

## T-Cell Responses Directed against Multiple HLA-A\*0201-Restricted Epitopes Derived from Wilms' Tumor 1 Protein in Patients with Leukemia and Healthy Donors: Identification, Quantification, and Characterization

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**Abstract** **Purpose:** Antigens derived from the Wilms' tumor (WT1) protein, which is overexpressed in leukemias, are attractive targets for immunotherapy. Four HLA-A\*0201-restricted WT1-derived epitopes have been identified: WT37, WT126, WT187, and WT235. We determined the natural immunogenicity of these antigens in patients with hematologic malignancies and healthy donor. **Experimental Design:** To detect very low frequencies of WT1-specific CD8<sup>+</sup> T cells, we used quantitative reverse transcription-PCR to measure IFN- $\gamma$  mRNA production by WT1 peptide-pulsed CD8<sup>+</sup> T cells from 12 healthy donors, 8 patients with chronic myelogenous leukemia, 6 patients with acute myelogenous leukemia, and 8 patients with acute lymphoblastic leukemia. **Results:** Responses were detected in 5 of 8 chronic myelogenous leukemia patients, 4 of 6 patients with acute myelogenous leukemia, and 7 of 12 healthy donors. No responses were detected in patients with acute lymphoblastic leukemia. The magnitude and extent of these CD8<sup>+</sup> T-cell responses was greater in patients with myeloid leukemias than in healthy donors. Clonotypic analysis of WT1-specific CD8<sup>+</sup> T cells directly *ex vivo* in one case showed that this naturally occurring population was oligoclonal. Using fluorescent peptide-MHC class I tetramers incorporating mutations in the  $\alpha 3$  domain (D227K/T228A) that abrogate binding to the CD8 coreceptor, we were able to confirm the presence of high-avidity T-cell clones within the antigen-specific repertoire. **Conclusion:** The natural occurrence of high-avidity WT1-specific CD8<sup>+</sup> T cells in the periphery could facilitate vaccination strategies to expand immune responses against myeloid leukemias.

The Wilms' tumor (WT1) gene is implicated in leukemogenesis and WT1 mRNA expression is identifiable in most cases of acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), and adult acute lymphoblastic leukemia (ALL; refs. 1–3). Recent reports of the *in vitro* generation of human WT1-specific CD8<sup>+</sup> CTLs suggest that WT1 could serve as a useful and broadly expressed antigenic target for immunotherapy of leukemia (4–6). Furthermore, mice immunized with WT1 peptides or WT1 cDNA generate WT1-specific CTLs and reject challenges with WT1-expressing tumors (7–9); this

indicates that the WT1 protein could serve *in vivo* as a tumor rejection antigen. We and others have shown the presence of WT1-specific CTL precursors in patients with myeloid leukemias (10, 11) and healthy volunteers (10). More recently, small clinical studies have shown the feasibility and potential efficacy of WT1 peptide vaccination in humans (12–14). Currently, four HLA-A\*0201-restricted WT1 epitopes have been described: WT37 (VLDFAPPGA), WT126 (RMFPNAPYL), WT187 (SLGEQQYSV), and WT235 (CMTWNQMNL; refs. 4, 6, 15, 16), although only WT126 has been extensively studied in myeloid leukemias (6, 10, 11). In this study, we determined the natural immunogenicity of these four HLA-A\*0201-restricted WT1 epitopes in healthy volunteers and patients with CML, AML, and ALL.

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### Materials and Methods

**Patients and healthy controls.** Fourteen HLA-A\*0201+ patients with myeloid leukemias (AML and CML), 8 patients with ALL, and 12 healthy volunteers were studied. All patients and donors gave written informed consent on treatment or cell collection protocols approved by the NIH Institutional Review Board. Cells obtained from leukapheresis products were separated using Ficoll-Hypaque density gradient centrifugation (Organon Teknika, Durham, NC) and subsequently frozen in RPMI 1640 complete medium (Life Technologies, Gaithersburg, MD)

supplemented with 20% heat-inactivated FCS and 10% DMSO according to standard protocols. Before use, frozen cells were thawed, washed, and suspended in RPMI 1640 complete medium + 10% pooled human AB serum (Sigma Chemical, St. Louis, MO). High-resolution HLA class I genotyping was done by sequence-specific PCR using genomic DNA (HLA Laboratory, Department of Transfusion Medicine, Warren G. Magnusson Clinical Center, NIH, Bethesda, MD). The presence of immunoglobulin G (IgG) and IgM cytomegalovirus (CMV) antibodies in the donors was analyzed by passive latex agglutination (CMVSCAN kit, Becton Dickinson Microbiology System, Cockeysville, MD).

**Cell lines.** The C1R cell line is a human plasma leukemia cell line that does not express endogenous HLA-A or HLA-B antigens. C1R-A2 cells are C1R cells that express a transfected genomic clone of HLA-A2.1 (17). These cells were maintained in RPMI 1640 complete medium/10% FCS.

**Peptide synthesis.** Peptides used in this study were prepared by Biosynthesis (Lewisville, TX) to a minimum purity of 95%. The identity of each of the peptides was confirmed by mass spectral analysis. The following peptides were tested: WT 37-45 (VLDFAPPGA; ref. 16), WT 126-134 (RMFPNAPYL), WT 187-195 (SLGEQQYSV), WT 235-243 (CMTWNQMNL; refs. 6, 8), CMV pp65 495-503 (NLVPMVATV; ref. 18), and the synthetically modified (to enhance HLA-A2 binding) gp100 (209-2M) melanoma self-antigen 209-217 (IMDQVPFSV; ref. 19).

**Direct quantitative reverse transcription-PCR assay of peptide-specific CD8<sup>+</sup> T-cell reactivity.** To screen for peptide-specific CD8<sup>+</sup> T cells, IFN- $\gamma$  mRNA production by CD8<sup>+</sup> T cells stimulated with test peptides was measured as described previously (10). In brief, after isolation,  $1 \times 10^6$  CD8<sup>+</sup> T cells per well were plated in a 96-well flat-bottomed plate with 200  $\mu$ L of RPMI 1640 complete medium supplemented with 10% human AB serum and incubated overnight at 37°C (humidity 90%, CO<sub>2</sub> 5%) to minimize background expression of IFN- $\gamma$  mRNA due to lymphocyte manipulation. CD8<sup>+</sup> T cells were then stimulated *in vitro* with  $1 \times 10^6$  C1R-A2 cells loaded with test peptides at the concentrations indicated. Control wells contained CD8<sup>+</sup> T cells in the presence of unloaded C1R-A2 cells, C1R-A2 cells pulsed with gp100 (209-2M) peptide (negative control), and CMV pp65<sub>495</sub> (positive control). After 3 hours of coculture at 37°C in 5% CO<sub>2</sub>, cells were harvested for RNA isolation and cDNA transcription. Quantitative reverse transcription-PCR (RT-PCR) was done for IFN- $\gamma$  mRNA expression and normalized to copies of CD8 mRNA from the same sample. Gene expression was measured using an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) as described previously (20, 21). We and others have previously validated the feasibility of this approach for the analysis of antigen-specific T-cell responses both in peripheral blood lymphocytes and in tumor tissues (10, 22). Primers for CD8 and IFN- $\gamma$  and TaqMan probes (Custom Oligonucleotide Factory, Foster City, CA) were designed to span exon-exon junctions to prevent amplification of genomic DNA. To create a standard curve, the cDNA was generated by reverse transcription with the same technique used for the preparation of test cDNA. IFN- $\gamma$  and CD8 cDNA was amplified by PCR using the same primers designed for the RT-PCR, purified, and quantified by UV spectrophotometry. The number of cDNA copies was calculated by using the molecular weight of each gene amplicon. Serial dilutions of the amplified genes at known concentrations were tested by RT-PCR. Quantitative RT-PCR reactions of cDNA specimens, cDNA standards, and water as negative control were conducted in a total volume of 25  $\mu$ L with TaqMan Master Mix (Applied Biosystems), 400 nmol/L primers, and 200 nmol/L probe. Primer sequences were as follows: IFN- $\gamma$  (forward), 5'-AGCTCTGCATC-GTTTTGGGT; IFN- $\gamma$  (reverse), 5'-GTTCCATTATCCGCTACATCTGAA; IFN- $\gamma$  (probe), FAM-TCTTGGCTGTACTGCCAGGACCCA-TAMRA; CD8 (forward), 5'-CCCTGAGCAACTCCATCATGT; CD8 (reverse), 5'-GTGGCTTCGCTGGCA; and CD8 (probe), FAM-CAGCCACTT-CGTGCCGCTCTT-TAMRA. Thermal cycler variables were 10 minutes at 95°C and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

