Identification of a Highly Immunogenic HLA-A*01-Binding T Cell Epitope of WT1

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Abstract

Purpose: The transcription factor Wilms tumor protein 1 (WT1) belongs to a new generation of tumor antigens, as it is essential for tumor cell proliferation and is highly expressed in various hematologic and solid malignancies. The aim of this study was to apply a modified reverse immunology strategy to identify immunogenic epitopes of WT1 which could be useful for immunotherapy.

Experimental Design: Potential HLA-A*01 epitopes predicted by a MHC binding algorithm were screened for recognition by peripheral blood mononuclear cells (PBMC) from patients with spontaneous T cell responses using intracellular cytokine cytometry. Epitope processing was shown by proteasomal cleavage. Epitope-specific T cells were generated from CD4+CD25+ regulatory T cell–depleted PBMC.

Results: One of five predicted HLA-A*01-binding candidate epitopes showed high immunogenicity as 5 of 14 hematologic malignancies had WT1.317-327–reactive T cells ranging from 0.4% to 1.5% of CD3+CD8+ T cells. Proteasomal degradation assays indicated the cleavage of WT1.317-327. The depletion of regulatory T cells from PBMCs enabled the rapid expansion of WT1.317-327–specific CTL, whereas no CTL could be generated from unfractionated PBMC. WT1.317-327–specific CTL efficiently lysed an autologous WT1-expressing tumor cell line but not HLA-A*01–negative WT1-expressing tumor cells. Immunogenicity of the epitope across histologies was verified by the demonstration of spontaneous ex vivo WT1.317-327–specific T cell responses in two of six patients with HLA-A*01–positive melanoma or lung cancer.

Conclusion: In this study, a modified reverse immunology strategy was employed to identify a first immunogenic HLA-A*01–restricted T cell epitope of the tumor antigen WT1, which is of considerable interest for use in vaccination trials.

The transcription factor Wilms tumor protein 1 (WT1) has recently attracted much interest as a target for the immunotherapy of cancer (1). WT1 is highly expressed in myeloid leukemias and various solid malignancies (2–7). WT1 is different from previously used tumor antigens, as it plays a key pathogenetic role in tumor progression. Treatment with WT1 antisense oligomers results in the growth inhibition of leukemic blasts as well as carcinoma cells (8, 9). HLA-A*0201- and HLA-A*24–restricted epitopes from WT1 have been identified and cytotoxic T cells raised against these epitopes have been shown to lyse carcinoma cell lines and leukemic blasts but not hematopoietic stem cells (10, 11). Several studies showed spontaneous WT1-specific T cell and antibody responses in patients with myeloid leukemias (12–16). First reports from vaccination trials with HLA-A*0201- or HLA-A*24–binding epitopes from WT1 showed the induction of T cell responses associated with clinical responses in patients with breast and lung cancer and acute myeloid leukemia (17, 18).

Thus far, only HLA class I-binding epitopes restricted to HLA-A*0201 and HLA-A*24 have been identified (19–21). The identification of WT1-derived T cell epitopes presented by other HLA class I molecules is of considerable interest for further development of WT1-based treatment strategies.

The search for new T cell epitopes in known tumor antigens using the classical “reverse immunology” strategy has led to the identification of numerous T cell epitopes, most of them restricted to HLA-A*0201 (22). The reverse immunology approach includes the prediction of potential T cell epitopes from known tumor antigens, their analysis for MHC-binding, followed by the in vitro generation of peptide-reactive T cells, and their testing for target cell recognition. This strategy is rather laborious, because of the need for T cell induction against multiple peptides, including many that may not be processed. In the setting of non–HLA-A*0201 alleles, target tumor cells expressing both the respective HLA allele and the
Materials and Methods

Patients and healthy controls. PBMC from patients with HLA-A*01–positive acute myeloid leukemia, melanoma, and lung cancer, and from healthy subjects were collected and cryopreserved. The investigation was approved by the Institutional Ethics Committee and informed consent was obtained from all individuals. In patients, high-resolution HLA typing was done molecularly by sequence-specific primers, whereas serologic HLA typing was done in healthy individuals.

Cell lines. The TF-1 acute myeloid leukemia cell line (DSMZ, accession no. 334; http://www.dsmz.de/) is HLA-A*01–negative and has strong WT1 expression (2 × 10⁻¹¹, calculated as a ratio of porphobilinogen deaminase, PBGD, housekeeping gene expression). Cell cultures were done in RPMI medium, supplemented with 10% FCS. The melanoma cell line, U87-MF-1 (HLA-A*01–positive; WT1 expression, 4 × 10⁻⁵), was established from a patient after surgical resection of a soft tissue metastasis and was maintained in Iscove's medium containing 10% FCS (30).

