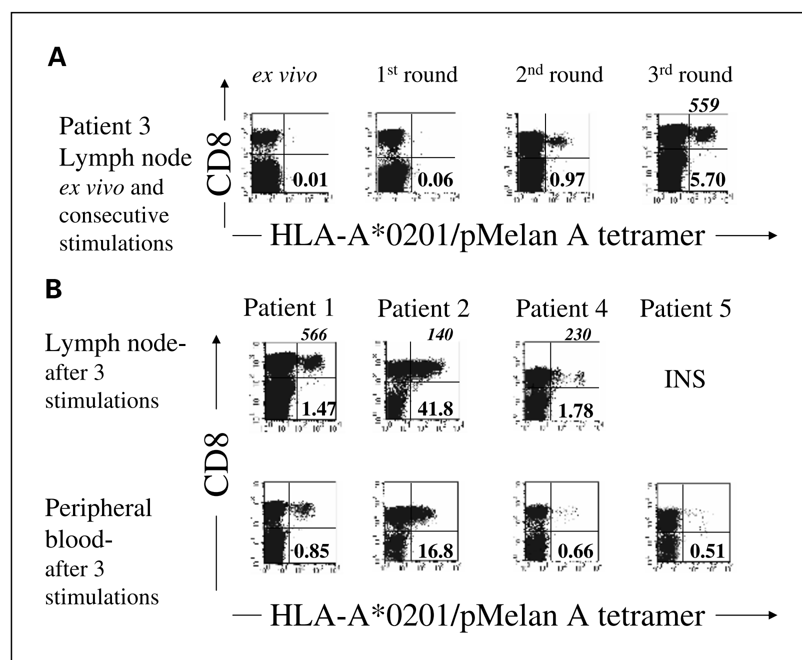

Fig. 2. HLA-A*0201/pMelan-A tetramer-binding T cells can be expanded from the lymph nodes and peripheral blood of patients with early breast cancer (*INS*, insufficient sample). Cells from lymph node and peripheral blood were stained with phycoerythrin-labeled HLA-A*0201/pMelan-A tetramers, anti-CD8-allophycocyanin, and anti-CD3-FITC antibodies and propidium iodide and analyzed by flow cytometry. FACS plots, gated viable CD3$^+$ lymphocytes. *A*, kinetics of pMelan-A tetramer – positive cell expansion in the lymph node of patient 3. Staining was done immediately *ex vivo* and after one, two, and three rounds of *in vitro* stimulation. Numbers in plots, percentage of viable CD3$^+$ cells that are CD8 and HLA-A*0201/pMelan-A tetramer positive; numbers in italics above the dot plots, mean fluorescence intensity of staining with the tetramer. *B*, results obtained from the lymph node and peripheral blood from patients 1, 2, 4, and 5 after three rounds of peptide-specific stimulation. pMelan-A-specific T cells were detectable, after 3 rounds of peptide-specific stimulation, in all lymph node and peripheral blood samples.

within the viable CD3$^+$ lymphocyte population used in the killing experiment shown in Fig. 3B. The T cells displayed cytotoxicity when target T2 cells were loaded with the relevant pWT126 peptide but not when T2 cells were loaded with the irrelevant pMelan-A peptide (Fig. 3B).

To determine whether it was the HLA-A*0201/pWT126 tetramer – positive cells within the T-cell cultures that had provided the pWT126-specific response, intracellular cytokine staining combined with tetramer staining was done to identify IFN-γ-producing cells. T cells were stimulated for 6 hours with T2 cells pulsed with either the relevant pWT126 peptide or the irrelevant pMelan-A peptide. pWT126 stimulation of lymphocytes was associated with a decrease in tetramer staining, suggesting T-cell receptor down-regulation upon antigen-specific T-cell activation (MFI 73 versus 304; Fig. 3E and C). In addition, when gating on to the HLA-A2/pWT126 tetramer – positive cells, there was over a 10-fold increase in the number of cells producing IFN-γ when the cultures had been stimulated with the relevant pWT126 peptide compared with the irrelevant pMelan-A peptide (41% versus 3.6%; Fig. 3F and D). These data confirmed that the tetramer – positive cells within the T-cell cultures were able to produce IFN-γ in a pWT126-specific manner.

