

Cancer Immunotherapy Targeting Wilms' Tumor Gene WT1 Product¹

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The Wilms' tumor gene *WT1* is expressed at high levels not only in acute myelocytic and lymphocytic leukemia and in chronic myelocytic leukemia but also in various types of solid tumors including lung cancers. To determine whether the WT1 protein can serve as a target Ag for tumor-specific immunity, three 9-mer WT1 peptides (Db126, Db221, and Db235), which contain H-2D^b-binding anchor motifs and have a comparatively higher binding affinity for H-2D^b molecules, were tested in mice (C57BL/6, H-2D^b) for in vivo induction of CTLs directed against these WT1 peptides. Only one peptide, Db126, with the highest binding affinity for H-2D^b molecules induced vigorous CTL responses. The CTLs specifically lysed not only Db126-pulsed target cells dependently upon Db126 concentrations but also WT1-expressing tumor cells in an H-2D^b-restricted manner. The sensitizing activity to the Db126-specific CTLs was recovered from the cell extract of WT1-expressing tumor cells targeted by the CTLs in the same retention time as that needed for the synthetic Db126 peptide in RP-HPLC, indicating that the Db126-specific CTLs recognize the Db126 peptide to kill WT1-expressing target cells. Furthermore, mice immunized with the Db126 peptide rejected challenges by WT1-expressing tumor cells and survived for a long time with no signs of autoaggression by the CTLs. Thus, the WT1 protein was identified as a novel tumor Ag. Immunotherapy targeting the WT1 protein should find clinical application for various types of human cancers. *The Journal of Immunology*, 2000, 164: 1873–1880.

The Wilms' tumor gene *WT1* was first reported as the gene responsible for Wilms' tumor, a pediatric renal cancer (1, 2). This gene encodes a zinc finger transcription factor involved in tissue development, in cell proliferation and differentiation, and in apoptosis, and is categorized as a tumor suppressor gene (3). The WT1 gene product regulates the expression of various genes either positively or negatively depending upon how it combines with other regulatory proteins in different types of cells.

Tumor Ags can be categorized into five groups: ubiquitous proteins such as mutated ras (4) or p53 (5); tumor-specific shared Ags such as P1A in mice (6) and MAGE in humans (7, 8); differentiation Ags with a good example of tyrosinase (9); overexpressed tumor Ags such as HER-2/neu (10); and Ags derived from oncogenic viruses with the best example of the E7 oncoprotein of human papilloma virus 16 (11).

We (12, 13) and others (14–17) have identified high expression levels of the wild-type *WT1* gene in leukemic cells regardless of the type of disease to clarify the essential role of the *WT1* gene in leukemogenesis. On the basis of accumulated evidence (13, 18–20), we have proposed that the wild-type *WT1* gene performs an oncogenic rather than a tumor suppressor gene function in hematopoietic progenitor cells. Moreover, we found that among 34 solid

tumor cell lines examined, 28 (82%), including lung, gastric, colon, and breast cancer cell lines, expressed the wild-type *WT1* gene (21). Cancer cells of lung cancer patients also expressed the *WT1* gene at high levels (21). Growth of WT1-overexpressing tumor cells was specifically inhibited by WT1 antisense oligodeoxynucleotides, thus suggesting a close relationship between WT1 overexpression and tumorigenesis. These results indicate that the WT1 gene product could be a promising tumor-specific Ag belonging to the fourth category of tumor Ags not only for leukemia but also for various types of solid tumors including lung cancers.

It is well known that tumor-specific CD8⁺ CTLs constitute the most important effectors for antitumor responses and recognize peptides derived from endogenous proteins presented on the cell surface in association with MHC class I molecules (22). It had been first demonstrated that patients who develop significant graft-versus-host disease (GVHD;⁴ grade \geq 2) after allogeneic bone marrow transplantation (BMT) have a significantly lower rate of relapse than patients with either no GVHD or grade 1 GVHD (23). The demonstration of such a graft-vs-leukemia effect after allogeneic BMT provides the most convincing clinical evidence for the effectiveness of tumor immunity.

Peptides that bind to a given MHC class I molecule have been shown to share common amino acid motifs (24). We (25) and others (26) have previously developed a peptide library-based method for predicting MHC class I-binding peptides. MHC-binding scores can be calculated for all of the peptides of eight or nine amino acids in a given protein sequence by using the experimentally obtained specificity profiles of MHC class I molecules. With few exceptions, the binding scores of naturally occurring MHC class I-binding epitopes for CTLs are as high as twice the SD from the mean score of all of the peptides present in the parental proteins.

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⁴ Abbreviations used in this paper: GVHD, graft-versus-host disease; BMT, bone marrow transplantation.

The present study shows that WT1-derived peptides, which were predicted to bind to H-2D^b molecules according to the peptide library-based scoring system of MHC class I-binding peptides, actually bind to H-2D^b molecules. Furthermore, one of the WT1 peptides induced peptide-specific CTLs as a result of in vivo immunization with the peptide of mice, which then rejected the challenges by WT1-expressing tumor cells.

Materials and Methods

Synthesis of peptides

Peptides were synthesized manually or with an ABI430A peptide synthesizer (Applied Biosystems, Foster City, CA) using Fmoc chemistry. They were then purified by RP-HPLC with a C₁₈ Microbondasphere column (Waters Japan, Osaka, Japan). Synthesis of the correct peptides was confirmed with the aid of an API III triple quadrupole mass spectrometer (Sciex, Thornhill, Toronto, Canada), and concentrations of the peptides were determined by means of a MicroBCA assay (Pierce, Rockford, IL) using BSA as the standard. Some peptides were also custom synthesized (Sawady Technology, Tokyo, Japan).

Cells and Abs

FBL3 is a Friend leukemia virus-induced erythroleukemia cell line originated from C57BL/6 (H-2D^b), and was generously provided by Dr. B. Chesebro (National Institutes of Health, Bethesda, MD) via Dr. M. Miyazawa (Kinki University, Japan). C1498 and EL4 are a WT1-nonexpressing leukemia or lymphoma cell line of C57BL/6 origin, respectively, and was obtained from American Type Culture Collection (ATCC, Rockville, MD). RMA is a Rauscher leukemia virus-induced lymphoma cell line, and RMAS is a TAP-deficient subline of RMA (27). These cell lines were kindly provided by Dr. K. Kärre (Karolinska Institute, Sweden) through Dr. H.-G. Rammensee (University of Tübingen, Germany) and maintained in DMEM containing 5% FBS. P815 is a mastocytoma originated from DBA/2 mice (ATCC). YAC-1 cells that were used as target cells for NK activity were obtained from ATCC. Murine WT1-expressing C1498-mWT1 and EL4-mWT1 were established by transfection of murine WT1 cDNA (a kind gift from Dr. D. Housman, Massachusetts Institute of Technology via Dr. H. Nakagama, National Cancer Center Research Institute, Japan).

mAbs B22.249 (anti-H-2D^b, a kind gift from Dr. J. Klein, Max Planck Institute of Biology, Germany) (28), 28.11.5S (anti-H-2D^b from ATCC), B8.24.3 (anti-H-2K^b from ATCC), 28.13.3S (anti-H-2K^b, a kind gift from Dr. D. Sachs, Massachusetts General Hospital), and MA143 (anti-H-2L^d, a kind gift from Dr. J. H. Stimpfling, McLaughlin Research Institute) were prepared as ascites and purified by DE52 anion exchange chromatography (Whatman, Maidstone, U.K.).

