

Peptide Epitopes from the Wilms' Tumor 1 Oncoprotein Stimulate CD4⁺ and CD8⁺ T Cells That Recognize and Kill Human Malignant Mesothelioma Tumor Cells

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Abstract Purpose: Wilms' tumor 1 protein (WT1), a transcription factor overexpressed in malignant mesothelioma, leukemias, and other solid tumors, is an ideal target for immunotherapy. WT1 class I peptide epitopes that were identified and shown to stimulate CD8⁺ T cells are being tested as vaccine candidates in several clinical trials. The induction and maintenance of a robust memory CD8⁺ cytotoxic T-cell response requires CD4⁺ T-cell help.

Experimental Design: Three HLA class II peptide epitopes of WT1 with high predictive affinities to multiple HLA-DRB1 molecules were identified using the SYFPEITHI algorithm. Due to the highly polymorphic nature of the HLA class II alleles, such reactivity is critical in the development of a broadly useful therapeutic. One of the WT1 CD4⁺ peptide epitopes, 122-140, comprises a previously identified CD8⁺ peptide epitope (126-134). By mutating residue 126 from an arginine to a tyrosine, we embedded a synthetic immunogenic analogue CD8⁺ epitope (126-134) inside the longer peptide (122-140). This analogue was previously designed to improve immunogenicity and induce a potent CD8⁺ response.

Results: WT1 peptides 328-349 and 423-441 are able to stimulate a peptide-specific CD4⁺ response that can recognize WT1⁺ tumor cells in multiple HLA-DRB1 settings as determined by IFN- γ enzyme-linked immunospot assays. The mutated WT1 peptide epitope 122-140 is able to induce CD4⁺ and cytotoxic CD8⁺ WT1-specific T-cell responses that can recognize the native WT1 epitopes on the surface of human WT1⁺ cancer cells. Cross-priming experiments showed that antigen-presenting cells pulsed with either mesothelioma or leukemia tumor lysates can process and present each of the CD4⁺ peptides identified.

Conclusions: These studies provide the rationale for using the WT1 CD4⁺ peptides in conjunction with CD8⁺ peptide epitopes to vaccinate patients with WT1-expressing cancers.

Wilms' tumor 1 protein (WT1) is a zinc finger transcription factor that is normally expressed in tissues of the mesodermal origin during embryogenesis, including the kidney, gonads, heart, mesothelium, and spleen. In normal adult tissues, WT1

expression is limited to low levels in the nuclei of normal CD34⁺ hematopoietic stem cells, myoepithelial progenitor cells, renal podocytes, and some cells in testis and ovary (1). Although originally described as a tumor suppressor gene, the WT1 protein also seems to be involved in tumorigenesis, as WT1 expression is up-regulated in leukemia cells of all lineages (2, 3) and in several solid tumors, including malignant mesothelioma, lung, breast, prostate, and ovarian carcinomas (4). Therefore, WT1 is an attractive target for immunotherapy.

Malignant mesothelioma is most often seen in patients with a history of occupational asbestos exposure. Although asbestos usage has been significantly reduced, incidence of malignant mesothelioma continues to rise due to the long latency between exposure and the onset of symptoms (5). To date, there is no standard curative therapy for mesothelioma, and the prognosis is poor. Surgical approaches, such as pleurectomy and extrapleural pneumonectomy, result in high recurrence rates, and chemotherapy and radiation therapy result in only limited improvements (6). There are reports describing the relationship between tumor-infiltrating lymphocytes and prognosis (7) as well as evidence of immune responsiveness in cases of spontaneous regression (8), suggesting that some patients can mount an immune response *in vivo* against these tumors. New immunotherapeutic approaches to malignant mesothelioma

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Table 1. Peptides from WT1 that are predicted to bind to HLA-DRB molecules

Name	Sequence	Score*					
		DRB1*0101	DRB1*0301	DRB1*0401	DRB1*0701	DRB1*1101	DRB1*1501
423	RSDELVRHHNMHQ [†] RNMTKL	15	17	20	14	10	24
328	PGCNKRYFKLSHLQMHSRKHTG [†]	28	11	28	18	25	20
122	SGQARMFNPAPYLPSCLES [‡]	22	18	22	16	16	18
122A1	SGQAYMFNPAPYLPSCLES [§]	27	17	22	18	16	18
Frequency in Caucasian population (49)		18.5	17.7	23.6	26.2	17.0	19.9

*SYFPEITHI prediction software available at <http://www.syfpeithi.de>.

[†] Peptide encompasses the sequence reported by ref. 29 (WT1 amino acids 331-345).

[‡] Peptide encompasses the sequence reported by Kobayashi et al. (WT1 amino acids 124-138; ref. 31).

[§] Residue Y in bold represents a modification from the native 122 sequence.

are being explored, including the use of mesothelioma tumor lysate-pulsed dendritic cells (9) and IFN- β gene therapy via an adenoviral vector (10). These studies were able to elicit CD8⁺ T cells against murine mesothelioma, illustrating the possibilities of successful immunotherapeutic approaches to this disease.

Expression of WT1 (11) and immunohistochemical detection of the WT1 protein (12) have been observed in most mesothelioma cell lines and primary tumor specimens, and WT1 expression is currently used as a diagnostic marker to distinguish mesothelioma from similar tumors, such as pulmonary adenocarcinoma (12). In addition to serving as a diagnostic marker, the unique overexpression of WT1 may also serve as a therapeutic target for this disease in particular.

The activation of WT1-specific CD8⁺ CTLs is critical to mounting an antitumor response. At least four native peptide nonamers from human WT1 have been identified and shown by others to generate a WT1-specific cytotoxic response in the context of HLA-A0201 and HLA-A2402 that is able to kill leukemic cell lines and blast cells from patients with acute myelogenous leukemia or acute lymphoblastic leukemia (13–18). In an effort to enhance the immunogenicity of these peptides and overcome possible problems with tolerance to this self-antigen, we have designed several synthetic analogue HLA class I peptide epitopes of WT1 via the introduction of specific amino acid point mutations that induce a more potent cytotoxic response to tumor cells (19).

Immunization with CD8-specific peptides alone induces a short-lived antigen-specific CTL response without generating T-cell memory (20). The need for antigen-specific CD4⁺ helper T cells is critical when CTL precursor frequency is low, as in the case of tumor-specific T cells (21). The presence of significant amounts of Th1-biased circulating anti-WT1 IgG antibodies in patients with hematopoietic malignancies compared with healthy volunteers suggests that the WT1 protein is immunogenic and that WT1-specific CD4⁺ T cells, which are necessary for immunoglobulin class switching, are activated in these patients (22). Herein, we report the identification of three HLA class II peptide epitopes of WT1 and their ability to stimulate an immune response reactive with human malignant mesothelioma cell lines as well as leukemia cell lines.