**Table 1.** Characteristics of 12 healthy donors and 8 CML, 6 AML, and 8 ALL patients

Healthy donor	Age/Sex
BD1 <input type="checkbox"/>	30/M
BD2 <input type="checkbox"/>	45/M
BD3 <input type="checkbox"/>	18/M
BD4 <input type="checkbox"/>	48/M
BD5 <input type="checkbox"/>	30/M
BD6 <input type="checkbox"/>	19/F
BD7 <input type="checkbox"/>	37/M
BD8 <input type="checkbox"/>	31/M
BD9 <input type="checkbox"/>	25/F
BD10 <input type="checkbox"/>	38/M
BD11 <input type="checkbox"/>	57/M
BD12 <input type="checkbox"/>	43/F

No. responders = 7/12

Patient (UPN)	Diagnosis	Treatment	Age
319 <input checked="" type="checkbox"/>	CML	HU	27/M
262 <input type="checkbox"/>	CML-CP	HU	40/M
365 <input type="checkbox"/>	CML-CP	HU	34/F
241 <input checked="" type="checkbox"/>	CML-CP	HU	39/F
17 <input checked="" type="checkbox"/>	CML-BC	IFN- $\alpha$ , HU	28/M
210 <input checked="" type="checkbox"/>	CML-BC	IFN- $\alpha$ , HU, Hyper-CVAD	64/M
28 <input type="checkbox"/>	CML-CP	Ara-c, IFN- $\alpha$ , HU	59/M
402 <input checked="" type="checkbox"/>	CML-CP	HU	31/M
No. responders = 5/8			
350 <input type="checkbox"/>	RAEB	None	45/F
378 <input type="checkbox"/>	RAEBT	Ida/ARA-C	63/M
104 <input type="checkbox"/>	AML-M2	HD ARA-C	34/F
370 <input type="checkbox"/>	AML-M4	Dauno/HD ARA-C	28/F
97 <input type="checkbox"/>	AML-M4	HD ARA-C, Myelotarg	53/F
389 <input type="checkbox"/>	AML-M2 ADE, HD	ARA-C	12/M
Total responders = 4/6			
119 <input type="checkbox"/>	B-ALL	Standard ALL therapy	16/F
449 <input type="checkbox"/>	B-ALL	Standard ALL therapy	18/F
444 <input type="checkbox"/>	B-ALL (2nd CR)	Standard ALL therapy	20/M
337 <input type="checkbox"/>	B-ALL	Standard ALL therapy	24/F
245 <input type="checkbox"/>	B-ALL (2nd CR)	Standard ALL therapy + HD ARA-C	29/M
274 <input type="checkbox"/>	Ph +ve ALL	Standard ALL therapy	36/F
002 <input type="checkbox"/>	B-ALL	Standard ALL therapy	
001 <input type="checkbox"/>	B-ALL (3rd CR)	Standard ALL therapy	12/M
Total responders = 0/8			

Abbreviations: BD, blood donor; CML-CP, CML chronic phase; CML-BC, CML blast crisis; Ph +ve, Philadelphia positive; RAEB, refractory anemia with excess of blasts; RAEBT, refractory anemia with excess of blasts in transformation; HU, hydroxyurea; Hyper CVAD, cyclophosphamide, doxorubicin, vincristine, and dexamethasone; HD ARA-C, high-dose cytosine-arabinoside; Ida ARA-C, idarubicin, cytosinearabinoside; dauno, daunorubicin; ADE, cytosine-arabinoside, daunorubicin, and etoposide; standard ALL treatment, cyclophosphamide, daunorubicin, prednisone, asparaginase, vincristine; CR, complete remission.

Standard curve extrapolation of copy number was done for both IFN- $\gamma$  and CD8. Sample data were normalized by dividing the number of copies of IFN- $\gamma$  transcripts by the number of copies of CD8 transcripts. All PCR assays were done in duplicate and reported as the mean. A 2-fold difference in gene expression was found to be within the discrimination range of the assay.

**Flow cytometry.** Intracellular cytokine detection was done as previously described (23). In brief, positively selected CD8<sup>+</sup> T cells ( $1 \times 10^6$ ) were incubated with C1R-A2 cells loaded with or without test peptides. After 2 hours, 10  $\mu$ g/mL Brefeldin A (Sigma) was added. After an additional 16 hours, CD8<sup>+</sup> T cells were stained with an anti-CD8 peridinin chlorophyll protein-conjugated antibody, fixed/permeabilized, and then stained with an anti-IFN- $\gamma$  FITC conjugate (all from BD/PharMingen).

**Peptide-HLA class I tetrameric complexes.** Tetramers were constructed as previously described with minor modifications (10, 24). The production of mutant tetramers containing the HLA-A\*0201  $\alpha$ 3 domain heavy chain substitutions D227K/T228A, which abrogate CD8 binding, was based on previous descriptions (25). These "CD8-null" tetramers were prepared afresh from frozen monomeric proteins within 48 hours of each experiment to minimize effects related to stability differences (26). Once prepared, tetramers were stored in the dark at 4°C.

**Flow cytometric cell sorting.** All sorts were done at 25 p.s.i. using a modified fluorescence-activated cell sorting (DIVA, Becton Dickinson, San Diego, CA). Instrument setup was done according to the instructions of the manufacturer. Antibody-capture beads (Becton Dickinson), stained separately with the individual monoclonal antibodies present in test samples, were used for electronic compensation. Post-sort purity was consistently >99%.