Measurement of binding affinity of WT1 peptides for H-2D^b molecules

Binding of WT1 peptides to MHC class I H-2D^b molecules was measured by a stabilization assay using RMAS cells as described previously (29). Briefly, RMAS cells were incubated at 26°C overnight to accumulate peptide receptive MHC class I molecules on the cell surface and then mixed with varying concentrations of WT1 peptides in 100 μl of DMEM containing 0.25% (w/v) BSA. After incubation for 30 min at room temperature, the temperature was raised to 37°C and the incubation was continued for 1 h. The cells were then washed and stained with FITC-labeled B22.249 mAb and analyzed by FACScan (Becton Dickinson, Mountain View, CA). The relative binding affinity in K_d values was calculated from the mean fluorescence intensities as described previously (25).

Induction of WT1 peptide-specific CTLs

WT1 peptide-specific CTL lines were induced in 4–12-wk-old C57BL/6 mice (H-2D^b) by immunization with LPS (from *Escherichia coli* 055:B5; Sigma, St. Louis, MO)-activated spleen cells pulsed with WT1 peptides as described elsewhere (Kagawa et al., *Submitted for publication*). Briefly, spleen cells were cultured for 3 days with 10 μg/ml LPS in 40 ml of DMEM containing 10% FBS, followed by pulsing with 1 μM WT1 peptide and 10 μM OVAII peptide (OVA 323–339, as a helper epitope) (30) for 2 h. The cells were then irradiated with 3000 rad and injected i.p. into mice. The immunization with LPS-activated spleen cells pulsed with the WT1 peptides was repeated three times at weekly intervals. After 1 wk from the third immunization, the spleen was resected from the immunized mice and the spleen cells were stimulated in vitro with LPS-activated spleen cells, which were pulsed with the WT1 peptide and then irradiated with 3000 rad.

After 5 days of the in vitro stimulation, the cells were tested for their killing activity.

Cytotoxicity assay

Cytotoxic activity was measured by means of ⁵¹Cr release assay. RMAS cells were incubated at 26°C overnight, labeled with ⁵¹Cr for 1 h, and pulsed with the WT1 peptides at room temperature for 30 min. Effector cells were then added to 1 × 10⁴ target cells at varying E:T ratios to a final volume of 200 μl in DMEM containing 5% FBS. After brief centrifugation at 1000 × g, cells were incubated at 37°C for 5 h. Relative cytotoxicity was calculated as follows from the radioactivity released in the culture supernatant: % specific lysis = (experimental release – spontaneous release)/(total release – spontaneous release) × 100. For Db126 peptide-dependent lysis assays, 50 μl of varying concentrations of Db126 peptide dissolved in DMEM was added to 50 μl of RMAS target cells in DMEM containing 0.5% BSA. After incubation for 30 min at room temperature, Db126-specific CTL lines were added at an E:T ratio of 4:1.

Purification of endogenously processed WT1 peptides from WT1-expressing tumor cells

A total of 1 × 10⁹ FBL3 cells were harvested and acid extracted with 1% trifluoroacetic acid. The Centricon 10 (Amicon, Beverly, MA)-passed fraction was loaded onto a Nova Pak C₁₈ RP-HPLC column (4.6 mm × 15 cm; Millipore and Waters Japan) and eluted at 1 ml/min with a shallow acetonitrile gradient. One-minute fractions were collected and dried by Speed Vac. An aliquot equivalent to 1 × 10⁸ FBL3 cells of the HPLC fractions was added to the wells of the ⁵¹Cr release assay, each of which contained 1 × 10⁴ target cells for screening of the peptides recognized by CTLs.

In vivo tumor challenges

C57BL/6 male mice were used to avoid male Ag (H-Y)-specific immune responses because the sex of C57BL/6 mice from which the FBL3 tumor cell line originated was not known. The inoculated dose of the tumor cells was determined by preliminary experiments and a lethal dose for nonimmune mice was used. To determine the effects of immunization, five to eight mice from each group were injected i.p. with PBS, LPS-activated spleen cells alone, or those pulsed with 1 μM Db126 peptide in combination with 10 μM of OVAII (30) at 37°C for 2 h. After three weekly immunizations, 3 × 10⁷ FBL3 leukemia cells were i.p. inoculated into 4–6-wk-old C57BL/6 mice.

Histology

The main organs, including kidney and lung, were removed from the surviving Db126-immunized mice that had rejected the tumor challenges and fixed in Bouin's solution. Paraffin sections of 8-μm thickness were stained with hematoxylin and eosin by means of standard methods.

Results

Identification of H-2D^b-binding peptides

Most CTL epitope peptides can be predicted by means of a peptide library-based scoring system for MHC class I-binding peptides (25, 26). Amino acid sequences of the murine WT1 protein were scanned for peptides with a potential binding capacity for H-2D^b molecules, and five peptides with comparatively high binding scores for H-2D^b molecules were identified (Table I). All five of these WT1 peptides with higher binding scores also exhibited a relatively higher binding affinity for H-2D^b molecules, and some correlation between binding scores and binding affinity (K_d) was established, thus indicating the utility of binding scores for finding peptides which bind to MHC class I molecules. Db126 peptide demonstrated the same order of binding affinity as that of viral Ags (24), which is the strongest Ags for CTL induction. Three peptides (Db126, Db221, and Db235) with anchor motifs for binding to H-2D^b molecules (24) were actually used for in vivo immunization.

Induction of CTLs against WT1 peptides

Whether specific CTLs against these three WT1 peptides could be induced by in vivo immunization with these peptides was examined (Fig. 1). Mice were immunized with LPS-activated spleen

Table I. Binding of WT1 peptides to H-2D^b molecules

Peptide	Amino Acid Sequence	Binding Score	K _d (M)
WT1 peptides			
Db126	aa 126–134 RMFPN ^a APYL	1.77	5.7 × 10 ⁻⁷
Db227	aa 227–235 YQMTSQLEEC	1.93	1.0 × 10 ⁻⁶
Db235	aa 235–243 CMTWNQMNL	1.20	1.3 × 10 ⁻⁶
Db221	aa 221–229 YSSDNL ^a YQM	1.05	2.6 × 10 ⁻⁶
Db136	aa 136–144 SCLESQPTI	1.61	3.7 × 10 ⁻⁶
Known epitope peptides			
SV40 T antigen	aa 223–231 CKGVNKEYL	1.85	1.9 × 10 ⁻⁷
Influenza A34 NP	aa 366–374 ASNENMETM	1.24	1.9 × 10 ⁻⁷

^a Bold letters represent anchor motifs.

cells pulsed with the peptides. The spleen cells of the immunized mice were then assayed for cytotoxic activity against peptide-pulsed RMA-S target cells. WT1 peptide-specific CTLs were induced by immunization with the Db126 peptide, whereas no CTLs were induced by immunization with the Db221 or Db235 peptide. Thus, only the Db126 peptide with the highest binding affinity for H-2D^b molecules could elicit CTL responses. Therefore, subsequent investigation focused on the CTLs against the Db126 peptide.