T cells from a breast cancer patient can kill IFN-γ-treated breast cancer cells. Four breast cancer cell lines were investigated as potential targets for use in the killing assays. The HLA-A2 status of the cell lines was determined by FACS staining using two monoclonal antibodies, HB-54 (anti HLA-A2, B17) and HB-117 (anti HLA-A2, A28). The MDA-MB-231 and ZR-75-1 cell lines stained with both antibodies and were HLA-A2 positive (molecular typing showed that both cell lines were HLA-A*0201 positive). The CAL-51 and BT-474 cell lines did not stain with either of these antibodies but did stain brightly with the monoclonal antibody HB-95, a pan MHC class I marker (American Type Culture Collection). Thus, both cell lines were HLA-A2 negative and had not merely down-regulated MHC class I expression (data not shown). Quantitative reverse transcription-PCR (RT-PCR) was used to determine the level of WT1 mRNA expression in the breast cancer cells using the leukemia K-562 cell line and purified CD8$^+$ T cells as positive and negative controls, respectively, for WT1 expression (Fig. 4A). All four breast cancer cell lines were found to express WT1 mRNA.

Tetramer staining of the bulk T cells before the killing assay revealed that HLA-A*0201/pWT126 tetramer – positive CTL made up 2.3% of the viable CD3$^+$ lymphocyte population (Fig. 4B). These T cells were not capable of killing the two HLA-A2-positive WT1 mRNA – expressing breast cancer cell lines (low-level killing of the ZR-75-1 cell line was seen in one experiment but this was not reproducible on repeating the experiment a further twice; Fig. 4C). However, killing was observed when the cell lines were pulsed with the pWT126 peptide, indicating that these breast cancer cells were indeed susceptible to CTL killing (Fig. 4C). As a control, the two HLA-A2-negative WT1 mRNA – expressing cell lines were not killed by the T-cell cultures even when pulsed with the relevant peptide (Fig. 4D). These data would suggest that the lack of killing of the HLA-A2-positive cell lines is likely to be due to insufficient expression of HLA-A2/pWT126 complexes on the cell surface.

Because IFN-γ can up-regulate MHC class I and immunoproteasome expression, the breast cancer cell lines were treated with IFN-γ (300 units/mL) for 3 days and the killing assay was repeated (34, 35). FACS analysis of the MDA-MB-231 and ZR-75-1 cell lines revealed that treatment with IFN-γ increased surface HLA-A2 expression, 3- and 10-fold, respectively (Fig. 5A). Tetramer staining done before the killing assay revealed a frequency of 0.35% of HLA-A*0201/pWT126 tetramer – positive CTL within the viable CD3$^+$ lymphocyte population (Fig. 5B). The IFN-γ treatment did result in an increase in the killing of the two HLA-A2-positive WT1 mRNA-expressing breast cancer cell lines (Fig. 5C) but not of the two HLA-A2-negative WT1 mRNA-expressing control cell lines (Fig. 5D).

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Discussion

WT1 was originally described as a tumor suppressor gene implicated in the pathogenesis of Wilms' tumors, a pediatric renal cancer (36). More recently, however, data have been published suggesting that WT1 may have an oncogenic role in various hematologic and solid organ malignancies, including breast cancer. WT1 protein and mRNA expression have been reported in ~90% of breast tumors (13, 14). Moreover, higher levels of WT1 mRNA in breast cancer tissue were associated with a significantly worse 5-year disease-free survival rate compared with lower WT1 mRNA levels (37). We have also done expression studies on breast cancer samples and, using conventional RT-PCR, found WT1 mRNA in 90% of primary tumors (33 of 37; data not shown). In this study, we could obtain fresh tumor tissue from only one patient and RT-PCR confirmed that the tumor expressed WT1 mRNA (data not shown). As fixed tumor tissues were available from all five patients, we attempted to analyze WT1 expression by immunohistochemistry using WT1 antibodies. However, this approach revealed staining of only a small number of cells in all five samples (0.2-10.6% of tumor cells), including the sample from the patient with good WT1 mRNA expression. One possible explanation is that immunohistochemistry is less sensitive than other methods that have been used to measure WT1 protein expression. For example, an immunohistochemistry study by Silberstein et al. (38) suggested that 43% (9 of 21) of breast cancer samples lacked WT1 protein expression, whereas Loeb et al. (13) using Western blotting detected WT1 protein in 87% (27 of 31) of breast cancer samples. The Western blotting results correlated with RT-PCR analysis showing WT1 mRNA in tumor samples, whereas normal breast epithelial tissues were WT1 negative by both Western blotting and RT-PCR. These results were confirmed by Miyoshi et al. (37) in a quantitative RT-PCR analysis of 99 breast cancer samples showing significant overexpression ($P < 0.0005$) in tumors compared with normal breast tissue. Together, the PCR data from two separate studies as well as our own data would suggest that WT1 mRNA expression is a feature of ~80% to 100% of primary breast cancers making it likely that our immunohistochemistry results have underestimated the degree of WT1 expression.