Epitope prediction. Epitope prediction was done as described previously (31). Briefly, potential HLA-A*01 ligands from the sequence of WT1 were selected using a matrix pattern suitable for the calculation of peptides fitting to the HLA-A*01 motif. Such motif predictions are available on our web page at http://www.syfpeithi.de.

Peptide synthesis. Peptides were synthesized in an Applied Biosystems 432A peptide synthesizer following standard protocols (Applied Biosystems, Foster City, CA). Synthesized products were analyzed by high-performance liquid chromatography (Varian Star; Zinsser Analytics, Munich, Germany) and MALDI-TOF mass spectrometry (G2025A; Hewlett-Packard, Waldbronn, Germany), and purified by preparative high-performance liquid chromatography to purities >93%.

Quantification of WT1 expression. Quantitative real-time reverse transcription-PCR was done to assess the expression of WT1 as previously described (32). The values were calculated as a ratio of PBGD housekeeping gene expression.

Analysis of antigen-specific T cells. Heparinized peripheral blood samples were obtained from each patient and mononuclear cells were isolated by density gradient centrifugation using Ficoll-Hypaque and cryopreserved. Following the thawing of cryopreserved samples and overnight resting, antigen-specific T cells were detected by intracellular cytokine staining after a short time of incubation with WT1 candidate epitopes or irrelevant peptides as described previously (13). For the analysis of cultured T cells, tetramer staining was done due to a high level of unspecific IFNγ production. In brief, PBMC (2 × 10⁶) were incubated with 10 μg/mL of each peptide. After 1 hour, 20 μg/mL of brefeldin A (Sigma, Deisenhofen, Germany) was added, and after an additional 5 hours, PBMC were stained extracellularly with fluorescence-conjugated monoclonal antibodies against CD8, CD3, and intracellularly with IFNγ fluorescence-conjugated monoclonal antibody (BD Bioscience, Heidelberg, Germany). Data acquisition was done on a FACSCalibur cytometer and analyzed using CellQuest Software (BD Bioscience). A T cell response was considered positive if the frequency of CD3+CD8+ T cells producing IFNγ in response to the candidate epitope was at least 2-fold higher compared with an irrelevant peptide, and if there was a minimum of 0.05% of IFNγ+CD3+CD8+ T cells after the subtraction of the irrelevant peptide background response.

Peptosomal processing. Purification of 20S proteasomes, in vitro degradation of the precursor peptide, WT1.313-336, and separation and analysis of cleavage products were done as described (35). In brief, proteasomes were isolated from the EBV-transformed B cell line, LCL-721.174 (constitutive proteasome), and the parental cell line, LCL-721 (immunoproteasome; ref. 35). WT1.313-336 (10 nmol/L, 27.6 μg) was incubated for 6 hours with 2 μg of immunoproteasome or with 2 μg of constitutive proteasome, respectively, in digestion buffer [20 mmol/L Tris-HCl (pH 7.6), 10 mmol/L NaCl, 10 mmol/L KCl, 2 mmol/L MgCl₂, 0.5 mmol/L DTT] and the reaction was stopped by freezing the reaction mixture at −80°C. A combination of Edman sequencing and MALDI-TOF mass spectrometry was subsequently done to determine the identity and quantity of peptides generated after proteasome digestion.

Generation of WT1.317-327–specific T cells. Unfractionated or CD4+CD25+ regulatory T cells–depleted PBMC from two patients with HLA-A*01–positive melanoma were used for the generation of WT1.317-327–specific T cells. CD4+CD25+ regulatory T cells were depleted from PBMC prior to T cell culture using the CD4+CD25+ regulatory T cell isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany), and used according to the manufacturer's instructions. CD4+ cells were separated with a biotinylated non–CD4 cell antibody cocktail. Following positive selection of CD4+CD25+ with CD25 MicroBeads, the CD4– fraction and the CD4+CD25– population were cocultured in the presence of 10 μg/mL of WT1.317-327 peptide or HLA-A*01–binding HIV.70-78 peptide as a negative control in complete medium supplemented with 10 IU/mL of interleukin 2 (II-2; Chiron, Munich, Germany) and 5 ng/mL of II-7 (R&D systems). Cells were plated in a 96-well U-bottomed plate (Costar/Corning, New York, NY) and placed in an incubator with 5% CO₂ and humidified atmosphere. Restimulation was done after 10 days with irradiated autologous PBMC pulsed with WT1.317-327 peptide in the presence of β2-microglobulin in a ratio of 5:1 CTL to autologous irradiated feeder cells. Every other day, II-2 was added at 10 IU/mL.