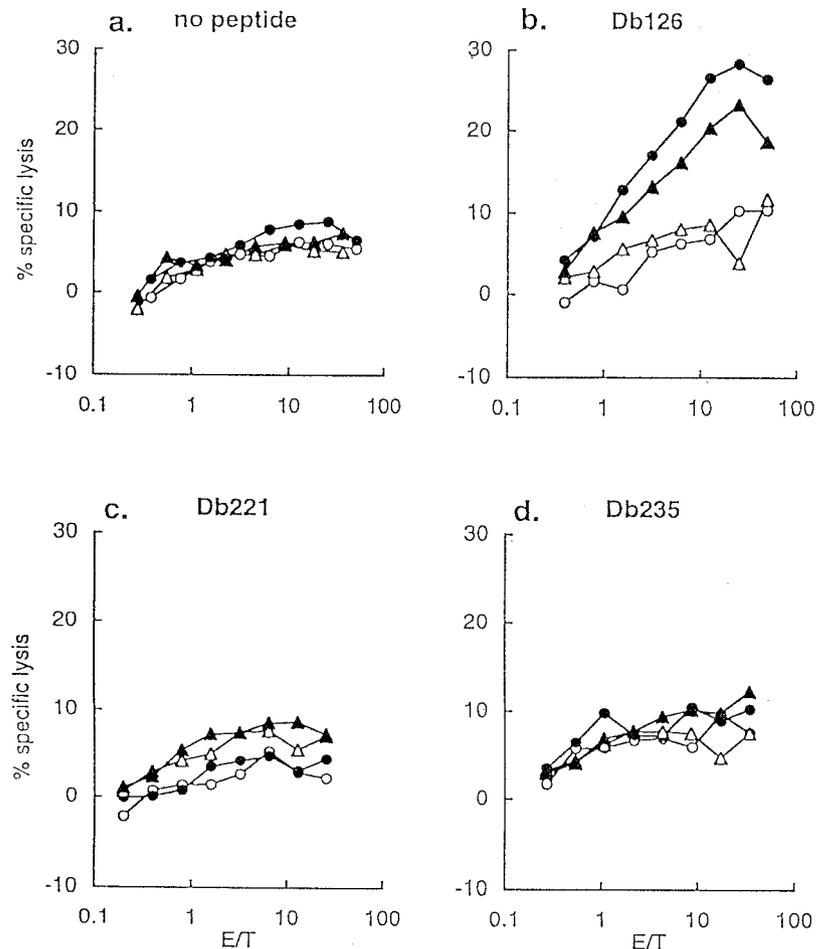
To confirm that the Db126 peptide-induced CTLs specifically recognize the Db126 peptide to kill the target cells, three different CTL lines against the Db126 peptide were assayed for cytotoxic activity against RMA-S target cells pulsed with increasing concentrations of the Db126 peptide (Fig. 2). Their cytotoxic activity was found to increase in parallel with an increase in peptide concen-

trations and to reach a plateau. Half the maximal lysis was observed in the range of nanomolar of the peptide. Lysis by the CTL lines of the RMA-S target cells pulsed with naturally occurring H-2D^b-binding peptide influenza A34 NP (Table I) was not observed (data not shown). These results proved that the cytotoxic activity of the CTL lines was specific for the Db126 peptide.

Lysis of endogenously WT1-expressing tumor cells by Db126-specific CTLs

We next investigated whether Db126-specific CTLs could recognize and lyse endogenously WT1-expressing tumor cells. As shown in Fig. 3, a panel of tumor cell lines was tested for lysis by Db126-specific CTL lines. Before the tests, specificity of the CTLs for the Db126 peptide was confirmed by specific lysis of Db126 peptide-pulsed RMA-S target cells (Fig. 3a). The Db126-specific

FIGURE 1. Induction of WT1 peptide-specific CTLs. Mice were immunized with LPS-activated spleen cells pulsed with WT1 peptides. Spleen cells of mice not immunized with any peptide (a), immunized with Db126 (b), Db221 (c), or Db235 (d) were stimulated in vitro with LPS-activated spleen cells not pulsed with any peptide (a) and with LPS-activated spleen cells pulsed with Db126 (b), Db221 (c), or Db235 (d). Their killing activity was tested against RMA-S cells pulsed (closed symbols) or unpulsed (open symbols) with the immunized peptides at different E:T ratios. Results from two mice (circles and triangles) are shown. At least three independently performed experiments for each peptide yielded similar results.



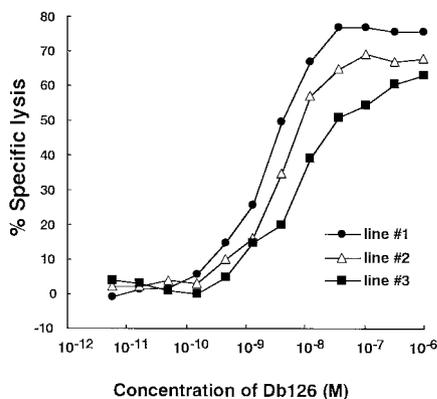


FIGURE 2. Db126 peptide-dependent lysis of RMA8 cells by Db126-specific CTL lines. Three CTL lines established from individual mice immunized with the Db126 peptide were tested for cytotoxic activity against RMA8 cells pulsed with the indicated concentrations of the Db126 peptide at an E:T ratio of 4:1. Lysis of RMA8 cells without the peptide was 2% for line #1, 4% for line #2, and 8% for line #3.