WT1 is overexpressed in the majority of primary breast tumors and thus may be a suitable target molecule for the immunotherapy of breast cancer. However, WT1 is expressed by some normal tissues, including hematopoietic progenitor cells, and immunologic tolerance to WT1 may therefore affect the magnitude and avidity of T-cell responses against WT1-expressing tumors. Vaccination strategies to enhance the immunity to WT1 may be effective should immunologic tolerance to WT1 be incomplete. Alternatively, adoptive therapy may be required if tolerance mechanisms have either deleted high-avidity WT1-specific CTL or rendered them unresponsive, leaving behind low-avidity CTL incapable of affording tumor protection.

The aim of this study has been to quantitate and characterize WT1-specific CTL in patients with breast cancer. Using fluorescent HLA-A*0201/WT1 tetramers, we detected T cells capable of binding this tetramer in all of the five tumor-draining lymph node samples analyzed. Interestingly, of these five samples, three were found to be HLA-A*0201 positive whereas the remaining two expressed different HLA-A2 subtypes. The WT1-tetramer-binding T cells became detectable only after at least one round of antigen-specific *in vitro*

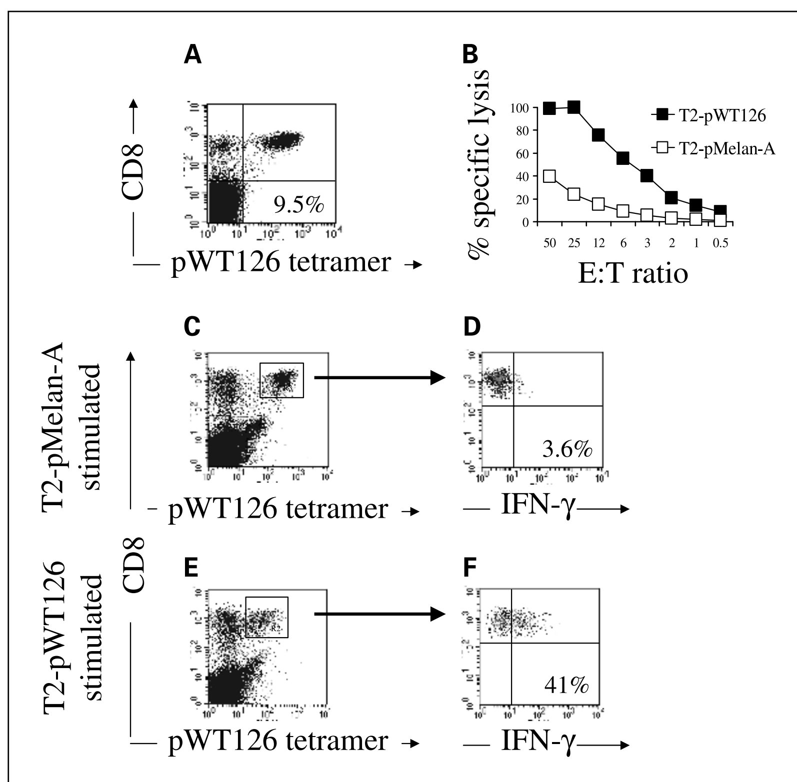

Fig. 3. T cells from a patient with breast cancer can kill and produce IFN-γ in a pWT126-specific manner. Functional assays were done on T cells from patient 3. Before the ^{51}Cr release killing assay, the T-cell cultures were stained with the HLA-A2/pWT126 tetramer and anti-CD8 and anti-CD3 antibodies and propidium iodide (*A*). Gating onto the viable CD3$^+$ lymphocytes revealed 9.5% of these cells to be tetramer-positive CTL. A 4-hour ^{51}Cr release killing assay was done using these T cells as effectors. T2 cells pulsed with either the relevant pWT126 peptide or the irrelevant pMelan-A peptide were used as targets. The T cells killed T2 cells only when pulsed with the relevant pWT126 peptide (*B*). E:T ratio, effector-to-target ratio. Intracellular cytokine staining, to identify IFN-γ-producing cells, was done following stimulation for 6 hours with T2 cells