Chromium release assay. The standard ⁵¹Cr chromium release assay was done as described previously (36). Target cells were labeled for 1 hour with 10 μCi ⁵¹Cr-sodium (Amersham Biosciences, Buckinghamshire, United Kingdom) for 1 hour at 37°C and 5% CO₂.
Labeled target cells (1 × 10^3 in 50 μL) were then added to effector cells (100 μL) at the indicated E/T ratios in V-bottomed microwells. The antigen specificity of tumor cell lysis was further assessed in a cold target inhibition assay by analyzing the capacity of unlabeled (cold) autologous PBMC pulsed with WT1.317-327 peptide to block the lysis of labeled (hot) WT1-expressing tumor cell line, UKBF-11. Chromium release was measured in the supernatant harvested after 4 hours of incubation at 37°C. The percentage of specific lysis was calculated as 

\[
\frac{\text{spontaneous release}}{(\text{spontaneous release} + \text{total release})} \times 100
\]

Results

**Predicted HLA-A*01–binding peptides.** Prediction of candidate 9-mer and 10-mer epitopes according to the HLA-A*01 motif from WT1 (Swiss Prot accession no. P19544) was done using the SYFPEITHI algorithm and 11- to 13-mer candidate epitopes were calculated according to the same prediction model as previously described (31). Using this approach, five peptides were selected as candidate T cell epitopes including one 11-mer (WT1.317-327; TSEKRPFMCAY), two 12-mer (WT1.37-48, VLDAPPAGASY; WT1.260-271, WTEQSNHGSTY), and two 13-mer (WT1.137-149, CLESEQPAIRNQGY; WT1.188-200, LGEQQYSVPVVY) peptides.

**Screening of T cell reactivity to predicted epitopes in patients.** Using functional flow cytometry detecting peptide-induced induction of intracellular IFNγ, peripheral blood T cells from HLA-A*01 patients were analyzed ex vivo for recognition of the five WT1 candidate epitopes. This patient group included 8 patients with myeloid leukemia and 6 patients with other hematologic malignancies, of whom 10 had received allogeneic stem cell transplantation. In addition to patients with HLA-A*01, we also included two patients expressing HLA-A*26, which had been grouped into the HLA-A*01 family due to very similar peptide specificity (37). No or less than 0.4% of CD3+CD8+ T cells (median 0.07%) produced IFNγ in the presence of an irrelevant HIV peptide. As shown in Fig. 1A, the candidate epitope WT1.317-327 showed high immunogenicity as >2-fold higher frequencies of T cells secreting IFNγ in response to this peptide were detected in 5 of 14 patients ex vivo. Following subtraction of the percentages of IFNγ+ CD3+CD8+ T cells in response to the HIV peptide, the calculated frequencies of WT1.317-327–reactive T cells were 1.5%, 0.8%, 0.6%, 0.6%, and 0.4% of CD3+CD8+ T cells, respectively. Figure 1B shows a representative dot blot of the T cell response to WT1.317-327 in a patient's sample detected by both intracellular IFNγ and tetramer staining. In addition, low-frequency T cell responses (0.24%, 0.27%, and 0.47% of CD3+CD8+ T cells) were seen in three patients against a minipool of WT1.188-200 and WT1.37-48. In contrast, no T cell response to WT1 epitopes was observed in 11 HLA-A*01–positive healthy subjects (Fig. 1A).

**In vitro proteasome digestion of WT1.313-336.** A crucial prerequisite for a peptide to be presented as a T cell epitope is its proper excision from the protein. To confirm the processing of WT1.317-327 as suggested by the T cell studies, we analyzed in vitro digestion of the 24-mer WT1-derived peptide 313-336 encompassing the WT1.317-327 peptide by constitutive as well as immunoproteasomes (Fig. 2). The COOH-terminal residue Tyr^{327} (Y) was generated by the immunoproteasome as the major cleavage event (313-327). The complementary fragment 328-336 represented the other major fragment in this digest. No processing of the NH2 terminus was observed in the immunoproteasome digest. This finding is not unexpected as immunoproteasomes most frequently produce an NH2-terminally extended precursor, which is trimmed to its final length by aminopeptidases (38). Using constitutive proteasomes, a cleavage site COOH-terminal to Tyr^{327} (Y) was also shown because the 328-336 fragment was efficiently generated. We failed, however, to detect the complementary fragment x-327, which most likely resulted from the formation of cysteine bridging. Because WT1.313-336 contains two cysteines at positions 325 and 330, which frequently results in disulfide bridging during high-performance liquid chromatography separation, an unequivocal assignment of each signal in the mass spectra to a peptide fragment was not possible. In the constitutive proteasome digest, a fragment was also found sharing the NH2 terminus Thr^{317} (T) of the WT1.317-327 peptide.