CTLs lysed endogenously WT1-expressing FBL3 cells, but not WT1-nonexpressing RMA cells (Fig. 3*b*). Furthermore, the Db126-specific CTLs killed murine WT1-transfected C1498 cells to a significant extent when compared with parental WT1-nonexpressing C1498 cells, confirming that the molecule targeted for killing by the CTLs is indeed the WT1 peptide (Fig. 3*c*). Similarly, a specific lysis of WT1-nonexpressing EL-4 cells used here (H-2D^b) was obtained as a result of transfection of the WT1 gene, although the lysis was weak because of low expression of H-2D^b molecules on the EL-4 cells used here (Fig. 3*d*). WT1-nonexpressing P815 cells with nonidentical H-2 molecules were not lysed by the CTLs (data not shown). RMA8 cells that were common targets for lymphokine-activated killer/NK cells (31) were not killed by the CTLs (Fig. 3*a*). However, when YAC-1 cells were used as

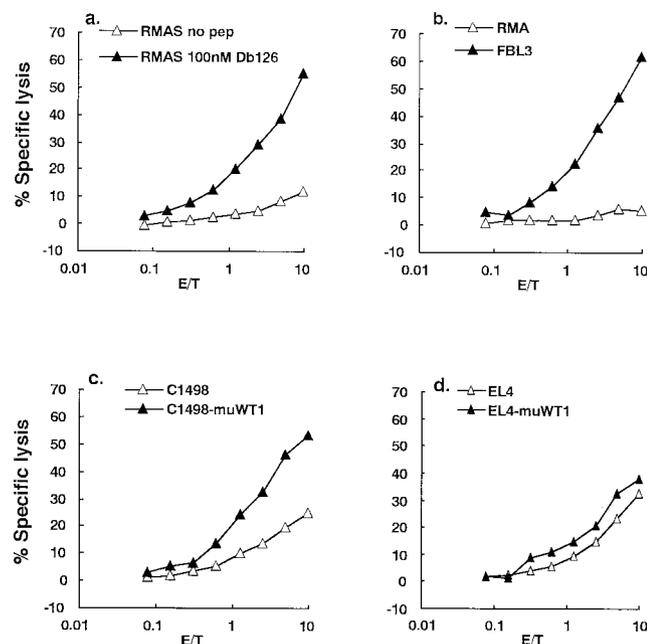


FIGURE 3. Specific lysis of WT1-expressing tumor cells by the Db126-specific CTL line. Specific lysis was tested for Db126-pulsed or -unpulsed RMA8 cells (*a*), WT1-expressing (FBL3) or nonexpressing (RMA) tumor cells (*b*), WT1-transfected or -untransfected C1498 cells (*c*), or WT1-transfected or -untransfected EL4 cells (*d*) at the indicated E:T ratios.

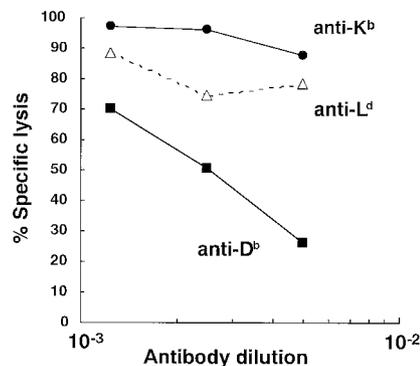


FIGURE 4. H-2D^b-restricted cytotoxic activity of the Db126-specific CTL line. Specific lysis of endogenous WT1-expressing FBL3 cells by the Db126-specific CTL line was tested in the presence of titer adjusted mAbs against H-2K^b (28.13.3S), H-2D^b (28.11.5S), or H-2L^d (MA143). Isotype-matched mAbs were used as control mAbs.

target cells for NK activity, the Db126-specific CTLs also lysed the cells. This phenomenon is reasonable, since it has been well known that CTLs frequently display an NK-like cytolytic activity in addition to Ag-specific cytolytic activity (32–34). The Db126-specific CTLs were also 99% positive for CD8 and virtually negative for NK1.1 (data not shown). Since various NK-activating/inhibitory receptors are expressed not only on NK cells but also on CTLs bearing TCR $\alpha\beta$ (31, 35, 36), these receptors may be responsible for the lysis of YAC-1 cells. Taken together, these results suggest that the Db126-specific CTLs can recognize Db126 or related peptides which are naturally produced through intracellular processing of the WT1 protein and are present on H-2D^b molecules in WT1-expressing cells.

Cytotoxic activity is H-2D^b restricted

Furthermore, to demonstrate that the cytotoxic activity of the CTLs is MHC restricted, it was assayed in the presence of Abs against H-2 class I molecules (Fig. 4). The suppression of cytotoxic activity of the CTLs against WT1-expressing FBL3 cells was found to depend upon an increase in the concentration of Abs against H-2D^b, but Abs against H-2K^b or H-2L^d did not show any suppressive effect on the cytotoxic activity of the CTLs. These results showed that the CTLs exert their cytotoxic activity in an H-2D^b-restricted fashion.

Presence of sensitizing activity to Db126-specific CTLs in cell extract of WT1-expressing cells

To confirm that endogenously WT1-expressing tumor cells express the Db126 peptide on their cell surface through intracellular processing of the WT1 protein and that Db126-specific CTL lines recognize this peptide for cell lysis, WT1-expressing FBL3 cells were lysed and peptide fractions were prepared (Fig. 5). The peptide fractions were further fractionated by RP-HPLC, and each fraction was assayed for its sensitizing activity to the Db126-specific CTLs. The sensitizing activity was recovered in the same retention time as that needed for the synthetic Db126 peptide. One additional sensitizing activity peak was detected. Such additional peaks have previously been observed by us and others when naturally processed endogenous peptides were prepared from whole-cell lysate. In such cases, some peaks represented the sensitizing activity of longer peptides harboring the minimal epitope peptide (37, 38), a tissue-specific variant peptide (39), or cross-recognized peptides bearing similar or unrelated amino acid sequences (40, 41). These results showed that the Db126 peptide targeted by the

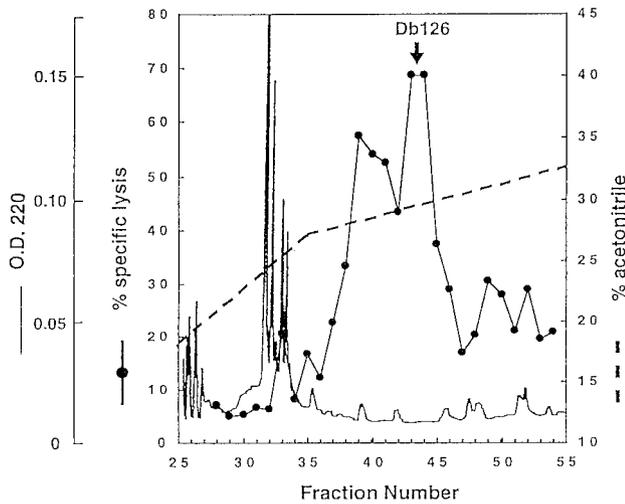


FIGURE 5. Recovery of sensitizing activity from acid extract of FBL3 cells. The acid extract of FBL3 cells was HPLC fractionated, and each fraction was tested for its sensitizing activity for specific lysis of RMA8 target cells by the Db126-specific CTL line. The profile of optical absorbance at 220 nm of the eluting peptides is shown in the background.

Db126-specific CTLs is naturally produced by intracellular processing of the WT1 protein in WT1-expressing cells.