pulsed with either the relevant pWT126 (*E* and *F*) or irrelevant pMelan-A peptide (*C* and *D*). Cells were stained with phycoerythrin-labeled HLA-A*0201/pWT126 tetramer, anti-CD8-allophycocyanin, and anti-IFN-γ FITC antibody. FACS plots (*C* and *E*), gated lymphocytes. Following pWT126-specific stimulation, there was a decrease in tetramer staining (mean fluorescence intensity: 73 versus 304; *E* and *C*). Gating onto the HLA-A*0201/pWT126 tetramer–positive cells revealed a 10-fold increase in numbers of IFN-γ-producing cells with peptide-specific stimulation (41% versus 3.6%; *F* and *D*).

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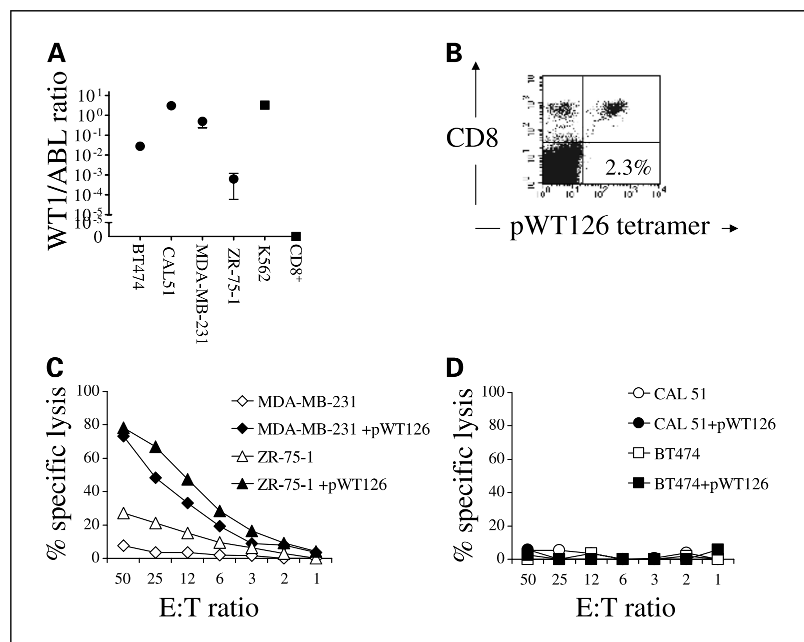

Fig. 4. T cells from a breast cancer patient can kill breast cancer cells when pulsed with the pWT126 peptide. Quantitative RT-PCR was done to determine whether four breast cancer cell lines expressed WT1 mRNA (see Materials and Methods). The K-562 leukemia cell line was used as a positive control for WT1 mRNA expression and cDNA from purified CD8$^+$ T cells was used as a negative control. Each experiment was carried out in triplicate and for each sample the WT1 expression levels were normalized to the ABL control gene (*A*). Points, WT1/ABL ratio for each cell line; bars, SD. All four breast cancer cell lines were found to express WT1 mRNA. Tetramer staining was done before the ^{51}Cr release killing assay. T-cell cultures from patient 3 were stained with the HLA-A*0201/pWT126 tetramer and anti-CD8 and anti-CD3 antibodies and propidium iodide. Gating onto the viable CD3$^+$ lymphocytes revealed 2.3% of these cells to be tetramer-positive CTL (*B*). A 4-hour ^{51}Cr release killing assay was done using these T cells as effectors. The two HLA-A2-positive cell lines, MDA-MB-231 and ZR-75-1, were not killed unless pulsed with the pWT126 peptide (although low level of killing of the ZR-75-1 cell line was seen in this experiment, this was not reproducible on repeating the experiment twice further; *C*). By contrast, the two HLA-A2-negative breast cancer cell lines, BT474 and CAL 51, were not killed even when pulsed with the pWT126 peptide (*D*).