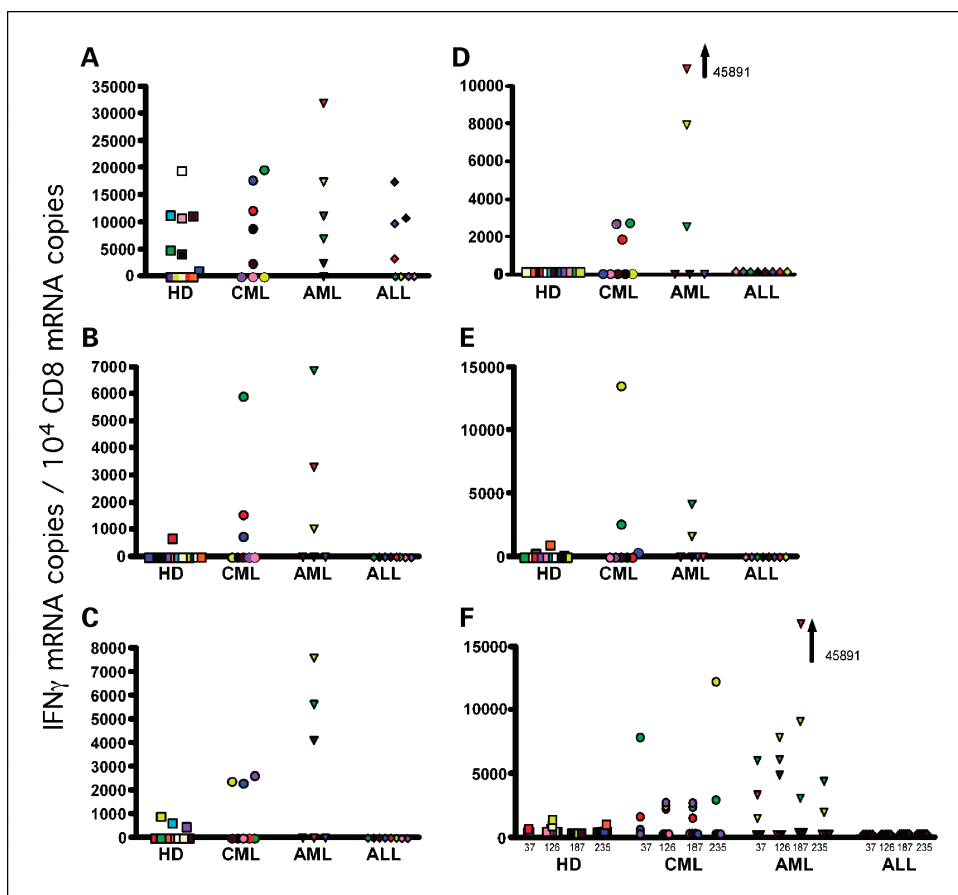
**Clonotypic analysis of specific CD8<sup>+</sup> T-cell populations.** Unbiased identification of specific clones in sorted T-cell populations was done as

previously described (27). Briefly, mRNA was extracted (Oligotex Kit, Qiagen, Chatsworth, CA) after cell lysis and subjected to template-switch anchored RT-PCR using a 3' T-cell receptor- $\beta$  constant region primer. Products were ligated into pGEM-T Easy vector (Promega, Madison, WI) and cloned by transformation of competent DH5 $\alpha$  *E. coli*. Selected colonies were amplified by PCR using standard M13 primers and then sequenced. A minimum of 50 clones was analyzed per sorted T-cell population. T-cell receptor- $\beta$  CDR3 consensus sequences were compiled and aligned using MacVector 7.2.

**Statistical analysis.** A cutoff value of 2.0 for the ratio of IFN- $\gamma$  mRNA (corrected for CD8 mRNA) obtained from CD8<sup>+</sup> T cells stimulated with C1R-A2 cells pulsed with the relevant epitope to that obtained from CD8<sup>+</sup> T cells stimulated with unpulsed C1R-A2 cells was considered to indicate a significant epitope-specific response. The cutoff value was derived by analyzing IFN- $\gamma$  mRNA transcripts detectable in CD8<sup>+</sup> T cells from both healthy donors and patients with CML stimulated with gp100 (209-2M) (irrelevant peptide) compared with background. Analysis of these CD8<sup>+</sup> T cells identified a mean ratio of 0.96 (range, 0.8-1.7) with 95% and 99% confidence intervals of  $0.96 \pm 0.76$  and  $0.96 \pm 1.16$ , respectively, a SE of 0.09, and a SD of 0.28. The cutoff ratio (stimulation index) was estimated by adding the mean to 3 SD, which equaled 1.8. To minimize the possibility of falsely considering CD8<sup>+</sup> T cells immunoreactive, we accepted a 2-fold increase in the stimulated-unstimulated IFN- $\gamma$  transcript ratio as evidence of epitope-specific reactivity.

The Mann-Whitney test was used to determine whether or not there was a statistically significant difference in IFN- $\gamma$  production in response to test peptides between patients with CML, AML, and ALL and healthy controls. Statistical significance was achieved when  $P < 0.05$ . The number of peptides recognized in each subject group was compared using the  $\chi^2$  test.

**Fig. 1.** CD8<sup>+</sup> T-cell responses to stimulation with the HLA-A\*0201 restricted peptides CMV pp65 (A), WT37 (B), WT126 (C), WT187 (D), and WT235 (E) and the cumulative CD8<sup>+</sup> T-cell response to stimulation with all four WT1 peptides, WT37, WT126, WT187, and WT235 (F), in healthy donors (square), patients with CML (circle), patients with AML (inverted triangle), and patients with ALL (diamond). CD8<sup>+</sup>-selected T cells were stimulated with C1R-A2 cells pulsed with three doses of peptide (0.1, 1, and 10  $\mu$ mol/L). Values represent copies of IFN- $\gamma$  mRNA per  $10^4$  copies of CD8 mRNA. The overall response magnitude was significantly greater in patients with AML and CML, but not ALL, compared with healthy donors.



## Results

**Ex vivo detection of CD8<sup>+</sup> T-cell responses directed against four HLA-A\*0201-restricted epitopes of Wilms' tumor 1.** Samples from all subjects in the cohort were studied for the presence of responses to WT37, WT126, WT187, and WT235. None of the patients had undergone allogeneic stem cell transplantation and all AML and ALL patients had received prior chemotherapy and were in remission. The clinical profiles of these donors and patients are presented in Table 1. The CD8<sup>+</sup> T-cell responses to stimulation with WT1 peptides were measured by quantitative RT-PCR for IFN- $\gamma$  mRNA production and are summarized in Fig. 1 and Table 2. Significant peptide-specific IFN- $\gamma$  mRNA expression was observed in 7 of 12 healthy donors, 5 of 8 patients with CML, and 4 of 6 patients with AML. There was no statistically significant difference in the frequency of positive results between these groups ( $P = 0.3$ ). However, a CD8<sup>+</sup> T-cell response to WT1 was not seen in any patient with ALL. Analysis of the responding population revealed that in healthy donors, the WT1-specific CD8<sup>+</sup> T-cell response was limited to a single peptide in each case. In contrast, all CML and three of four AML patients had a response to more than one of the four WT1-derived peptides. This difference was highly statistically

significant ( $P = 0.0015$ ), suggesting that the presence of myeloid leukemia cells induces T-cell responses to several different WT1 epitopes.