Eradication of tumor challenges by preimmunization with the Db126 peptide

We next investigated whether active immunization with the Db126 peptide elicited *in vivo* tumor immunity. Mice were immunized once a week for 3 wk with LPS-activated spleen cells pulsed with the Db126 peptide and then inoculated *i.p.* with a lethal number of FBL3 leukemia cells. As shown in Fig. 6, all five mice immunized with LPS-activated spleen cells pulsed with Db126 peptide, none of five mice immunized with LPS-activated spleen cells alone, and one of eight mice inoculated with PBS were alive after tumor challenges. A statistical significance ($p < 0.01$) was found between the group immunized with the WT1 peptide and the group immunized with LPS-activated spleen cells alone, or the group inoculated with PBS alone. This experiment was repeated with similar results. In both the immune and nonimmune mice, ascites was observed 3 days after the *i.p.* inoculation of the tumor cells. In the nonimmune mice, the ascites continued to increase and the mice died. On the other hand, in the immune mice, the ascites

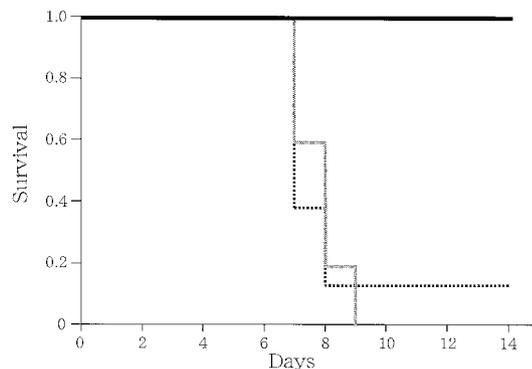


FIGURE 6. Rejection of tumor challenges by immunization with Db126 peptide. Mice were immunized once a week with LPS-activated spleen cells pulsed with Db126 peptide (solid line), LPS-activated spleen cells alone (shaded line), or PBS alone (dashed line). After immunization for 3 wk, 3×10^7 FBL3 leukemia cells were *i.p.* injected.

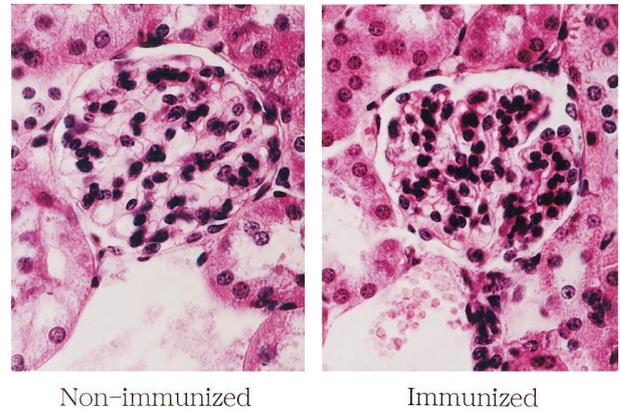


FIGURE 7. No pathological changes of kidney in the immunized mice that rejected tumor challenges. Hematoxylin and eosin staining of the glomeruli of the kidney is shown. No pathological changes such as lymphocyte infiltration or tissue destruction and repair are observed.

gradually decreased afterward, and the mice completely rejected tumor challenges and survived. Spontaneous regression was occasionally observed in nonimmune mice. This regression is presumed to be due to spontaneous induction of CTLs specific for the Friend leukemia virus (FBL3 leukemic cells are transformed by this virus), since such CTL induction was not infrequently observed in C57BL/6 mice (42).

No evidence of autoaggressive reactions in surviving mice that rejected tumor cell challenges

WT1 expression in normal adult mice is limited to a few cell types in several tissues. Moreover, WT1 expression levels in these tissues are considerably lower than those in leukemia (13) and solid tumor (21) cells, suggesting a low risk of normal tissue damage as a result of immune responses to the WT1 protein. To evaluate the risk of autoaggression by immunization against self-WT1 peptide, the tissues of immunized mice were pathologically examined a few weeks after tumor cells had been eradicated. The lung and kidney of three mice were intensively examined because WT1 was mainly expressed in the mesothelial cells of the lung capsule and in the podocytes of the kidney glomeruli (Fig. 7). Both tissues showed normal structure and cellularity in all three mice examined, and no pathological changes caused by immune response, such as lymphocyte infiltration or tissue destruction and repair, were observed. These results showed that the CTLs against the Db126 peptide were ignorant of normal self-cells that express WT1 at physiological levels.

Discussion

The rationale for the efficacy of immunotherapy for cancer patients has been clinically demonstrated by the following findings (23): patients who develop significant GVHD (grade ≥ 2) after allogeneic BMT have a significantly lower rate of relapse than patients with either no GVHD or grade I GVHD; patients who receive T cell-depleted marrow also have a higher rate of relapse after allogeneic BMT than patients who receive unmodified marrow; and donor lymphocyte infusion is effective for complete remission induction of relapsed leukemia patients after allogeneic BMT. This graft-vs-leukemia effect after allogeneic BMT provides the most convincing clinical evidence for the effectiveness of tumor immunology for cancer treatment.

The search for widely expressed tumor Ags as targets for MHC class I-restricted CTLs is of great importance for the development

of T cell-mediated immunotherapy for cancer patients. Reports on such tumor Ags have been increasing exponentially in recent years, and the results indicate that these Ags can be categorized into five groups (43, 44). Ags of the first category correspond to peptides derived from regions of ubiquitous proteins such as mutated ras (4) or p53 (5). Chimeric proteins that result from chromosomal translocation are also unique to tumor cells (45, 46). The second group of tumor Ags consists of tumor-specific shared Ags such as P1A in mice (6) and MAGE in humans (7, 8). The third group of tumor Ags includes differentiation Ags. A good example is tyrosinase, a melanocyte protein that gives rise to different peptides that are presented by either MHC class I or class II molecules (9). The fourth group of tumor Ags is made up of overexpressed tumor Ags. An Ag that is expressed in some normal tissues and overexpressed in tumors is HER-2/neu (10), which is found at high levels in about 30% of breast and ovarian cancers. The last group of tumor Ags includes Ags derived from oncogenic viruses. Thus, the WT1 protein is thought to be a tumor Ag corresponding to the fourth group of tumor Ags.

In the murine models of immunotherapy against WT1-expressing tumors described here, surviving mice that rejected tumor challenges by the immunization of the Db126 peptide did not demonstrate obvious organ damage. These results demonstrate that the CTLs against the WT1 protein can discriminate differences in WT1 expression levels between abnormally WT1-overexpressing tumor cells and physiologically WT1-expressing normal cells, resulting in the killing of tumor cells with no damage to normal tissues. As for the application of immunotherapy with WT1 protein to human cancers, the following evidence suggests that this immunotherapy is promising without damage to normal organs. We (12, 13) and others (14–17) have demonstrated that the wild-type *WT1* gene is aberrantly overexpressed in almost all leukemia cells regardless of type of leukemia: whether it is acute myeloid leukemia, acute lymphoid leukemia, or chronic myeloid leukemia. The WT1 expression levels in leukemic cells are at least 10 times higher than those in normal CD34⁺ hematopoietic progenitor cells (13). This striking difference in WT1 expression levels between leukemic cells and normal hematopoietic progenitor cells is the basis for the reasoning that CTLs induced against the WT1 protein would not cause damage to normal hematopoietic progenitor cells. Furthermore, we have recently reported WT1 expression in 28 (82%) of 34 various types of solid tumor cell lines, including lung, gastric, colon, breast, and ovary cancer cell lines. High WT1 expression in fresh lung cancer tissues has also been reported (21). Our reports demonstrate that WT1 expression is significantly higher in cancer cell-rich tissues than in tissues appearing to be normal, confirming the abnormal overexpression of the *WT1* gene not only in cultured tumor cells but also in fresh lung cancer cells. As mentioned earlier, the striking difference in WT1 expression levels between tissues appearing to be normal and cancer cell-rich tissues is also the basis for the reasoning that CTLs induced against WT1 protein would not cause damage to normal lung tissue.