stimulation. One possible explanation for the apparent lack of WT1-specific T cells *ex vivo* is an initial inability of the CTL to bind the HLA-A*0201/WT1 tetramer. The existence of such antigen-specific CTL, which were unable to bind tetramers, was clearly documented in patients with chronic hepatitis B virus infection (39). Despite their ability to produce cytokine and induce cytotoxicity, it was only after repeated *in vitro* stimulation that the hepatitis B virus–specific T cells were capable of binding the relevant tetramer. One possible mechanism for this initial inability to bind tetramer may be related to the expression pattern of lipid rafts because it was shown recently that efficient tetramer binding is dependent on the integrity of lipid rafts on the CTL membrane (40).

An alternative explanation for our inability to detect *ex vivo* CTL is that WT1-specific T cells are indeed present but at a frequency below the limit of detection of the tetramer assay. Rezvani et al. (22) developed quantitative PCR to measure IFN-γ mRNA production in response to peptide stimulation and found it to be ~10 times more sensitive than tetramer staining. Using this method, they were able to detect pWT126-specific responses *ex vivo* in the peripheral blood of patients with chronic myelogenous leukemia and of healthy HLA-A2-positive individuals. Reduced sensitivity of tetramer staining is supported by our analysis of five normal HLA-A2-positive individuals showing that tetramer-positive T cells were not detectable in the peripheral blood (data not shown).

One final possible explanation for the lack of WT1-specific CTL *ex vivo* is that the stimulation protocol has merely led to *in vitro* priming of naïve T cells. This might also explain the observation that WT1-specific CTL could be expanded from lymph node samples but not from the corresponding peripheral blood, reflecting a higher precursor frequency of

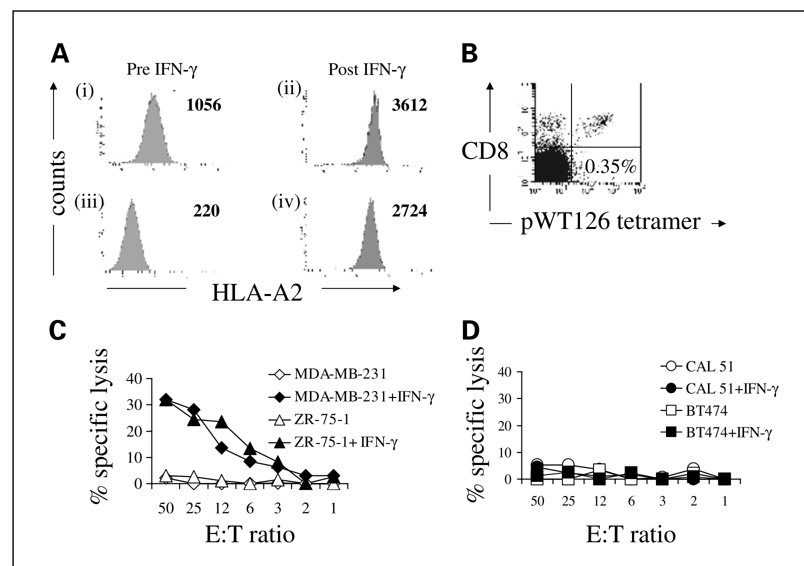

Fig. 5. T cells from a breast cancer patient can kill IFN-γ-treated breast cancer cells. Four breast cancer cell lines, previously shown to express WT1 mRNA, were used as targets in a killing assay either with or without treatment with IFN-γ (300 units/mL). FACS staining was done to look at the effects of IFN-γ treatment on surface HLA expression (*A*). Results are shown of staining with the HB-54 monoclonal antibody for the HLA-A2-positive cell lines MDA-MB-231 (*i* and *ii*) and ZR-75-1 (*iii* and *iv*) pretreatment and posttreatment with IFN-γ. Following treatment with IFN-γ, there was a 3-fold and 10-fold increase in the level of staining with the HB-54 antibody for the MDA-MB-231 and ZR-75-1 cell lines, respectively (similar results were seen for the HB-117 and HB-95 antibodies). Numbers, mean fluorescence intensity. Tetramer staining was done before the ^{51}Cr release killing assay. T-cell cultures from patient 3 were stained with the HLA-A*0201/pWT126 tetramer and anti-CD8 and anti-CD3 antibodies and propidium iodide. Gating onto the viable CD3$^+$ lymphocytes revealed 0.35% of these cells to be tetramer-positive CTL (*B*). A 4-hour ^{51}Cr release killing assay was done using T cells from patient 3 as effectors. Following treatment with IFN-γ, there was an increase in the killing of the two HLA-A2-positive breast cancer cell lines, MDA-MB-231 and ZR-75-1 (*C*). This was not seen with the two HLA-A2-negative breast cancer cell lines, BT474 and CAL 51 (*D*). Representative data from one of two experiments is shown.