Materials and Methods

Synthetic peptides. Each of the peptides used in this study was purchased and synthesized by Genemed Synthesis, Inc. using fluo-

renylmethoxycarbonyl chemistry and solid-phase synthesis and purified by high-pressure liquid chromatography. The quality of the peptides was assessed by high-performance liquid chromatography analysis, and the expected molecular weight was observed using matrix-assisted laser desorption mass spectrometry. Peptides were sterile and 70% to 90% pure. The peptides were dissolved in DMSO and diluted in PBS (pH 7.4) or saline at 5 mg/mL and stored at -80°C.

Amino acid sequences and predicted binding of putative CD4⁺ epitopes to HLA-DRB1 molecules were identified using the predictive algorithm of the SYFPEITHI database³ (Table 1; ref. 23). Irrelevant control peptides used in *in vitro* experiments were the following: RAS (TEYKLVVVGAPGVGKSAITIQ) or chronic myelogenous leukemia (CML) b2a2 (VHSIPLTINKEEALQRPVASFDFE) for class II and HIV pol (ILKEPVHGV) or CML F (YLKALQRPY) for class I.

Cell lines. Human mesothelioma cell lines, including sarcomatoid (VAMT, H2373, and H28), epithelioid (H2452), and biphasic (JMN, MSTO, and H-Meso1A) were cultured as described (24, 25).

The cell lines were obtained from the following sources: H-Meso1A (National Cancer Institute, Bethesda, MD), JMN and VAMT (a gift of Dr. Sirotiak, Memorial Sloan-Kettering Cancer Center, New York, NY), H2452 and H2373 (a gift of Dr. Pass, Karmanos Cancer Institute, Wayne State University, Detroit, MI), and H28 and MSTO (American Type Culture Collection). Mesothelioma cell lines Meso 11, Meso 34, Meso 37, Meso 47, and Meso 56 were provided to us by Dr. M. Gregoire (Institute of Biology, Nantes, France) and cultured as described (26). All cells were HLA typed by the Department of Cellular Immunology at Memorial Sloan-Kettering Cancer Center. Melanoma cell line MeWo (WT1⁻, A201⁺) was obtained from the American Type Culture Collection and cultured as directed. SKRC-52 renal cell carcinoma was obtained from L. Old (Ludwig Institute, New York, NY). Leukemia cell lines were cultured in RPMI 1640 supplemented with 10% FCS, penicillin, streptomycin, 2 mmol/L glutamine, and 2-mercaptoethanol at 37°C/5% CO₂. LAMA81, BV173, and 697, Ph⁺ leukemias that are all WT1⁺, A201⁺ were kindly provided by Dr. H.J. Stauss (University College London, London, United Kingdom). SKLY16 is a human B-cell lymphoma (WT1⁺, A201⁺); K562, RwLeu4, and HL60, all WT1⁺ leukemias, were obtained from the American Type Culture Collection.

In vitro immunization and human T-cell cultures. After informed consent on Memorial Sloan-Kettering Cancer Center Institutional Review Board protocols, peripheral blood mononuclear cells (PBMC) from HLA-typed healthy donors were obtained by Ficoll density centrifugation. Monocyte-derived dendritic cells were generated from PBMCs using a plastic adherence technique. The adherent cells were cultured for 7 days in RPMI 1640/1% to 5% autologous plasma, 500 units/mL recombinant human interleukin (IL)-4 (R&D Systems), and 1,000 units/mL recombinant human granulocyte macrophage

³ <http://www.syfpeithi.de>

colony-stimulating factor (Immunex). On days 2 and 4 of incubation, fresh medium with IL-4 and granulocyte macrophage colony-stimulating factor was added. On day 5, 10 µg/mL peptide was added to the immature dendritic cells. On day 6, maturation cytokine cocktail was added [IL-4, granulocyte macrophage colony-stimulating factor, 500 IU/mL IL-1β (R&D Systems), 1,000 IU/mL IL-6 (R&D Systems), 10 ng/mL tumor necrosis factor-α (R&D Systems), and 1 µg/mL prostaglandin E2 (Sigma)]. On day 8, either CD4⁺ or CD3⁺ T lymphocytes were isolated from the same donors using negative selection by depletion with anti-CD11b, anti-CD56, anti-CD19, and/or anti-CD8 monoclonal antibodies (Miltenyi) and stimulated at a 10:1 E:T ratio with the monocyte-derived dendritic cells. The mature dendritic cells expressed dendritic cell-associated antigens, such as CD80, CD83, CD86, and HLA class I and class II, on their cell surfaces (data not shown). CD4⁺ or CD3⁺ cells were stimulated for 7 days in the presence of RPMI 1640/5% autologous plasma, 10 µg/mL WT1 synthetic peptides, 1 µg/mL β2-microglobulin (Sigma), and 10 ng/mL IL-15 (R&D Systems). On day 7, T cells were restimulated with either a 5:1 E:T ratio of freshly isolated CD14⁺ cells or a 50:1 E:T ratio of monocyte-derived dendritic cells. In some cases, cells were restimulated after another 6 to 7 days in the same manner. After the second or third stimulation, IFN-γ secretion of these cells was examined by enzyme-linked immunospot (ELISPOT). In some cases, total PBMCs were stimulated with 10 µg/mL WT1 synthetic peptide and 10 ng/mL IL-15. PBMCs were restimulated every 7 days by spinning down the cells and resuspending in fresh medium containing 10 µg/mL peptide and 10 ng/mL IL-15. After two or three rounds of stimulation, the cells were used in an IFN-γ ELISPOT assay.

IFN-γ ELISPOT. HA-Multiscreen plates (Millipore) were coated with 100 µL of mouse anti-human IFN-γ antibody (10 µg/mL; clone 1-D1K; Mabtech) in PBS, incubated overnight at 4°C, washed with PBS to remove unbound antibody, and blocked with RPMI 1640/10% autologous plasma for 2 h at 37°C. Purified CD4⁺, CD8⁺, or CD3⁺ T cells (>95% pure) were plated with either autologous CD14⁺ (5:1 E:T ratio) or autologous dendritic cells (10:1 E:T ratio). Various test peptides were added to the wells at 40 µg/mL. Negative control wells

contained antigen-presenting cells (APC) with or without T cells or T cells alone plus peptides. Positive control wells contained T cells plus APC plus 10 µg/mL phytohemagglutinin (Sigma). All conditions were done in triplicate. Blocking experiments were done whereby 50 µg/mL of anti-HLA-DR monoclonal antibody clone L243 (a gift from Dr. Denzin, Memorial Sloan-Kettering Cancer Center) were incubated with 1×10^6 CD14⁺ target cells for 1 h at 37°C. Microtiter plates were incubated for 20 h at 37°C and then extensively washed with PBS/0.05% Tween and 100 µL/well biotinylated detection antibody against human IFN-γ (2 µg/mL; clone 7-B6-1; Mabtech) was added. Plates were incubated for an additional 2 h at 37°C and spot development was done as described (27). Spot numbers were automatically determined with the use of a computer-assisted video image analyzer with KS ELISPOT 4.0 software (Carl Zeiss Vision).