**Quality and extent of CD8<sup>+</sup> T-cell response to Wilms' tumor 1.** The overall CD8<sup>+</sup> T-cell response to WT1 peptide stimulation as measured by IFN- $\gamma$  mRNA expression was greater in patients with AML and CML compared with healthy controls. In view of the small number of subjects, it was not possible to compare the responses to each individual peptide although the response to all four peptides in sum was significantly greater in patients with CML and AML (289-13,584 and 418-45,891 IFN- $\gamma$  mRNA/ $10^4$  CD8 copies with stimulation index values of 2.4-8.8 and 2.1-52.7, respectively) compared with healthy donors (160-2,683 IFN- $\gamma$  mRNA/ $10^4$  CD8 copies with stimulation index values of 2.5-6.5;  $P = 0.028$  and  $P = 0.008$ , respectively; Fig. 1F). The response was not statistically different between CML and AML patients ( $P = 0.31$ ). Of note, no response to WT1 was detected in patients with ALL although this lack of response was not significantly different when compared with healthy donors ( $P = 0.43$ ). However, the response was significantly greater in patients with CML and AML when compared with patients with ALL ( $P = 0.017$  and  $P = 0.07$ , respectively). Importantly, the CD8<sup>+</sup> T-cell response to the CMV peptide

**Table 2.** Reactivity of CD8<sup>+</sup> T cells from healthy donors and patients with CML, AML, and ALL to test peptides and comparison of overall WT1-specific CD8<sup>+</sup> T-cell responses

Healthy donors	CMV ( $\mu\text{mol/L}$ )		WT37 ( $\mu\text{mol/L}$ )		WT126 ( $\mu\text{mol/L}$ )		WT187 ( $\mu\text{mol/L}$ )		WT235 ( $\mu\text{mol/L}$ )	
	0.1	10	0.1	10	0.1	10	0.1	10	0.1	10
BD1 □	–	–	–	–	6.5	2.9	–	–	–	–
BD2 ■	–	–	–	–	–	–	–	–	–	–
BD3 ■	5.6	–	–	–	–	–	–	–	2.1	2
BD4 ■	–	–	–	–	–	–	–	–	–	–
BD5 ■	12.3	–	–	–	–	–	–	–	2.1	–
BD6 ■	–	–	–	–	–	–	–	–	–	–
BD7 ■	24.6	–	6.5	–	–	–	–	–	–	–
BD8 ■	8.4	–	–	–	–	4.8	–	–	–	–
BD9 □	10.7	–	–	–	–	–	–	–	–	–
BD10 □	–	–	–	–	2.2	–	–	–	–	–
BD11 ■	3.8	–	–	–	–	–	–	–	–	–
BD12 □	–	–	–	–	–	–	–	–	6.1	–
Responses = 7/12			1	1	3				3	1
CML										
319 ●	28.2	–	4.4	–	–	–	–	–	2.7	–
262 ○	–	–	–	–	2	–	–	–	–	8.8
365 ○	13.2	–	4.7	–	–	–	–	–	2.6	2.6
241 ●	18.8	–	–	–	–	–	–	–	–	–
17 ●	12.2	–	–	–	–	–	–	–	–	–
210 ●	–	–	2.3	–	2.2	2.2	5.5	2.3	–	–
28 ○	–	–	–	–	–	–	–	–	–	–
402 ●	120	–	–	–	–	2.9	–	–	–	3.1
Responses = 5/8			3	2	2	1	3			3
AML										
350 ▼	37	–	4.7	1	–	–	–	–	52.7	–
378 ▼	41.9	–	–	3.418.8	–	19.8	19	2.2	4.9	–

(Continued on the following page)

(Fig. 1A) was not significantly different in the four groups; this mitigates against a generalized immune deficiency in ALL as the explanation for the absence of WT1-specific CD8<sup>+</sup> T-cell responses.

All four peptides were immunogenic in patients with myeloid leukemias (Table 2). In the healthy donor population, however, the peptides differed in their ability to induce a response. WT126 and WT235 were highly antigenic, each eliciting responses in 3 of 12 healthy donors and in 6 of 14 and 5 of 14 patients with myeloid leukemias, respectively. Peptides WT187 and WT37 were highly specific for patients; none of the healthy donors exhibited responses to the former and only one responded to the latter. This difference, however, did not reach statistical significance.

We also examined the WT1-specific CD8<sup>+</sup> T-cell response to the same panel of peptides by *ex vivo* intracellular IFN- $\gamma$  staining. Representative data on patient UPN-365 are shown in Fig. 2. CD8<sup>+</sup> T-cell responses were detected against WT37, WT187, and WT235 but not against WT126. This is in keeping with quantitative RT-PCR data (Table 2).

**CD8<sup>+</sup> T-cell response to different peptide concentrations as a measure of functional avidity.** To determine functional avidity, the response of CD8<sup>+</sup> T cells to stimulation with three concentrations of peptide (0.1, 1, and 10  $\mu$ mol/L) was measured by quantitative RT-PCR. High- and low-avidity T cells have been previously shown to possess different requirements for both peptide-MHC density and CD8 interaction (28, 29). In our

experiments, high-avidity CD8<sup>+</sup> T cells were defined as those capable of producing IFN- $\gamma$  in response to a lower concentration of peptide (0.1  $\mu$ mol/L) whereas intermediate- and low-avidity CD8<sup>+</sup> T cells were those that produced IFN- $\gamma$  in response to a higher concentration of peptide (1 and 10  $\mu$ mol/L, respectively). To examine the issue of functional avidity, the ratio of high-avidity and low-avidity CD8<sup>+</sup> T-cell responses for each WT1 epitope was determined by comparing the stimulation indices of low or higher peptide concentrations, respectively (Fig. 3).

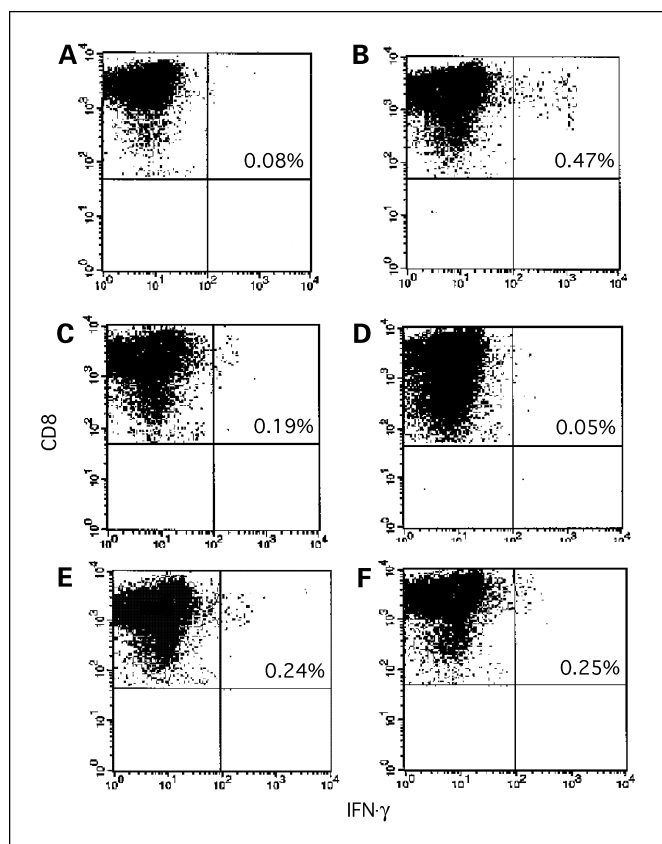
Patients with AML and CML were analyzed together. Interestingly, in patients with myeloid leukemias, the avidity of the CD8<sup>+</sup> T-cell response to WT1 depended on the peptide tested. Whereas the CD8<sup>+</sup> T-cell response to WT37 was mostly high avidity (median ratio of high avidity/low-avidity response, 3.4), both high- and low-avidity responses to WT126 were detected (median ratio of high avidity/low-avidity response, 1.4). In contrast, the response to WT187 and WT235 was mostly low avidity (median high/low avidity ratios of 0.4 in both cases). In view of the small sample size, it was not possible to compare the avidity of response to individual WT1 epitopes in healthy donors.