The *WT1* gene has been categorized as a tumor suppressor gene (3). However, we have proposed that the *WT1* gene has basically two functional aspects, namely, that of a tumor suppressor gene and that of an oncogene, but that in leukemic cells it performs an oncogenic rather than a tumor suppressor gene function (18–20). The following findings support our proposal: 1) high expression of wild-type WT1 in almost all leukemic cells (12, 13), 2) an inverse correlation between WT1 expression levels and prognosis (12), 3) an increased WT1 expression at relapse compared with that at diagnosis in acute leukemia (47), 4) growth inhibition of leukemic cells by WT1 antisense oligomers (18), and 5) blocking of differentiation but induction of proliferation in response to G-CSF in

myeloid progenitor cell line 32D cl3 (19) and normal myeloid progenitor cells (20), both of which constitutively express WT1 by the transfection with the *WT1* gene. Furthermore, it is suggested that the wild-type *WT1* gene also has an oncogenic function in WT1-expressing solid tumors, since the *WT1* gene is overexpressed in various types of solid tumor cells, including lung cancers, and since the suppression of *WT1* gene expression by WT1 antisense oligomers inhibits the growth of solid tumor cells (21).

The loss of tumor-specific Ags followed by the escape from immune surveillance by CTLs is one of the major obstacles of the host's immunological warfare against tumors. Since the WT1 protein plays an essential role in the growth of leukemic and solid tumor cells, loss of the expression of the WT1 protein, i.e., loss of the WT1 Ag, results in cessation of the proliferation of leukemic and solid tumor cells. Thus, immunotherapy directed against the WT1 protein would have little risk of escape from immune surveillance following loss of the WT1 Ag.

It has been well documented that tolerance to self-peptides is induced by deletion of self-reactive T cells in the thymus (48) as well as by deletion or exhaustion of such cells in the periphery (49), and that self-reactive T cells which have escaped deletion are functionally anergized or silenced by down-regulation of coreceptor molecules (50, 51). Since WT1 is a self-protein, it is considered to become tolerant in classical immunology. However, increasing evidence promoted us to accept that a large quantity of antigenic determinants of the self have not induced self-tolerance and thus that a substantial number of self-reactive clones must exist in healthy individuals and have the potential to elicit immune responses directed against tumors. These potentially self-reactive T cell clones are either anatomically secluded (52) or can be simply ignorant of their targets (53–55). It is probably possible to break tolerance especially if the self-proteins are not expressed at sufficient levels at the time and place of tolerance induction. The WT1 peptides are likely to be subdominant self-peptides so that the epitopes are probably ignored by the immune system under physiological conditions, although CTL precursors responsible for the WT1 peptides are present.

WT1 peptides that were predicted on the basis of the peptide library-based scoring system of MHC class I-binding peptides (25, 26, 29) actually showed comparatively higher binding affinity for H-2D^b molecules, confirming that this scoring system is useful for finding candidates for MHC class I-binding peptides. Dyall et al. (56) designed a few artificial variants of MHC class I-binding self-peptides. Since these variant peptides are obviously foreign to the host immune system, a strong CTL response can be induced. Unlike weak T cell responses to self-MHC complexes, CTL responses to variant peptides can be sustained for a longer period without causing annihilation of the clones due to insufficient signals for cell division or survival (57, 58). Since a substantial fraction of such CTLs cross-reacts against nonmutated self-peptides expressed in tumor cells in much smaller amounts, immunization with variant peptides may be a more efficient method to induce CTLs against tumors. The scoring system for MHC class I-binding peptides should provide a convenient design of cross-reactive self-mimicking peptides for immunization.

We have recently reported that in vitro stimulation of HLA-A2.1-positive PBMC with WT1 peptides, Db126, RMFPNAPYL, or WH187, SLGEQQYSV, both of which contain anchor motifs needed for binding to HLA-A2.1 molecules and actually bind to HLA-A2.1 molecules, elicits CTLs against each WT1 peptide (59). The CTLs specifically killed the WT1 peptide-pulsed target cells and endogenous WT1-expressing leukemic cells in an HLA-A2.1-restricted fashion. Thus, the WT1 peptide Db126 that was a shared

sequence between murine and human WT1 protein was immunogenic for the induction of CTLs in both mice and humans. These accumulated data obtained from both human and murine settings suggested a successful clinical application of WT1 protein-directed immunotherapy for patients with leukemia and solid tumors. We are now planning clinical trials of this immunotherapy for patients with leukemia or lung cancer.