Cross-priming experiments. A CD3⁺ *in vitro* stimulation was done as described above. Immature dendritic cells (2×10^6) were incubated with total cellular lysate from 2×10^6 tumor cells that was previously prepared by three freeze/thaw cycles. Following an 18-h incubation, maturation cytokines were added to the dendritic cells as described above. CD3⁺ cells were stimulated thrice with these autologous mature dendritic cells, after which the T cells were tested in an IFN-γ ELISPOT assay, as described above, for reactivity against autologous, mature dendritic cells that had been previously pulsed with individual CD4⁺ peptides when in the immature state. These dendritic cells were exposed to peptide again during the ELISPOT assay as described above.

Chromium-51 cytotoxicity assay. The presence of specific CTLs was measured in a standard 4-h chromium release assay as described (27). Briefly, target cells are pulsed with 10 µg/mL of synthetic peptides overnight at 37°C, after which they are labeled with 300 µCi of Na²⁵¹CrO₄ (NEN Life Science Products, Inc.). After extensive washing, target cells are incubated with T cells at E:T ratios ranging from 100:1 to 10:1. All conditions were done in triplicate. Plates were incubated for 4 h at 37°C in 5% CO₂. Supernatant fluids were harvested and radioactivity was measured in a gamma counter. Percentage specific lysis was determined from the following formula: $100 \times [(experimental$

Table 2. Summary of *in vitro* stimulation experiment and IFN-γ ELISPOT results

Experiment no.	WT1DR 328		WT1DR 423		WT1DR 122		WT1DR 122A1	
	Donor HLADRB*	Response [†]	Donor HLADRB	Response	Donor HLADRB	Response	Donor HLADRB	Response
1	1001/1501	+++	1001/1501	+++	1401/15XX	—	1401/15XX	+ (heteroclitic) [‡]
2	301/901	+++	101/1201	—	1401	+	1401	++ (heteroclitic)
3	1001/1501	++	1001/1501	—	1401	+	1401	+ (heteroclitic)
4	407/1302	NA [§]	407/1302	NA	104/1104	—	104/1104	+++ (heteroclitic)
5	407/1302	+++	407/1302	+	405/1302	—	301/1601	NA
6	1001/1501	—	1001/1501	—	301/1601	++	405/1302	—
7	407/1302	—	407/1302	—	1401/15XX	+++	15XX	+++ (heteroclitic)
8	1001/1501	++	1001/1501	—	15XX	+	422/13XX	+++ (heteroclitic)
9	407/1302	++	407/1302	+	1401/15XX	+++	422/13XX	++ (heteroclitic)
10	701/1202	++	701/1202	—			301/1601	—
11	1401/15XX	++	1401/15XX	—			1401/15XX	+
12	407/1302	+++	422/13XX	—			1401/15XX	+
13	422/13XX	+	422/13XX	+++			15XX	+++ (heteroclitic)
14	422/13XX	+++	1001/1501	+++				
15	1001/1501	+++						
No. donors	6	80%	6	35.7%	6	66.6%	7	76.9%

*Healthy donor CD3⁺/CD4⁺ T cells were stimulated twice with individual WT1 peptide as described in Materials and Methods. Stimulated T cells were challenged in an IFN-γ ELISPOT.

[†]ELISPOT results comparing T cell reactivity with autologous CD14⁺ cells pulsed with stimulating peptide versus autologous CD14⁺ cells pulsed with irrelevant peptide. -, negative results; +, two times background; ++, three times background; +++, four times background.

[‡]T cells stimulated with WT1DR peptide 122A1 recognized CD14⁺ cells pulsed with stimulating peptide and CD14⁺ cells pulsed with WT1DR peptide 122.

[§]Experiment failed.

||Percentage of experiments with a significant positive results ($P \leq 0.05$).

release - spontaneous release) / (maximum release - spontaneous release)]. Maximum release was determined by lysis of radiolabeled targets in 2.5% Triton X-100.

Quantitative reverse transcription-PCR for WT1 transcripts. Total RNA was isolated from the various cell lines using a phenol/chloroform extraction method. RNA purity was confirmed by absorbance at 260 nm. The reverse transcription reaction was adapted from protocols supplied by Applied Biosystems. Beginning with 1 μ g of total RNA, random hexamers and reverse transcriptase were used to isolate cDNA. For the PCR, cDNA was mixed with the following WT1 primers and probe: 5'-CAGGCTGCAATAAGAGATATTITAAGCT-3' (forward primer, located on exon 7), 5'-GAAGTCACACTGGTATGGTTTCTCA-3' (reverse primer, located on exon 8), and 5'-CTTACAGATGCACAGCAGGAAGCACACTG-3' (Taqman probe, located on exon 7). The fluorescent WT1 probe 5'-56-FAM/CTTACAGATGCACAGCAGGAAGCACACTG/3BHQ_1/-3' was labeled with 6-carboxyfluorescein phosphoramidate as reporter dye at the 5'-end and with the quencher dye carboxytetramethylrhodamine at the 3'-end (Integrated DNA Technologies). The PCR has been described (28). Briefly, 2 min at 50°C, 10 min at 95°C followed by 50 cycles of 15 s at 95°C and 60 s at 62°C. Each reaction was done in triplicate, and discrepancies >1 Ct in one of the wells were excluded. The quantitative reverse transcription-PCR and fluorescence measurements were made on the Applied Biosystems 7500 Real-Time PCR System. Control ABL primers and probes were 5'-TGGAGATAACACTCTAAGCATAACTAAAGGT-3' (forward primer, located on ENF-10030), 5'-GATGTAGTTGCTTGGGACCCA-3' (reverse primer, located on ENR-1063), and 5'-56-FAM/CCATTTTGGTTGGGCTTCACAC-CATT/3BHQ_1/-3' (fluorescent probe, located on ENPr-1043).

Statistics. Statistical analyses were done on StatView software (SAS Institute) using a two-tailed unpaired *t* test, with the level of statistical significance set at 0.05.

Results

Identification of peptides with a high predictive binding to multiple HLA class II molecules. Due to the permissive nature of the HLA class II binding pocket, designing a peptide that displays high affinity to multiple class II molecules, rather than binding limited to a single DR type, is possible. Such peptides would therefore be capable of stimulating T cells from a larger percentage of the target population. The WT1 protein sequence was screened using the SYFPEITHI algorithm, and three WT1DR peptides (423, 328, and 122) were identified that had predicted high-affinity binding to multiple HLA-DRB1 molecules that are highly expressed in the Caucasian population (Table 1). Peptide 423 spans WT1 amino acids 423-441, peptide 328 spans WT1 amino acids 328-349, and peptide 122 spans WT1 amino acids 122-140. The maximal scores to each HLA protein are variable, with HLA-A0201 maximal score being 36. Three WT1 native 15-mer peptides that were predicted to bind to HLA-DRB1*0401 (29) and HLA-DP501 (30) and HLA-DR1, HLA-DR4, and HLA-DR7 (31) have been identified by others. These peptides were capable of stimulating CD4⁺ T cells that were restricted to HLA-DRB1*0401 (29), HLA-DP501 (30), and HLA-DR53 (31). Peptide 328 is an extended version of the HLA-DRB1*0401-specific peptide (29), and peptide 122 is similar to the peptide reported by Kobayashi et al. (31).