**Clonal identity of Wilms' tumor 1-specific CD8<sup>+</sup> T cells.** CD8<sup>+</sup> T cells specific for the WT126 epitope from healthy donor BD1 were sorted by flow cytometry after staining with HLA-A\*0201 tetrameric complexes folded around the relevant peptide and subjected to clonotype analysis without *in vitro* manipulation as described in Materials and Methods. Three

**Table 2.** Reactivity of CD8<sup>+</sup> T cells from healthy donors and patients with CML, AML, and ALL to test peptides and comparison of overall WT1-specific CD8<sup>+</sup> T-cell responses (Cont'd)

104 ▽	21	–	21	17.4	–	–	8.6	–	3.3
370 ▽	24	–	–	–	–	–	–	–	–
97 ▽	–	–	–	–	–	–	–	–	–
389 ▽	8.4	–	–	2.3	13.6	–	–	–	–
Responses = 4/6		1	3	3	1	1	3	1	2
ALL									
119 ◇	–	–	–	–	–	–	–	–	–
449 ◇	89	–	–	–	–	–	–	–	–
444 ◇	29	–	–	–	–	–	–	–	–
337 ◇	51	–	–	–	–	–	–	–	–
245 ◇	–	–	–	–	–	–	–	–	–
274 ◇	4.9	–	–	–	–	–	–	–	–
002 ◇	–	–	–	–	–	–	–	–	–
001 ◇	–	–	–	–	–	–	–	–	–
Responses = 0/8									
	Percentage positive responders				Percentage recognizing ≥2 epitopes	Median no. antigens recognized per subject (range)			
	WT37	WT126	WT187	WT235					
CML	38	38	38	38	100	2 (0-3)			
AML	50	50	50	33	75	2 (0-4)			
Healthy donor	14	43	0	43	0.1 (0-1)				

NOTE: Data are represented as stimulation index that corrects IFN- $\alpha$  mRNA copy number expressed by CD8<sup>+</sup> T cells exposed to relevant peptide to constitutive expression of the same cytokine (exposure to unloaded C1R-A2 cells) as measured by quantitative RT-PCR. Values of >100 IFN- $\alpha$  mRNA copies/10<sup>4</sup> copies of CD8 and at least twice that of background were defined as positive responses. Stimulation indices scored as (–) represent reactivities <2 over background. The (–) scores for CMV stimulation were only seen in CMV seronegative individuals. Due to limited availability of material, not all peptides and concentrations were tested in every case.



**Fig. 2.** CD8<sup>+</sup> T-cell responses defined by analysis of intracellular cytokine production in UPN-365. CD8<sup>+</sup> T cells (10<sup>6</sup>) were stimulated with peptide-pulsed C1R-A2 cells and intracellular cytokine analysis for IFN- $\gamma$  was done as described in Materials and Methods. Plots are gated on CD8<sup>+</sup> T cells. Numbers in the top right quadrant represent percentage of IFN- $\gamma$  producing CD8<sup>+</sup> T cells. A, unpulsed C1R-A2 (negative control). B, CMV pp65 – pulsed C1R-A2 (positive control). C, WT37-pulsed C1R-A2. D, WT126-pulsed C1R-A2. E, WT187-pulsed C1R-A2. F, WT235-pulsed C1R-A2.

populations of WT1-specific CD8<sup>+</sup> T cells were sorted: (a) WT1/HLA-A\*0201 tetramer-binding CD8<sup>+</sup> T cells (WT1-A2<sup>+</sup>CD8<sup>+</sup>), (b) WT1/HLA-A\*0201 tetramer-binding CD8<sup>dim</sup> T cells (WT1-A2<sup>+</sup>CD8<sup>dim</sup>), and (c) WT1/HLA-A\*0201  $\alpha$ 3 D227K/T228A tetramer-binding CD8<sup>+</sup> T cells (WT1-A2<sup>+</sup>D227K/T228A<sup>+</sup>CD8<sup>+</sup>; Fig. 4). Tetramers containing the  $\alpha$ 3 D227K/T228A mutation have been shown previously to identify high-avidity CD8<sup>+</sup> T cells; it should be noted that this assay does not rely on the detection of a biological outcome and therefore can be considered as a purer measurement of true avidity for antigen (30, 31). The WT1-A2<sup>+</sup>CD8<sup>+</sup> population was oligoclonal, comprising only four clones. The WT1-A2<sup>+</sup>CD8<sup>dim</sup> population was monoclonal; this clone was also present in the WT1-A2<sup>+</sup>CD8<sup>+</sup> population as confirmed by nucleotide sequence identity (data not shown). Interestingly, clonotypic analysis of the WT1-A2<sup>+</sup>D227K/T228A<sup>+</sup>CD8<sup>+</sup> T cells revealed the same monoclonal population. This clone therefore exhibited a high level of functional avidity for cognate antigen, which could explain the preferential down-regulation of the CD8 coreceptor compared with the other three clones present in the WT1-A2<sup>+</sup>CD8<sup>+</sup> population (Fig. 4). To substantiate this finding, the avidity of the WT1-specific CD8<sup>+</sup> T-cell response was also assessed by stimulating CD8<sup>+</sup> T cells with different concen-

trations of WT1 peptide and subsequently measuring IFN- $\gamma$  mRNA production by quantitative RT-PCR. These experiments confirmed that the WT1-specific response consisted of both high- and low-avidity CD8<sup>+</sup> T cells (Table 2).

## Discussion

Recent developments in the ability to detect and quantify antigen-specific T cells have led to an appreciation of the strength and specificity of T cells directed against leukemia antigens. Here, quantitative RT-PCR was used to study CD8<sup>+</sup> T-cell responses to the leukemia self-antigen WT1. To broaden our understanding of T-cell responses to WT1, we studied four HLA-A\*0201-restricted epitopes derived from this protein. We have previously shown a strong correlation between IFN- $\gamma$  copy number and the frequency of peptide-HLA-A\*0201 tetramer-binding CD8<sup>+</sup> T cells specific for an immunodominant CMV pp65-derived antigen, suggesting that these two assays measure the same cell populations (10). However, it should be noted that this relationship is not necessarily absolute because of variations in the strength of different antigenic stimuli, differences in IFN- $\gamma$  production on a per cell basis (29), and interindividual polymorphisms within the IFN- $\gamma$  gene (32, 33). Further, it was not possible to compare these assays in the setting of WT1-specific CD8<sup>+</sup> T-cell responses because the frequencies of such cells directly *ex vivo* are generally below the detection limit for tetramer staining.