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naïve T cells in lymph nodes (41). However, preliminary data in patients with stage IV metastatic breast cancer has revealed that, in marked contrast to patients with early breast cancer, WT1-specific T cells can be expanded from the peripheral blood of patients with advanced disease (data not shown). This suggests a correlation between tumor burden and the presence of expandable WT1-specific T cells. This possibility is supported by the observation that the patient with the greatest degree of lymph node involvement at presentation (patient 3) yielded the highest frequency of pWT126-specific CTL. Together, the data obtained from the analysis of patients with local stage I/II tumors and advanced stage IV disease are in agreement with the possibility that WT1-specific CTL were primed *in vivo* leading initially to a local response in the draining lymph nodes, followed by an increase in CTL numbers in the peripheral blood of patients with systemic disease. Similar observations were made in patients with metastatic melanoma where the frequency of T cells in the peripheral blood directed against differentiation antigens, including Melan-A and gp100, was shown to be significantly increased in patients with stage III and IV disease compared with stage I and II disease (42).

Our functional analysis of WT1-specific T cells expanded from a breast cancer patient revealed that they were able to kill breast cancer cells but only after the tumor cells had been exposed to IFN-γ. This increase in susceptibility to killing of the tumor cells was associated with an increase in surface HLA-A2 expression. This suggests that the T cells require a certain threshold of peptide/MHC complexes to be present on the surface of target tumor cells otherwise killing will not take place and therefore these T cells may only be effective against cancers with high HLA class I expression. To obtain some information on the avidity of pWT126-specific CTL, tetramer staining was carried out using tetramers that possess an amino acid substitution in the α3 domain of the HLA-A*0201 molecule, which reduces the ability of the CD8 coreceptor to bind to the MHC class I molecule (43). It has been shown previously that it is possible to identify high-avidity, antigen-specific T cells by their ability to bind tetramers independently of CD8 (44). Similar patterns of staining were seen for the patient T cells with the "native" HLA-A*0201/pWT126 tetramer and the "mutated" HLA-A*0201/pWT126 tetramer, suggesting that these T cells were of high avidity (data not shown). Thus, it is likely that the tumor cells were not killed because of the low level of MHC class I expression.

Recently, a phase I WT1 peptide vaccination study in patients with a variety of malignancies reported clinical and radiological responses in two patients with stage IV breast cancer (12). In one patient, this was associated with an increase in the numbers of WT1 tetramer–positive T cells in the peripheral blood, although the functional avidity of these T cells was not determined. It would be interesting to explore if clinical responses correlate with the ability of vaccine-induced T cells to kill tumor cells.

Our results suggest that the induction of autologous WT1-specific CTL may be of limited benefit for tumors with low HLA expression. In this case, the introduction of a WT1-specific T-cell receptor into the patient's own T cells offers an attractive alternative immunotherapeutic approach. This strategy allows WT1 specificity to be passively acquired and should allow for the generation of CTL capable of killing breast cancer cells without the requirement for pretreatment with IFN-γ. Recently, we have isolated an HLA-A2-restriced WT1-specific TCR from a healthy allogeneic donor. Retroviral gene transfer resulted in TCR expression and the gene-modified $CD8^+$ T cells showed efficient killing activity against human leukemia cells without IFN-γ treatment (45).

Together, this study suggests that whereas induction of autologous WT1-specific CTL may be of clinical benefit in tumors with high HLA expression, they may not be capable of eradicating tumors with low HLA expression. Thus, strategies that allow high level of peptide/MHC complex presentation and/or improve CTL avidity may be of clinical benefit.

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Clinical Cancer Research

Detection of Wilms' Tumor Antigen–Specific CTL in Tumor-Draining Lymph Nodes of Patients with Early Breast Cancer

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