WT1DR peptide 122 comprises within it a known CD8⁺ epitope (126-134; ref. 14) We previously reported the use of amino acid substitutions at HLA-A0201 binding anchor motifs to increase the antigenicity and binding affinity of native WT1 9-mer peptides. One such modified peptide, termed WT1A1, spans the WT1 amino acid residues 126-134 and contains a modified amino acid at position 126, in which we substituted a

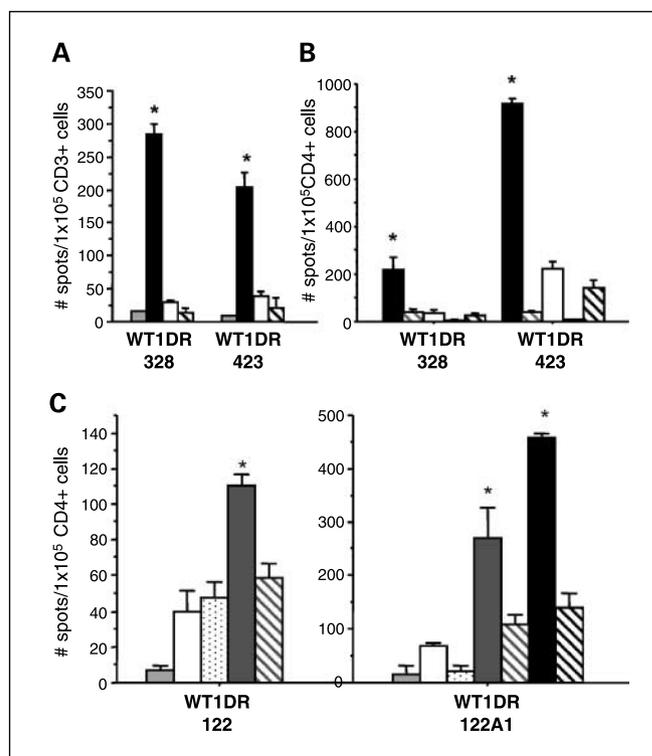


Fig. 1. IFN- γ ELISPOT with WT1DR peptides 328, 423, 122, and 122A1. **A.** CD3⁺ T cells from an HLA-DRB1*1001/1501 donor were stimulated twice with either peptide WT1DR 328 or WT1DR 423 as described in Materials and Methods. Stimulated T cells were challenged in an IFN- γ ELISPOT assay with the following: unchallenged control (gray columns), CD14⁺ cells pulsed with stimulating peptide (either WT1DR 328 or WT1DR 423; black columns), CD14⁺ cells pulsed with irrelevant CD4⁺ peptide epitope (RAS; white columns), and unpulsed CD14⁺ cells (hatched columns). *, *P* < 0.05, compared with controls. Y axis, number of spots per 1×10^5 CD3⁺ T cells; X axis, peptide used for T-cell stimulations. **B.** CD4⁺ T cells from an HLA-DRB1*1001/1501 donor were stimulated twice with either peptide WT1DR 328 or WT1DR 423 as described in Materials and Methods. Stimulated T cells were challenged in an IFN- γ ELISPOT assay with the following: CD14⁺ cells pulsed with stimulating peptide (either WT1DR 328 or WT1DR 423; black columns), CD14⁺ cells pulsed with stimulating peptide (either WT1DR 328 or WT1DR 423) and incubated with anti-HLA-DR blocking antibody (gray hatched columns), CD14⁺ cells pulsed with irrelevant CD4⁺ peptide epitope (CML b2a2; white columns), CD14⁺ cells pulsed with irrelevant CD4⁺ peptide epitope (CML b2a2) and incubated with anti-HLA-DR blocking antibody (checkerboard columns), and unpulsed CD14⁺ cells (black hatched columns). *, *P* < 0.04, compared with irrelevant peptide controls and compared with CD14⁺ T cells in the presence of anti-HLA-DR monoclonal antibody. Y axis, number of spots per 1×10^5 CD4⁺ T cells; X axis, peptide used for T-cell stimulations. **C.** CD4⁺ T cells from an HLA-DRB1*1501 donor were stimulated twice with either peptide WT1DR 122 or WT1DR 122A1 as described in Materials and Methods. Stimulated T cells were challenged in an IFN- γ ELISPOT assay with the following: unpulsed CD14⁺ cells (light gray columns), CD14⁺ cells pulsed with irrelevant CD4 peptide epitope (CML b2a2; white columns), CD14⁺ cells pulsed with irrelevant CD4 peptide epitope (CML b2a2) and incubated with anti-HLA-DR blocking antibody (dark gray hatched columns), CD14⁺ cells pulsed with peptide WT1DR 122 (dark gray columns), CD14⁺ cells pulsed with peptide WT1DR 122 and anti-HLA-DR blocking antibody (dark gray hatched columns), CD14⁺ cells pulsed with peptide WT1DR 122A1 (black columns), and CD14⁺ cells pulsed with peptide WT1DR 122A1 and incubated with anti-HLA-DR blocking antibody (black hatched columns). *, *P* < 0.05, compared with irrelevant peptide controls and compared with CD14⁺ T cells in the presence of peptide and anti-HLA-DR monoclonal antibody. Y axis, number of spots per 1×10^5 CD4⁺ T cells; X axis, peptide used for T-cell stimulations.

tyrosine for the native arginine. This peptide induces a more potent cytotoxic CD8⁺ T-cell response against WT1⁺ tumors (19). Due to the location of peptide 126 within the longer CD4⁺ 122 peptide epitope, we investigated whether the inclusion of the mutated residue 126 would confer both a CD4 and a potent CD8⁺ epitope within the long 122 CD4⁺

epitope. This mutated peptide, termed 122A1, did not significantly alter the predictive binding score of the native WT1DR 122 peptide to the HLA-DRB1 molecules (Table 1) but offered the possibility of inducing simultaneously a robust CD4⁺ and CD8⁺ WT1-specific T-cell response if the long 122A1 peptide could be processed by the cell into the shorter 126-134 CD8⁺ epitope.

Induction of a peptide-specific CD4 immune response as detected by IFN- γ assay. Web-based predictive algorithms are only 50% reliable in identifying peptide epitopes capable of stimulating T cells (23). Therefore, *in vitro* stimulation assays are necessary to confirm those peptides that can stimulate CD4⁺ T cells. Table 2 lists the results of CD3⁺ or CD4⁺ cells from healthy donors that were stimulated with peptide-loaded autologous dendritic cells. WT1DR peptide 328 stimulated a significant peptide-specific CD4⁺ response in a variety of HLA-DRB1 settings, a representative sample of which is shown in Fig. 1A, whereas responses to WT1DR peptide 423 were more variable. The peptide-specific response seen is HLA-DR restricted because the addition of anti-HLA-DR antibody blocked the peptide-specific response (Fig. 1B).