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References

- Call, K. M., T. Glaser, C. Y. Ito, A. J. Buckler, J. Pelletier, D. A. Haber, E. A. Rose, A. Kral, H. Yeger, W. H. Lewis, et al. 1990. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell* 60:509.
- Gessler, M., A. Poustka, W. Cavenee, R. L. Nevel, S. H. Orkin, and G. A. P. Bruns. 1990. Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. *Nature* 343:774.
- Menke, A. L., A. J. Van der Eb, and A. G. Jochemsen. 1998. The Wilms' tumor 1 gene: oncogene or tumor suppressor gene? *Int. Rev. Cytol.* 181:151.
- Gjertsen, M. K., J. Bjorheim, I. Saeterdal, J. Myklebust, and G. Gaudemack. 1997. Cytotoxic CD4⁺ and CD8⁺ T lymphocytes, generated by mutant p21-ras (12 Val) peptide vaccination of a patient, recognize 12 Val-dependent nested epitopes present within the vaccine peptide and kill autologous tumour cells carrying this mutation. *Int. J. Cancer* 72:784.
- Theobald, M., J. Biggs, D. Dittmer, A. J. Levine, and L. A. Sherman. 1995. Targeting p53 as a general tumor antigen. *Proc. Natl. Acad. Sci. USA* 92:11993.
- Ramarathinam, L., S. Sarma, M. Maric, M. Zhao, G. Yang, L. Chen, and Y. Liu. 1995. Multiple lineages of tumors express a common tumor antigen, P1A, but they are not cross-protected. *J. Immunol.* 155:5323.
- Traversari, C., P. Van der Bruggen, I. F. Luescher, C. Lurquin, P. Chomez, A. Van Pel, E. De Plaen, A. Amar-Costesec, and T. Boon. 1992. A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. *J. Exp. Med.* 176:1453.
- Gaugler, B., B. Van den Eynde, P. Van der Bruggen, P. Romero, J. J. Gaforio, E. De Plaen, B. Lethé, F. Brasseur, and T. Boon. 1994. Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. *J. Exp. Med.* 179:921.
- Topalian, S. L., M. I. Gonzales, M. Parkhurst, Y. F. Li, S. Southwood, A. Sette, S. A. Rosenberg, and P. F. Robbins. 1996. Melanoma-specific CD4⁺ T cells recognize nonmutated HLA-DR-restricted tyrosinase epitopes. *J. Exp. Med.* 183:1965.
- Fisk, B., T. L. Blevins, J. T. Wharton, and C. G. Ioannides. 1995. Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. *J. Exp. Med.* 181:2109.
- Rensing, M. E., A. Sette, R. M. P. Brandt, J. Ruppert, P. A. Wentworth, M. Hartman, C. Oseroff, H. M. Grey, C. J. M. Melief, and W. M. Kast. 1995. Human CTL epitopes encoded by human papilloma virus type 16 E6 and E7 identified through in vivo and in vitro immunogenicity studies of HLA-A*0201-binding peptides. *J. Immunol.* 154:5934.
- Inoue, K., H. Sugiyama, H. Ogawa, M. Nakagawa, T. Yamagami, H. Miwa, K. Kita, A. Hiraoka, T. Maseoka, K. Nasu, et al. 1994. WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. *Blood* 84: 3071.
- Inoue, K., H. Ogawa, Y. Sonoda, T. Kimura, H. Sakabe, Y. Oka, S. Miyake, H. Tamaki, Y. Oji, T. Yamagami, et al. 1997. Aberrant overexpression of the Wilms' tumor gene (WT1) in human leukemia. *Blood* 89:1405.
- Miwa, H., M. Beran, and G. F. Saunders. 1992. Expression of the Wilms' tumor gene (WT1) in human leukemias. *Leukemia* 6:405.
- Miyagi, T., H. Ahuja, T. Kubota, I. Kubonishi, H. P. Koeffler, and I. Miyoshi. 1993. Expression of the candidate Wilms tumor gene, WT1, in human leukemia cells. *Leukemia* 7:970.
- Brieger, J. E., Weidmann, K. Fenchel, P. S. Mitrou, D. Hoelzer, and L. Bergmann. 1994. The expression of the Wilms' tumor gene in acute myelocytic leukemias as a possible marker for leukemic blast cells. *Leukemia* 8:2138.
- Menssen, H. D., H. J. Renkl, U. Rodeck, J. Maurer, M. Notter, S. Schwarz, R. Reinhardt, and E. Thiel. 1995. Presence of Wilms' tumor gene (WT1) transcripts and the WT1 nuclear protein in the majority of human acute leukemias. *Leukemia* 9:1060.
- Yamagami, T., H. Sugiyama, K. Inoue, H. Ogawa, T. Tatekawa, M. Hirata, T. Kudoh, T. Akiyama, A. Murakami, T. Maekawa, and T. Kishimoto. 1996. Growth inhibition of human leukemic cells by WT1 (Wilms tumor gene) antisense oligonucleotides: Implications for the involvement of WT1 in leukemogenesis. *Blood* 87:2878.
- Inoue, K., H. Takami, H. Ogawa, Y. Oka, T. Soma, T. Tatekawa, Y. Oji, A. Tsuboi, E. H. Kim, M. Kawakami, et al. 1998. Wilms' tumor gene (WT1) competes with differentiation-inducing signal in hematopoietic progenitor cells. *Blood* 91:2969.
- Tsuboi, A., Y. Oka, H. Ogawa, O. A. Elisseeva, H. Tamaki, Y. Oji, E. H. Kim, T. Soma, T. Tatekawa, M. Kawakami, et al. 1999. Constitutive expression of the Wilms' tumor gene WT1 inhibits the differentiation of myeloid progenitor cells but promotes their proliferation in response to granulocyte-colony stimulating factor (G-CSF). *Leuk. Res.* 23:499.
- Oji, Y., H. Ogawa, H. Tamaki, Y. Oka, A. Tsuboi, E. H. Kim, T. Soma, T. Tatekawa, M. Kawakami, M. Asada, T. Kishimoto, and H. Sugiyama. 1999. Expression of the Wilms' tumor gene WT1 in solid tumors and its involvement in tumor cell growth. *Jpn. J. Cancer. Res.* 90:194.
- Melief, C. J. M., and W. M. Kast. 1995. T-cell immunotherapy of tumors by adoptive transfer of cytotoxic T lymphocytes and by vaccination with minimal essential epitopes. *Immunol. Rev.* 145:167.
- Ritz, J. 1994. Tumor immunity: Will new keys unlock the door. *J. Clin. Oncol.* 12:237.
- Rammensee, H.-G., T. Friede, and S. Stevanović. 1995. MHC ligands and peptide motifs: first listing. *Immunogenetics* 41:178.
- Udaka, K., K.-H. Wiesmüller, S. Kienle, G. Jung, and P. Walden. 1995. Tolerance to amino acid variations in peptides binding to the MHC class I protein H-2Kb. *J. Biol. Chem.* 270:24130.
- Stryn, A., L. Pedersen, T. Romme, C. B. Holm, A. Holm, and S. Buus. 1996. Peptide binding specificity of major histocompatibility complex class I resolved into an array of apparently independent subspecificities: quantitation by peptide libraries and improved prediction of binding. *Eur. J. Immunol.* 26:1911.
- Ljunggren, H.-G., and K. Kärre. 1985. Host resistance directed selectively against H-2-deficient lymphoma variants. *J. Exp. Med.* 162:1745.
- Lemke, H., G. J. Hämmerling, and U. Hämmerling. 1979. Fine specificity analysis with monoclonal antibodies of antigens controlled by the major histocompatibility complex and by the Qa/TI region in mice. *Immunol. Rev.* 47:175.
- Udaka, K., K.-H. Wiesmüller, S. Kienle, G. Jung, and P. Walden. 1995. Deciphering the structure of MHC-I restricted CTL epitopes with complex peptide libraries. *J. Exp. Med.* 181:2097.
- Shimonkevitz, R., S. Colon, J. W. Kappler, P. Marrack, and H. M. Grey. 1984. Antigen recognition by H-2-restricted T cells. II. A tryptic ovalbumin peptide that substitutes for processed antigen. *J. Immunol.* 133:2067.
- Chang, C. S., L. Brossay, M. Kronenberg, and K. P. Kane. 1999. The murine nonclassical class I major histocompatibility complex-like CD1.1 molecule protects target cells from lymphokine-activated killer cell cytotoxicity. *J. Exp. Med.* 189:483.
- Moretta, A., G. Pantaleo, L. Moretta, J. C. Cerottini, and M. C. Mingari. 1983. Direct demonstration of the clonogenic potential of every human peripheral blood T cells: clonal analysis of HLA-DR expression and cytolytic activity. *J. Exp. Med.* 157:743.
- Harris, D. T., L. Jaso-Friedmann, R. B. Devlin, H. S. Koren, and D. L. Evans. 1992. The natural killer cell-like lytic activity expressed by cytolytic T lymphocytes is associated with the expression of a novel function-associated molecule. *Scand. J. Immunol.* 35:299.
- Mingari, M. C., C. Vitale, A. Cambiaggi, F. Schiavetti, G. Melioli, S. Ferrini, and A. Poggi. 1995. Cytolytic T lymphocytes displaying natural killer (NK) - like activity: expression of NK-related functional receptors for HLA class I molecules (p58 and CD94) and inhibitory effect on the TCR-mediated target cell lysis or lymphokine production. *Int. Immunol.* 7:697.
- Kozlowski, M., J. Schorey, T. Potis, V. Grigoriev, and J. Kornbluth. 1999. NK lytic-associated molecules: a novel gene selectively expressed in cells with cytolytic function. *J. Immunol.* 163:1775.
- Speiser, D. E., M. J. Pittet, D. Valmori, R. Dunbar, D. Rimoldi, D. Lienard, H. R. MacDonald, J. C. Cerottini, V. Cerundolo, and P. Romero. 1999. In vivo expression of natural killer cell inhibitory receptors by human melanoma-specific cytolytic T lymphocytes. *J. Exp. Med.* 190:775.
- Udaka, K., T. J. Tsomides, P. Walden, N. Fukusen, and H. N. Eisen. 1993. A ubiquitous protein is the source of naturally occurring peptides that are recognized by a CD8⁺ T cell clone. *Proc. Natl. Acad. Sci. USA* 90:11272.
- Uenaka, A., T. Ono, T. Akisawa, H. Wada, T. Yasuda, and E. Nakayama. 1994. Identification of a unique antigen peptide pRL1 on BALB/c RL male 1 leukemia recognized by cytotoxic T lymphocytes and its relation to the Akt oncogene. *J. Exp. Med.* 180:1599.
- Wu, M. X., T. J. Tsomides, and H. N. Eisen. 1995. Tissue distribution of natural peptides derived from a ubiquitous dehydrogenase, including a novel liver-specific peptide that demonstrates the pronounced specificity of low affinity T cell reactions. *J. Immunol.* 154:4495.
- Nanda, N. K., K. K. Arzoo, H. M. Geysen, A. Sette, and E. E. Sercarz. 1995. Recognition of multiple peptide cores by a single T cell receptor. *J. Exp. Med.* 182:531.
- Udaka, K., K.-H. Wiesmüller, S. Kienle, G. Jung, and P. Walden. 1996. Self MHC-restricted peptides recognized by an alloreactive T lymphocyte clone. *J. Immunol.* 157:670.
- Cheever, M. A., R. A. Kempf, and A. Fefer. 1977. Tumor neutralization, immunotherapy, and chemoimmunotherapy of a Friend leukemia with cells secondarily sensitized in vitro. *J. Immunol.* 119:714.
- Urban, J. L., and H. Schreiber. 1992. Tumor antigens. *Annu. Rev. Immunol.* 10:617.
- Van den Eynde, B., and P. Van der Bruggen. 1997. T cell defined tumor antigen. *Curr. Opin. Immunol.* 9:684.