Our results confirm earlier findings that healthy individuals frequently have detectable numbers of circulating T cells that recognize WT1 (10). The persistence of WT1-specific T cells in nonleukemic individuals may be attributed to tolerance established by the relatively low expression of WT1 in normal tissue (3). We also show here that the magnitude and breadth of the WT1-specific CD8<sup>+</sup> T-cell response determined by peptide-specific IFN- $\gamma$  mRNA expression was significantly higher in patients with CML and AML compared with healthy donors. Overall, a CD8<sup>+</sup> T-cell response to the previously identified HLA-A\*0201-restricted epitopes was detected in more than 60% (9 of 14) of patients with myeloid leukemia and 58% (7 of 12) of healthy donors tested. Whether or not the WT1-reactive T cells detected by our assay measuring IFN- $\gamma$  production mediate antileukemic cytotoxicity cannot directly be assessed *ex vivo*. However, these results are in keeping with previous studies by our group and others, which show expansion of WT126-specific CD8<sup>+</sup> T cells in patients with AML and CML (6, 10, 11). These findings seem paradoxical because it might be anticipated that the emergence of a leukemia expressing large amounts of WT1 should cause the extinction of high-avidity WT1-specific T cells and a narrowing of the T-cell response to WT1. Our results indicate the opposite; i.e. that the WT1-specific T-cell response is increased both with respect to the magnitude of the IFN- $\gamma$  response and the number of WT1 epitopes that are recognized. This latter observation may reflect epitope spreading. Epitope spreading consists of diversification of the immune response from the initial dominant epitope to other epitopes derived from the same protein (intramolecular spreading) and then to epitopes from different proteins (intermolecular spreading) within the target tissue and is thought to play a crucial role in several autoimmune disease models (34–36). It is noteworthy that

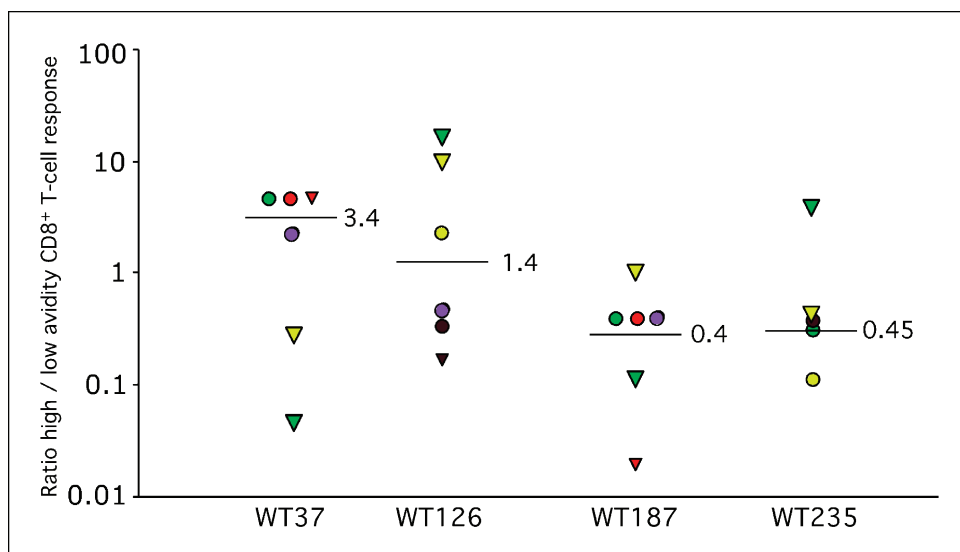
many of the recently isolated tumor antigens, including WT1, are nonmutated differentiation antigens inappropriately expressed or overexpressed in the tumor. Effective cancer immunotherapy can similarly be considered a "controlled" induction of autoimmunity (37) and some of the salient features of autoimmunity, including epitope spreading, are being recognized in the field of tumor immunology. It is therefore conceivable that, in the context of tumor rejection, T cells that react specifically to a given immunodominant epitope may induce an inflammatory cascade at the site of antigen encounter, resulting in tissue destruction. Tissue debris processed and cross-presented by host antigen-presenting cells may amplify the response due to the recruitment of a diversified repertoire of T cells that react to subdominant and/or cryptic tumor-derived epitopes. It is possible to test this hypothesis, using WT1 as a model tumor antigen, by vaccinating patients with a single MHC class I-restricted CTL peptide epitope and investigating for responses to other class I and class II epitopes derived from WT1 (intramolecular spreading) and to epitopes from different proteins within the target tissue such as proteinase 3 (intermolecular spreading). Indirect evidence for the existence of intermolecular spreading comes from our previous work showing that the majority of patients with myeloid leukemia respond to multiple antigens (PR1, WT1, and BCR-ABL; ref. 10). A study of both CD8<sup>+</sup> and CD4<sup>+</sup> T-cell responses to every potential epitope of WT1 without assumptions of immunodominance is currently under way to define the overall extent of the adaptive immune response to WT1 in healthy donors and patients with leukemia.

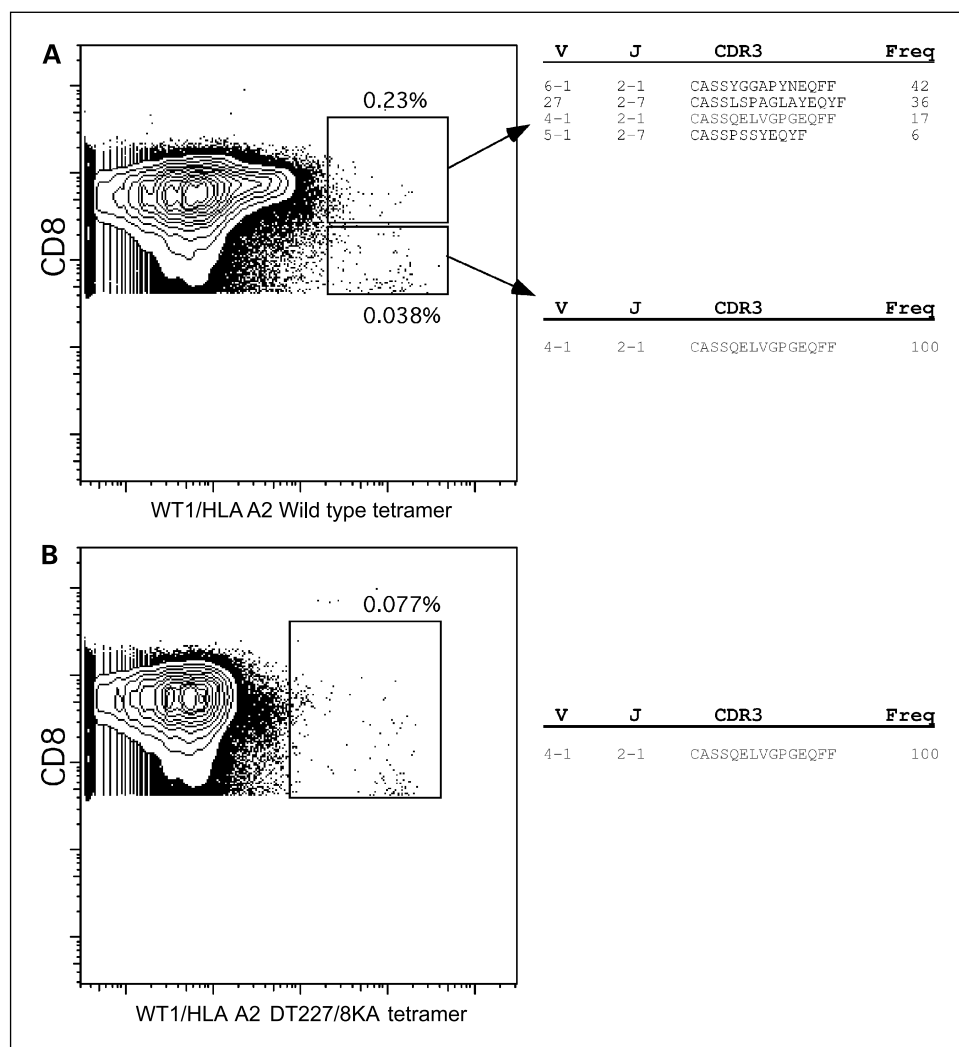
The failure to detect an autoimmune response in patients with ALL recapitulates clinical observations showing that, compared with myeloid leukemias, the alloimmune graft-versus-leukemia effect in B-ALL is modest, resulting in greater risk of disease relapse and weak graft-versus-leukemia effects of donor lymphocyte infusions which achieve remission in <10% in patients with B-ALL (38). Minor histocompatibility antigens can serve as target antigens for donor T cells in the allogeneic setting (39–41). However, very little is known about the role of an auto-logical immune response in ALL. In keeping with our findings, a