Consistent with previously published results (31), WT1DR peptide 122 induced a peptide-specific response that was HLA-DR mediated. CD4⁺ T cells stimulated with WT1DR peptide 122A1 displayed a more robust heteroclitic T-cell response in that the stimulated T cells not only recognized the mutated immunizing peptide 122A1 but also the native 122 peptide (Fig. 1C, note the scale difference in the Y axis and Table 2). Here, too, the effect was shown to be HLA-DR mediated. This cross-reactivity to the native peptide is critical because only the native sequence would be expressed on the surface of tumor cells.

These stimulation experiments were reproduced multiple times in up to seven healthy donors, each with a different HLA-DRB1 type to assess the efficacy of the peptides in various HLA-DR settings (Table 2). Up to 15 separate experiments were done with each WT1DR peptide. Peptide-specific T-cell responses were seen in 12 of 15 experiments with WT1DR 328, 5 of 14 experiments with WT1DR 423, and 6 of 9 experiments with WT1DR122. WT1DR 122A1 stimulated T cells that recognized the immunizing WT1DR 122A1 peptide and the native WT1DR 122 peptide in 10 of 13 experiments. Although the number of experiments is too small, and the number of HLA-DRB1 alleles tested is too great to draw any statistically significant

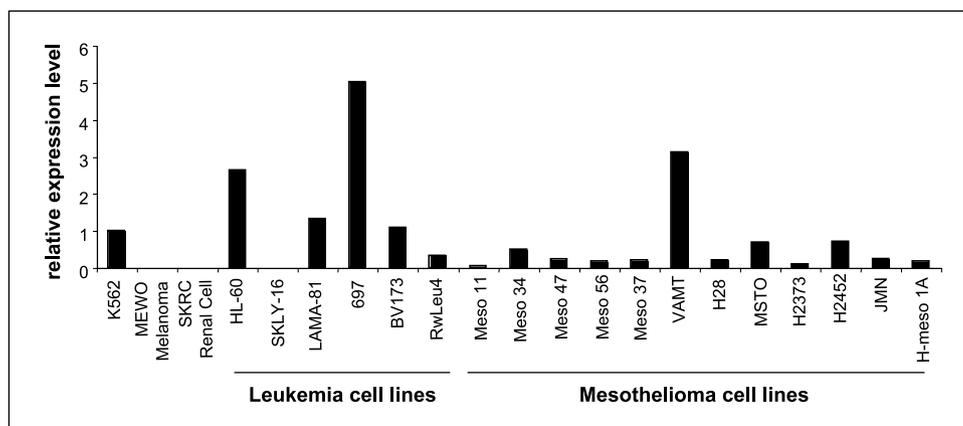
conclusions, there is considerable variation in how the predicted binding scores correlate to actual efficacy for each of the HLA-DRB1 alleles.

WT1 expression in human mesothelioma cell lines. Whereas many groups have been focused on developing WT1 peptide immunotherapy to hematopoietic malignancies, only a few have studied the feasibility of WT1 vaccine-based therapy of solid tumors (32, 33). In particular, we were interested in developing a WT1-based vaccine for patients with malignant mesothelioma, a disease with a high WT1 expression and poor prognosis. The WT1 transcript levels in several human mesothelioma cell lines (sarcomatoid, epithelioid, and biphasic) were quantified by reverse transcription-PCR and compared with various leukemia cell lines with known WT1 expression (Fig. 2). Although 12 of 12 mesothelioma cell lines expressed WT1 message, in most cases, the WT1 level was lower in these cell lines compared with the leukemic cell lines. Melanoma (MeWo) and lymphoma (SKLY16) cell lines were WT1 negative. Interestingly, SKRC-52, a human renal cell carcinoma cell line, did not express WT1 despite the low expression of WT1 in adult renal podocytes (1). Flow cytometry analysis confirmed that the mesothelioma cell lines express class II molecules (data not shown), confirming previous studies (34). However, only mesothelioma cell lines JMN and H2452 expressed class I molecules (data not shown).

Processing and presentation of WT1 epitopes. Peptides that are presented on the surface of target cells bound to either HLA class I or class II molecules must first be processed either in the cytosol for class I peptides or in endocytic vesicles for class II peptides. Because the peptides we are studying were identified first by computer algorithms, it was necessary to determine whether these peptides were properly processed from the WT1 protein and presented in the context of HLA molecules. In addition, most tumor cells have low MHC II molecule expression, and it was critical to show that these peptides derived from dying tumor cells can be processed by APCs and presented to CD4⁺ T cells. "Cross-priming" experiments were done to determine if all the WT1DR peptides under study (423, 328, and 122) were presented and recognized by CD4⁺ T cells.

Total tumor lysates were prepared from three different cell lines: 697 (WT1⁺, HLA-A0201⁺), an e1a2 leukemia cell line; JMN (WT1⁺, HLA-A0201⁺), a biphasic mesothelioma cell line; and as a control, MeWo (WT1⁻, HLA-A0201⁺), a malignant melanoma cell line. Dendritic cells from healthy A0201⁺

Fig. 2. Quantitative reverse transcription-PCR. Relative WT1 expression levels in a variety of hematopoietic and mesothelioma cell lines. WT1 levels are shown as relative values compared with the human leukemia cell line K562, which is defined as 1.0 as described in ref. 28.



donors were incubated for 18 h with the tumor lysates and then used to stimulate autologous CD3⁺ T cells. Following three stimulations, the T cells were tested for their reactivity to autologous dendritic cells pulsed with the various WT1 peptides. T cells that had been stimulated with WT1⁺ tumor lysates recognized the individual HLA class II peptides (Fig. 3A and B), whereas T cells stimulated by dendritic cells pulsed with the WT1⁻ lysate (MeWo) did not stimulate WT1-specific T cells. In addition, T cells stimulated with dendritic cells pulsed with 697 tumor lysate recognized the native short class I peptide WT1A (126-134) and the analogue WT1A1 peptide (Fig. 3A). These experiments were repeated in five separate donors. Stimulated T cells could recognize WT1DR peptide 328 and WT1DR peptide 122A1 in three of five experiments, whereas stimulated T cells recognized WT1DR 423 in each experiment. Therefore, despite the low expression of WT1 transcript in the mesothelioma cell lines, the newly identified WT1 CD4 epitopes seem to be processed and presented in HLA class II molecules.