45. Yotnda, P., H. Firat, F. Garcia-Pons, Z. Garcia, G. Gourru, J.-P. Vemant, F. A. Lemonnier, V. Leblond, and P. Langlade-Demoyen. 1998. Cytotoxic T cell response against the chimeric p210 BCR-ABL protein in patients with chronic myelogenous leukemia. *J. Clin. Invest.* 101:2290.
46. Yotnda, P. F. Garcia, M. Peuchmaur, B. Grandchamp, M. Duval, F. Lemonnier, E. Vilmer, and P. Langlade-Demoyen. 1998. Cytotoxic T cell response against the chimeric ETV6-AML1 childhood acute lymphoblastic leukemia. *J. Clin. Invest.* 102:455.
47. Tamaki, H., H. Ogawa, K. Inoue, T. Soma, T. Yamagami, S. Miyake, Y. Oka, Y. Oji, T. Tatekawa, A. Tsuboi, et al. 1996. Increased expression of the Wilms tumor gene (*WT1*) at relapse in acute leukemia. *Blood* 88:4396.
48. Kappler, J. W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell* 49:273.
49. Webbs, S., C. Morris, and J. Sprent. 1990. Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. *Cell* 63:1249.
50. Burke, L. C., D. Lo, O. Kanagawa, R. Brinster, and R. A. Flavell. 1989. T-cell tolerance by clonal anergy in transgenic mice with nonlymphoid expression of MHC class II I-E. *Nature* 342:564.
51. Schönrich, G., U. Kalinke, F. Momburg, M. Malissen, A.-M. Schmitt-Verhulst, B. Malissen, G. Hämmerling, and B. Arnold. 1991. Down-regulation of T cell receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction. *Cell* 65:293.
52. Mackay, C. R. 1993. Homing of native, memory and effector lymphocytes. *Curr. Opin. Immunol.* 5:423.
53. Ohashi, P. S., S. Oehlen, K. Buerki, H. Pircher, C. T. Ohashi, B. Odermatt, and B. Malissen. 1991. Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell* 65:305.
54. Oldstone, M. B., M. Nerenberg, P. Southern, J. Price, and H. Lewicki. 1991. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. *Cell* 65:319.
55. Heath, W. R., F. Karamalis, J. Donoghue, and J. F. Miller. 1995. Autoimmunity caused by ignorant CD8⁺ T cells is transient and depends on avidity. *J. Immunol.* 155:2339.
56. Dyall, R., W. B. Bowne, L. W. Weber, J. LeMaout, P. Szabo, Y. Moroi, G. Piskun, J. J. Lewis, A. N. Houghton, and J. Nikolic-Zugic. 1998. Heteroclitic immunization induces tumor immunity. *J. Exp. Med.* 188:1553.
57. Kaneko, T., T. Moriyama, K. Udaka, K. Hiroishi, H. Kita, H. Okamoto, H. Yagita, K. Okumura, and M. Imawari. 1997. Impaired induction of cytotoxic T lymphocytes by antagonism of a weak agonist borne by a variant hepatitis C virus epitope. *Eur. J. Immunol.* 27:1782.
58. Kurts, C., H. Kosaka, F. R. Carbone, J. F. A. P. Miller, and W. R. Heath. 1997. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8⁺ T cells. *J. Exp. Med.* 186:239.
59. Oka, Y., O. A. Elisseeva, A. Tsuboi, H. Ogawa, H. Tamaki, H. Li, Y. Oji, E. H. Kim, T. Soma, M. Asada, et al. Human cytotoxic T lymphocyte responses specific for peptides of wild-type Wilms' tumor gene WT1 product. *Immunogenetics*, In press.