recent study found increased production of IgG1, IgG2, and IgG3 antibodies and, therefore, Th1-biased humoral responses against WT1 in patients with CML and AML but not ALL (42). The lack of an IFN- $\gamma$  response to WT1, despite overexpression of the antigen in most cases of ALL (1–3), may be related to defective antigen presentation in ALL or to a direct suppressive effect of ALL cells on T-cell function (43, 44). It is also possible that other cytokines such as interleukin 4 or interleukin 10 are induced in response to stimulation with WT1 in patients with ALL. More work is therefore needed to determine the relevance of WT1-directed immunotherapy in this patient group.

To study the functional avidity of the CD8<sup>+</sup> T-cell response against individual WT1 epitopes, CD8<sup>+</sup> T cells were stimulated with three logarithmically increasing peptide concentrations; an arbitrary "functional avidity ratio" was calculated based on these readouts. In patients with myeloid leukemias, the avidity was dependent on the individual peptides tested. Whereas the WT37-specific CD8<sup>+</sup> T cells were high avidity, both high- and low-avidity CD8<sup>+</sup> T cells were detected against WT126 and the CD8<sup>+</sup> T cells specific for WT187 and WT235 were mostly of low avidity. This suggests that the repertoire of leukemia antigen-specific CD8<sup>+</sup> T cells is diverse, both in terms of clonal composition and efficiency of peptide recognition. Indeed, clonotypic segregation according to avidity was confirmed in one donor using a mutant WT1/HLA-A\*0201  $\alpha$ 3 D227K/T228A tetramer that does not bind the CD8 coreceptor to isolate and characterize WT126-specific CD8<sup>+</sup> T cells directly *ex vivo* (Fig. 4). This approach determines avidity for antigen as a physical variable independent of any downstream biological outcome; it is distinct from functional avidity, defined as the response sensitivity in peptide titration assays, albeit related in a nonlinear manner. Importantly, these qualitatively different assays revealed heterogeneity within the corresponding WT126-specific CD8<sup>+</sup> T-cell populations. Overall, these findings are of clinical relevance as functional avidity is emerging as a key factor that determines the effectiveness of antigen-specific T cells (45). Identification of antigens that induce high-avidity CTLs should help in the design of more effective immunotherapeutic and vaccination strategies.

**Fig. 3.** High- and low-avidity CD8<sup>+</sup> T-cell responses determined by sensitivity to peptide concentration in patients with myeloid leukemias. Stimulation of CD8<sup>+</sup> T cells with 0.1 and 10  $\mu$ mol/L of WT37, WT126, WT187, and WT235 determined high- and low-avidity responses, respectively. Ratios of high-avidity and low-avidity CD8<sup>+</sup> T-cell responses calculated for patients with CML (circle) and AML (inverted triangle). Ratios were obtained by the following calculation: stimulation index with 0.1  $\mu$ mol/L peptide / stimulation index with 10  $\mu$ mol/L peptide. Bars, median high/low avidity ratio for each condition.





**Fig. 4.** Amino acid sequences of T-cell receptor- $\beta$  CDR3 regions from three populations of WT1-specific CD8<sup>+</sup> T cells isolated directly *ex vivo* from donor BD1 with HLA-A\*0201 tetrameric complexes folded around the WT126 peptide. **A**, WT1/HLA-A\*0201 tetramer-binding CD8<sup>+</sup> T cells (WT1-A2<sup>+</sup>CD8<sup>+</sup>) and WT1/HLA-A\*0201 tetramer-binding CD8<sup>dim</sup> T cells (WT1-A2<sup>+</sup>CD8<sup>dim</sup>). **B**, WT1/HLA-A\*0201  $\alpha$ 3 D227K/T228A tetramer-binding CD8<sup>+</sup> T cells (WT1-A2<sup>+</sup>D227K/T228A<sup>+</sup>CD8<sup>+</sup>).

In summary, these results show that CD8<sup>+</sup> T cells specific for WT1 are polyclonal and detectable in patients with CML and AML whereas the corresponding response is narrowly focused and less frequently found in healthy donors. These findings support the development of WT1 peptide-based immunotherapy. The WT1 epitope used for vaccination may be more effective in patients with preexisting CD8<sup>+</sup> T-cell populations specific for the peptide antigen. All four epitopes tested were found to be immunogenic in patients with

myeloid leukemias and it is possible that vaccination of leukemic patients with a cocktail of peptides could lead to the induction of strong and diverse WT1-specific CD8<sup>+</sup> T-cell responses that mediate better clinical outcomes.

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## References

- Brieger J, Weidmann E, Fenchel K, Mitrou PS, Hoelzer D, Bergmann L. The expression of the Wilms' tumor gene in acute myelocytic leukemias as a possible marker for leukemic blast cells. *Leukemia* 1994;8: 2138–43.
- Inoue K, Sugiyama H, Ogawa H, et al. WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. *Blood* 1994;84:3071–9.
- Menssen HD, Renkl HJ, Rodeck U, et al. Presence of Wilms' tumor gene (*wil1*) transcripts and the WT1 nuclear protein in the majority of human acute leukemias. *Leukemia* 1995;9:1060–7.
- Oka Y, Elisseeva OA, Tsuboi A, et al. Human cytotoxic T-lymphocyte responses specific for peptides of the wild-type Wilms' tumor gene (WT1) product. *Immunogenetics* 2000;51:99–107.
- Ohnishi H, Yasukawa M, Fujita S. HLA class I-restricted lysis of leukemia cells by a CD8(+) cytotoxic T-lymphocyte clone specific for WT1 peptide. *Blood* 2000;95:286–93.
- Gao L, Bellantuono I, Elsasser A, et al. Selective elimination of leukemic CD34(+) progenitor cells by cytotoxic T lymphocytes specific for WT1. *Blood* 2000;95: 2198–203.
- Tsuboi A, Oka Y, Ogawa H, et al. Cytotoxic T-lymphocyte responses elicited to Wilms' tumor gene WT1 product by DNA vaccination. *J Clin Immunol* 2000; 20:195–202.
- Oka Y, Udaka K, Tsuboi A, et al. Cancer immunotherapy targeting Wilms' tumor gene WT1 product. *J Immunol* 2000;164:1873–80.
- Gaiger A, Reese V, Disis ML, Cheever MA. Immunity to WT1 in the animal model and in patients with acute myeloid leukemia. *Blood* 2000;96: 1480–9.
- Rezvani K, Grube M, Brenchley JM, et al. Functional leukemia-associated antigen-specific memory CD8<sup>+</sup> T cells exist in healthy individuals and in