The reverse experiment was next conducted to determine if stimulation with the peptides could result in recognition of the tumor cells by the T cells, as should occur after vaccination with the peptides. A sufficient amount of WT1 peptide is presented on the surface of the WT1⁺ mesothelioma tumor cell for T cells stimulated with individual WT1DR peptides to recognize mesothelioma tumor cells compared with the control WT1⁻ melanoma cells (Fig. 3C). In another experiment, T cells were stimulated by the mutated WT1DR 122A1 and challenged with pulsed and unpulsed adherent cell targets. When control WT1⁻ target cells are pulsed with additional WT1DR 122A1 peptide, the amount of IFN- γ spots increases. When WT1⁺ target cells are pulsed with additional WT1DR 122A1 peptide, spots do not increase, implying that a maximal response has been achieved with the native processed peptides (Fig. 3D).

Peptide 122A1 induces a CD4⁺ and a cytotoxic CD8⁺ T-cell response. Although a strong CD8⁺ epitope is nested within the longer WT1DR 122 and WT1DR 122A1 peptides, it was critical to determine if the long peptide could be processed to stimulate CD8⁺ T cells. Both WT1DR 122 and WT1DR 122A1 were able to activate CD8⁺ T cells against the native short epitope (termed WT1A, amino acids 126-134) and CD4⁺ cells against the long peptide (Fig. 4A and B; ref. 15). The mutated WT1DR 122A1 sequence was a more potent stimulator. WT1DR 122A1, but not WT1DR 122, stimulated a sufficient amount of CD8⁺ cells to be cytotoxic to 697, a WT1⁺ leukemia cell line that expresses native WT1 protein. These stimulated CD8⁺ T cells did not recognize SKLY16, a WT1⁻ B-cell lymphoma, unless it had been pulsed with peptide WT1A (Fig. 4C). T cells stimulated with WT1DR 122 were unable to kill WT1⁺ target tumor cell lines (data not shown). These experiments have been repeated in four different A0201⁺ donors, each with a distinct HLA-DRB1 type. In three of four experiments, the long WT1DR 122A1 peptide stimulated CD8⁺ responses (data not shown). The magnitude of the response varied, indicating that the potency of the WT1DR 122A1 peptide is donor dependent.

CD8⁺ T cells reactive with the WT1A epitope are cytotoxic to leukemia cell lines (14, 19) that express high levels of WT1. We have previously shown that T cells stimulated with the WT1A1 analogue can kill WT1⁺ leukemia cells (19). In addition, human T cells stimulated twice with either the native WT1A or the analogue WT1A1 peptide can kill human

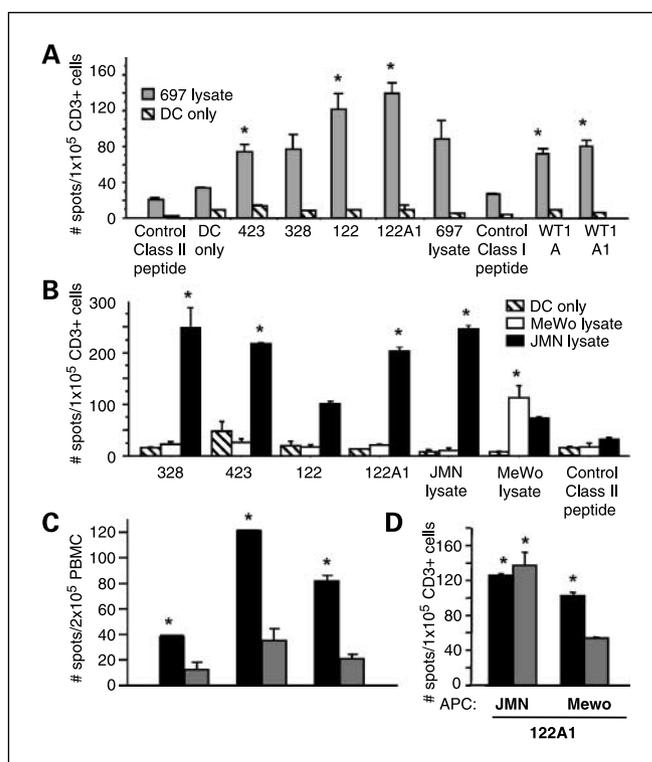


Fig. 3. Processing and presentation of WT1DR peptides. *A* and *B*, cross-priming experiments. *A*, CD3⁺ T cells from an HLA-A0201/301 DRB1*1301/1302 healthy donor were stimulated with autologous dendritic cells (DC) previously incubated with 697 tumor lysates. 697 is a WT1⁺ leukemia cell line. Stimulated T cells were challenged in an IFN- γ ELISPOT assay with autologous dendritic cells previously incubated with either 697 tumor lysate, individual WT1 peptides, control peptides, or unpulsed dendritic cells, as indicated on the X axis. Hatched columns, background level of spots from autologous dendritic cells incubated in the absence of T cells. *, $P < 0.05$, compared with control peptides. Y axis, number of spots per 1×10^5 CD3⁺ cells. *B*, CD3⁺ T cells from an HLA-A0201/101 DRB1*0301/1601 healthy donor were stimulated with autologous dendritic cells previously incubated with tumor lysates from either JMN, a WT1⁺ mesothelioma cell line (black columns), or MeWo, a WT1⁻ melanoma cell line (white columns). Stimulated T cells were challenged in an IFN- γ ELISPOT assay with autologous dendritic cells previously incubated with either JMN or MeWo tumor lysates, individual WT1DR peptides, or control class II peptide, as indicated on the X axis. Hatched columns, background level of spots from autologous dendritic cells incubated in the absence of T cells. *, $P < 0.05$, compared with control peptides. Y axis, number of spots per 1×10^5 CD3⁺ cells. *C* and *D*, CD3⁺ IFN- γ ELISPOT against mesothelioma cell lines. *C*, total PBMCs from an HLA-DRB1*13XX donor were stimulated twice with the different WT1DR peptides as described in Materials and Methods. Stimulated T cells were challenged in an IFN- γ ELISPOT assay with the following: mesothelioma H-Meso1A cell line (WT1⁺, HLA-DRB1*1301; black columns) and control melanoma MeWo cell line (WT1⁻, HLA-DRB1*15XX; gray columns). *, $P \leq 0.01$, compared with MeWo controls. Y axis, number of spots per 2×10^5 PBMCs; X axis, peptide used for T-cell stimulation. *D*, CD3⁺ T cells from an HLA-A0201/DRB1*1501 donor were stimulated twice with WT1DR 122A1 as described in Materials and Methods. Stimulated T cells were then challenged in an IFN- γ ELISPOT assay with the following target cells: JMN, an A0201/DRB1*1505 WT1⁺ mesothelioma cell line, or MeWo, an A0201/DRB1*15XX WT1⁻ melanoma cell line. The target cells were either pulsed with WT1DR 122A1 (black columns) or not pulsed (gray columns). *, $P < 0.05$, compared with the unpulsed MeWo target cell. Y axis, number of spots per 1×10^5 CD3⁺ T cells; X axis, different cell lines used as target cells.

WT1⁺ mesothelioma cell lines compared with WT1⁻ control cell lines (9.2% lysis of MeWo versus 19% lysis of JMN for WT1A-stimulated T cells; 22.2% lysis of MeWo versus 44.8% lysis of JMN for WT1A1-stimulated T cells). We were able to show in this study that CD8⁺ T cells stimulated with WT1DR 122A1 are cytotoxic to the A0201⁺, WT1⁺ JMN human mesothelioma cell line and not the A0201⁺, WT1⁻ MeWo melanoma cell line (Fig. 4D). In contrast, CD4⁺ cells

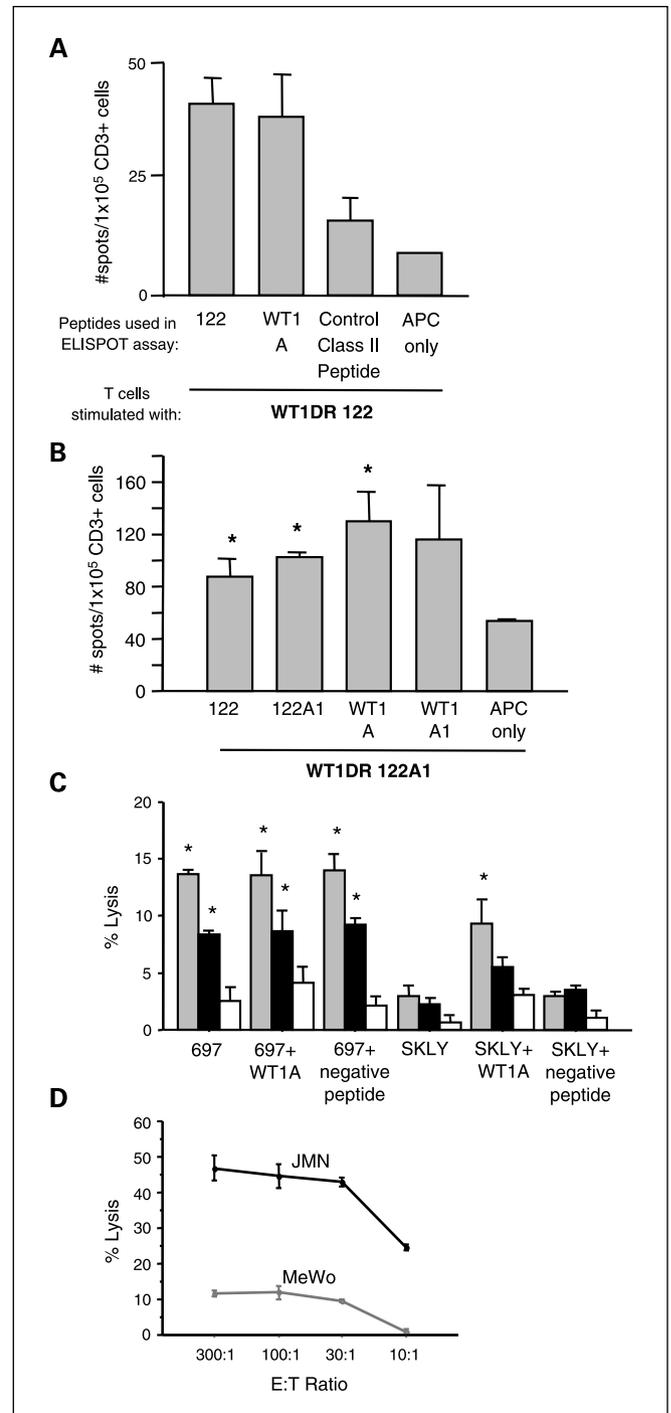
stimulated with WT1DR 122A1 showed no cytotoxicity to either WT1⁺ mesothelioma or WT1⁻ melanoma cells. However, we were unable to define the exact CD8⁺ peptide epitope of 122A1-stimulated CTLs from these experiments. These findings together provide a rationale to further study WT1-based immunotherapy for malignant mesothelioma.

Discussion

An approach to improving vaccine potency is to use analogue peptide epitopes of native tumor antigens, which are created by introducing amino acid point mutations into certain MHC anchor motifs (19). These novel peptides, which are homologous to the native peptide, can generate CTL responses that recognize the immunizing epitope and the native sequence. Immunogenic analogue peptides to human CD33 (35), WT1 (19), and bcr-abl (27) and others have been described. However, the induction and maintenance of a robust memory CTL response requires CD4⁺ T-cell help (20). CD4⁺ T helper cells recognize peptides ranging from 16 to 19 amino acids that are bound to the HLA class II molecule. Once activated, CD4⁺ cells enhance immunity by producing IFN- γ and IL-2 and licensing dendritic cells, thereby maintaining the activation, proliferation, and survival of potent antitumor activity mediated by CTLs (36, 37). Some *in vitro* studies have shown that CTLs at low E:T ratios are not cytotoxic unless CD4⁺ cells are added, underscoring the importance of T-cell help at the tumor site (38). Although some tumor cells are able to process and present antigens in the context of HLA class I and class II molecules (34), many tumors typically express low levels of class II molecules and cannot stimulate antigen-specific CD4⁺ cells on their own. Therefore, when designing peptide vaccines for those tumors, it may be valuable to also include CD4⁺ peptide epitopes. These peptides will be processed and presented by APCs, which in turn will activate CD4⁺ T cells and help induce a potent CD8⁺ antitumor T-cell response. The absence of activated CD4⁺ T-cell help will likely result in the induction of anergic CTL T-cell clones (39), which may explain

the failure of some vaccination strategies with class I peptides alone. To overcome this problem, some groups have vaccinated patients with short tumor-specific CD8⁺ epitopes along with universal nonspecific MHC class II-restricted epitopes, such as keyhole limpet hemocyanin (40) or the promiscuous epitopes of tetanus toxoid (41). Although a robust CD4⁺ response to the peptide is induced, the response to the tumor antigen often remains limited. An alternative approach to more selectively enhance the tumor-specific CD8⁺ response would be to find CD4⁺ epitopes directly from the tumor antigen.