- patients with chronic myelogenous leukemia before and after stem cell transplantation. *Blood* 2003;102:2892–900.
11. Scheibenbogen C, Letsch A, Thiel E, et al. CD8 T-cell responses to Wilms tumor gene product WT1 and proteinase 3 in patients with acute myeloid leukemia. *Blood* 2002;100:2132–7.
  12. Mailander V, Scheibenbogen C, Thiel E, Letsch A, Blau IW, Keilholz U. Complete remission in a patient with recurrent acute myeloid leukemia induced by vaccination with WT1 peptide in the absence of hematological or renal toxicity. *Leukemia* 2004;18:165–6.
  13. Oka Y, Tsuboi A, Murakami M, et al. Wilms tumor gene peptide-based immunotherapy for patients with overt leukemia from myelodysplastic syndrome (MDS) or MDS with myelofibrosis. *Int J Hematol* 2003;78:56–61.
  14. Oka Y, Tsuboi A, Taguchi T, et al. Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. *Proc Natl Acad Sci U S A* 2004;101:13885–90.
  15. Bellantuono I, Gao L, Parry S, et al. Two distinct HLA-A0201-presented epitopes of the Wilms tumor antigen 1 can function as targets for leukemia-reactive CTL. *Blood* 2002;100:3835–7.
  16. Smithgall M, Misher L, Spies G, Cheever MA, Gaiger A. Identification of a novel WT1 HLA-A\*0201-restricted CTL epitope using whole gene in vitro priming. *ASH meeting* 2001.
  17. Storkus WJ, Howell DN, Salter RD, Dawson JR, Cresswell P. NK susceptibility varies inversely with target cell class I HLA antigen expression. *J Immunol* 1987;138:1657–9.
  18. Diamond DJ, York J, Sun JY, Wright CL, Forman SJ. Development of a candidate HLA A\*0201 restricted peptide-based vaccine against human cytomegalovirus infection. *Blood* 1997;90:1751–67.
  19. Boon T, Coulie PG, Van den EB. Tumor antigens recognized by T cells. *Immunol Today* 1997;18:267–8.
  20. Kruse N, Pette M, Toyka K, Rieckmann P. Quantification of cytokine mRNA expression by RT-PCR in samples of previously frozen blood. *J Immunol Methods* 1997;210:195–203.
  21. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 1996;6:986–94.
  22. Kammula US, Lee KH, Riker AI, et al. Functional analysis of antigen-specific T lymphocytes by serial measurement of gene expression in peripheral blood mononuclear cells and tumor specimens. *J Immunol* 1999;163:6867–75.
  23. Hensel N, Melenhorst JJ, Bradstock K, et al. Flow cytometric quantitation and characterization of the T-lymphocyte memory response to CMV in healthy donors. *Cytotherapy* 2002;4:29–40.
  24. Altman JD, Moss PA, Goulder PJ, et al. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 1996;274:94–6.
  25. Purbhoo MA, Boulter JM, Price DA, et al. The human CD8 coreceptor effects cytotoxic T cell activation and antigen sensitivity primarily by mediating complete phosphorylation of the T cell receptor  $\zeta$  chain. *J Biol Chem* 2001;276:32786–92.
  26. Hutchinson SL, Wooldridge L, Tafuro S, et al. The CD8 T cell coreceptor exhibits disproportionate biological activity at extremely low binding affinities. *J Biol Chem* 2003;278:24285–93.
  27. Douek DC, Betts MR, Brechley JM, et al. A novel approach to the analysis of specificity, clonality, and frequency of HIV-specific T cell responses reveals a potential mechanism for control of viral escape. *J Immunol* 2002;168:3099–104.
  28. Alexander-Miller MA, Leggett GR, Berzofsky JA. Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. *Proc Natl Acad Sci U S A* 1996;93:4102–7.
  29. Alexander MA, Damico CA, Wietes KM, Hansen TH, Connolly JM. Correlation between CD8 dependency and determinant density using peptide-induced, Ld-restricted cytotoxic T lymphocytes. *J Exp Med* 1991;173:849–58.
  30. Choi EM, Chen JL, Wooldridge L, et al. High avidity antigen-specific CTL identified by CD8-independent tetramer staining. *J Immunol* 2003;171:5116–23.
  31. Pittet MJ, Rubio-Godoy V, Bioley G, et al.  $\alpha 3$  domain mutants of peptide/MHC class I multimers allow the selective isolation of high avidity tumor-reactive CD8 T cells. *J Immunol* 2003;171:1844–9.
  32. Gray PW, Goeddel DV. Human immune interferon (IFN- $\gamma$ ) gene sequence and structure. *Basic Life Sci* 1983;25:35–61.
  33. Hoffmann SC, Stanley EM, Darrin CE, et al. Association of cytokine polymorphic inheritance and in vitro cytokine production in anti-CD3/CD28-stimulated peripheral blood lymphocytes. *Transplantation* 2001;72:1444–50.
  34. Kaufman DL, Clare-Salzler M, Tian J, et al. Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature* 1993;366:69–72.
  35. McRae BL, Vanderlugt CL, Dal Canto MC, Miller SD. Functional evidence for epitope spreading in the relapsing pathology of experimental autoimmune encephalomyelitis. *J Exp Med* 1995;182:75–85.
  36. Tisch R, Yang XD, Singer SM, Liblau RS, Fugger L, McDevitt HO. Immune response to glutamic acid decarboxylase correlates with insulinitis in non-obese diabetic mice. *Nature* 1993;366:72–5.
  37. Nanda NK, Sercarz EE. Induction of anti-self-immunity to cure cancer. *Cell* 1995;82:13–7.
  38. Sullivan KM, Storb R, Buckner CD, et al. Graft-versus-host disease as adoptive immunotherapy in patients with advanced hematologic neoplasms. *N Engl J Med* 1989;320:828–34.
  39. Fontaine P, Roy-Proulx G, Knafo L, Baron C, Roy DC, Perreault C. Adoptive transfer of minor histocompatibility antigen-specific T lymphocytes eradicates leukemia cells without causing graft-versus-host disease. *Nat Med* 2001;7:789–94.
  40. Mutis T, Gillespie G, Schrama E, Falkenburg JH, Moss P, Goulmy E. Tetrameric HLA class I-minor histocompatibility antigen peptide complexes demonstrate minor histocompatibility antigen-specific cytotoxic T lymphocytes in patients with graft-versus-host disease. *Nat Med* 1999;5:839–42.
  41. Pion S, Fontaine P, Baron C, Gyger M, Perreault C. Immunodominant minor histocompatibility antigens expressed by mouse leukemic cells can serve as effective targets for T cell immunotherapy. *J Clin Invest* 1995;95:1561–8.
  42. Wu F, Oka Y, Tsuboi A, et al. Th1-biased humoral immune responses against Wilms tumor gene WT1 product in the patients with hematopoietic malignancies. *Leukemia* 2005;19:268–74.
  43. Cardoso AA, Schultze JL, Bousiotis VA, et al. Pre-B acute lymphoblastic leukemia cells may induce T-cell anergy to alloantigen. *Blood* 1996;88:41–8.
  44. Han P, Story C, McDonald T, Mrozik K, Snell L. Immune escape mechanisms of childhood ALL and a potential countering role for DC-like leukemia cells. *Cytotherapy* 2002;4:165–75.
  45. Molldrem JJ, Lee PP, Wang C, et al. Evidence that specific T lymphocytes may participate in the elimination of chronic myelogenous leukemia. *Nat Med* 2000;6:1018–23.