Fig. 4. WT1DR peptide 122 and 122A1 stimulate CD8⁺ T-cell responses. **A**, CD3⁺ T cells from an HLA-A0201/DRB1*1401 donor were stimulated twice with WT1DR 122 as described in Materials and Methods. Stimulated T cells were challenged in an IFN- γ ELISPOT assay with autologous CD14⁺ cells in the presence of different peptides. Y axis, number of spots per 1×10^5 CD3⁺ cells; X axis, different test peptides used in the ELISPOT. **B**, CD3⁺ T cells from an HLA-A0201/DRB1*1401 donor were stimulated twice with WT1DR 122A1 as described in Materials and Methods. Stimulated T cells were challenged in an IFN- γ ELISPOT assay with control melanoma cell line MeWo (A0201/DRB1*15XX, WT1⁻) in the presence of different peptides. *, $P < 0.05$, compared with no peptide controls. Y axis, number of spots per 1×10^5 CD3⁺ cells; X axis, different test peptides used in the ELISPOT. **C**, CD3⁺ T cells from an HLA-A0201/DRB1*0101/15XX donor were stimulated twice with WT1DR 122A1 as described in Materials and Methods. After two rounds of stimulation, CD8⁺ T cells were isolated by negative selection and used as effector cells in a ⁵¹Cr release cytotoxicity assay as described in Materials and Methods. CD8⁺ T cells were incubated with various radiolabeled target cells [pulsed or unpulsed 697 (A0201⁺, WT1⁺) or SKLY16 (A0201⁺, WT1⁻)] at three different E:T ratios: 100:1 (gray columns), 30:1 (black columns), and 10:1 (white columns). Y axis, percentage of cytotoxicity; X axis, different target cell conditions. *, $P < 0.05$, compared with SKLY16 controls at the same E:T ratio. **D**, CD3⁺ T cells from an HLA-A0201/DRB1*0101/15XX donor were stimulated twice with WT1DR 122A1 as described in Materials and Methods. After two rounds of stimulation, CD8⁺ T cells were isolated by negative selection and used as effector cells in a ⁵¹Cr release cytotoxicity assay as described in Materials and Methods. CD8⁺ T cells were incubated with radiolabeled JMN (A0201⁺, WT1⁺; black line) or MeWo (A0201⁺, WT1⁻; gray line) target cells at four different E:T ratios. Y axis, percentage of cytotoxicity; X axis, different E:T ratios. $P < 0.001$, compared with MeWo controls.



We have identified three WT1 CD4⁺ epitopes that can stimulate T cells from a broad group of HLA-DRB types to recognize target cells pulsed with the stimulating peptide but not with an irrelevant peptide. Most important, these T cells also recognized leukemia and mesothelioma cell lines as assayed by IFN- γ ELISPOT.

WT1DR peptide 328 is a modified version of a previously reported peptide that has high binding affinity to HLA-DRB1*0401 and has been shown to stimulate CD4⁺ T cells that could recognize HLA-DRB1*0401-restricted CML tumor cells (29). We have added three amino acids to the NH₂-terminal ends and four amino acids to the COOH-terminal ends, as flanking residues outside the minimal epitope have been shown to greatly influence processing of the peptide (42) and increase the predictive binding scores. In addition, because class II molecules have a more permissive binding pocket, the elongated 328 peptide was determined to be a more promiscuous epitope and a potent stimulator of T cells from donors expressing several HLA-DRB1 alleles.

WT1DR peptide 423 is a new WT1 CD4⁺ epitope identified using the SYFPEITHI binding algorithm. A similar peptide from WT1 (418-432) was recently identified by Rezvani et al. (43) after screening a WT1 peptide library consisting of overlapping 15-mer peptides and testing the ability of these peptides to stimulate CD4⁺ T cells. The cross-priming experiments described here confirmed that this peptide is properly processed and presented by APCs and that the peptide itself could adequately stimulate T cells to recognize the native processed protein.

A recent study by Kobayashi (31) identified WT1 peptide 124-138 as a potential CD4⁺ epitope based on the presence of HLA-DR1, HLA-DR4, and HLA-DR7 binding motifs. T cells stimulated with this peptide secreted IFN- γ in the presence of autologous APCs in an HLA-DR53-restricted fashion. In addition, peptide 124-138 was processed by APC after pulsing with tumor lysate and it is expressed on the surface of HLA-DR⁺ tumor cells. Because CD4⁺ helper T cells play an essential role in maintenance of CTL antitumor responses, a peptide encompassing both a CD8⁺ and CD4⁺ epitope would be an advantageous immunogen. However, the reactivity of T cells stimulated by peptide 124-138 was inhibited by anti-HLA-DR antibodies, but not anti-HLA class I antibodies, indicating that CD8⁺ T cells were not activated (31) despite the presence of the

WT1 CTL epitope (126-134; ref. 14) within the longer peptide. Therefore, in an attempt to create a peptide that would simultaneously induce a robust CD4⁺ and CD8⁺ T-cell response, we mutated amino acid 126 from an arginine to a tyrosine, thereby embedding our previously described (19) heteroclitic WT1A1 peptide within the longer CD4⁺ epitope. In addition, we extended the peptide by several amino acids at the NH₂-terminal and COOH-terminal ends to increase the likelihood of better processing and increasing the HLA-DR binding promiscuity. This new long peptide, 122A1, is properly processed and presented by APC and can stimulate CD4⁺ and CD8⁺ T cells that are cytotoxic to cells expressing the native WT1 antigen.

Vaccines containing a combination of a CD8⁺ and CD4⁺ epitopes result in a more robust CTL response (44). Chimeric peptides have been made by linking CD4⁺ and CD8⁺ epitopes (45). However, such peptides may not be processed correctly due to the possible modification of natural protease cleavage sites, and chimeric peptides contain nonnatural sequences that are potentially immunogenic. Other investigators have made peptides in which the CD4⁺ and CD8⁺ epitopes overlap and have shown this elongated peptide to be more effective than administering separate CD4⁺ and CD8⁺ peptides (46, 47). We describe a different approach wherein a peptide containing a heteroclitic analogue CD8⁺ epitope is nested inside a CD4⁺ epitope. We believe that such a peptide is an ideal vaccine candidate because it has been shown by others to induce a CD8⁺ immune response that is greater in magnitude and durability than immunizing with the short CD8⁺ peptide alone (48). The mutated CD8⁺ WT1 epitope seems to be a more potent than its native parent.

This is the first report establishing the *in vitro* activity of WT1-based CD4⁺ and CD8⁺ T-cell stimulation reactive with human malignant mesothelioma. The three WT1DR CD4⁺ epitopes stimulate T cells that recognize human mesothelioma cell lines, and WT1-stimulated CTLs are able to kill these tumor cells despite the lower expression levels of WT1. These studies provide a rationale for clinical testing of these peptides in patients with WT1⁺ cancers. Whether patients with mesothelioma or other solid tumors will have the capacity to respond to vaccination in a manner similar to that seen *in vitro* with healthy donor cells will require a clinical trial. Such a trial is now open at Memorial Sloan-Kettering Cancer Center.

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Article on Use of Single-Dose Intrapleural IFN- β gene Transfer for Malignant Pleural Mesothelioma and Metastatic Pleural Effusions

In the article on single-dose intrapleural IFN- β gene transfer for malignant pleural mesothelioma and metastatic pleural effusions, beginning on page 4456 of the August 1, 2007, issue of Clinical Cancer Research, Michelle Kanther was omitted from the list of authors.

Clinical Cancer Research

Peptide Epitopes from the Wilms' Tumor 1 Oncoprotein Stimulate CD4⁺ and CD8⁺ T Cells That Recognize and Kill Human Malignant Mesothelioma Tumor Cells

Rena J. May, Tao Dao, Javier Pinilla-Ibarz, et al.

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