![Fig. 1. Flow cytometric analysis of ex vivo T cell responses to HLA-A*01–binding candidate epitopes from WT1 in 14 patients with hematologic malignancies and 11 healthy subjects. A, the frequencies of antigen-specific T cells were detected by intracellular IFNγ staining of CD3+CD8+ T cells after 18 hours of coinoculation with the five WT1 peptides and an irrelevant HLA-A*01–binding peptide as negative control (HIV). Five of 14 patients showed a T cell response to WT1.317-327, whereas in 11 healthy subjects, no response to either of the five candidate WT1 peptides was observed. Due to the paucity of samples, T cell responses were tested against minipools consisting of WT1.37-48 + WT1.188-200, and WT1.137-149 + WT1.260-271, respectively. B, example of the T cell response analysis against WT1.317-327 in one HLA-A*01–positive patient using intracellular IFNγ and A*01/WT1.317-327 tetramer staining. The CD8+IFNγ profile of CD3-gated lymphocytes in PBMC in response to an HLA-A*01–binding HIV peptide (left), and to WT1.317-327 (middle). Tetramer staining of CD3-gated T cells (right).](image-url)
WT1.317–327–specific CTL could rapidly be generated from regulatory T cell–depleted PBMC and efficiently lyse HLA-A*01–positive WT1 expressing autologous tumor cells. To verify recognition of the naturally processed WT1.317-327 epitope, specific T cells were generated. In the first set of experiments, we failed to expand WT1.317-327–reactive T cells from unfractionated PBMC from a total of eight HLA-A*01–positive patients following 14 days of \textit{in vitro} stimulation.

**Fig. 2.** Proteasome-mediated digestion of the precursor peptide WT1.313–336. \textit{In vitro} digestion of 24-mer peptide WT1.313–336 encompassing the WT1.317–327 epitope, TSEKRPFMCAY, by constitutive as well as immunoproteasome digests was analyzed. First column, the amount of fragments detected. The NH2-terminally extended precursor peptide 313–327 was found as the major fragment in the immunoproteasome digest. Detection of fragments WT1.328–336 and WT1.317–323... in the constitutive digest also suggests the cleavage of the epitope WT1.317–327. The COOH-terminally cleaved peptide 317–327 could not be detected, most likely due to cysteine bridging.

**Fig. 3.** WT1.317–327–specific CTL can be generated \textit{in vitro} from PBMC after CD4+CD25+ regulatory T cell depletion and efficiently lyse HLA-A*01–positive WT1 expressing autologous tumor cells. A, in the PBMC of a patient with melanoma depleted of CD4+CD25+, regulatory T cell stimulation with peptide WT1.317–327, IL-2, and IL-7 resulted in the \textit{in vitro} generation of 26.54% of WT1.317–327–tetramer+ T cells after 14 days (right), whereas no detectable WT1.317–327–specific T cells were obtained from unfractionated PBMC (left). B, in another patient with melanoma, 7.7% WT1.317–327–tetramer+ T cells were generated in a regulatory T cell–depleted culture in the presence of WT1.317–327 (right) but not when incubated under the same conditions but without WT1 peptide (left). C, the autologous melanoma cell line UKBF–11 were used as target cells in a standard \textit{51} chromium release assay (\textbullet;). WT1.317–327–specific CTL efficiently lyse HLA-A*01–positive WT1 expressing autologous tumor cells, but not the HLA-A*01–negative WT1 expressing acute myeloid leukemia line TF–1 (\textdagger;). Stimulated control PBMC (\textbullet; left) did not lyse the autologous tumor cells, confirming the specificity of the T cell recognition (\textdagger). Points, mean of triplicate values; bars, SE; one out of three experiments with concordant results. D, a cold target inhibition assay was done to further assess the specificity of the autologous WT1-specific CTL. The lysis of UKBF–11 could be inhibited by the addition of unlabeled WT1 peptide – loaded autologous PBMC (cold target) but not by HIV peptide – loaded PBMC. \textit{51}Chromium-labeled WT1 peptide loaded autologous PBMC (hot) but not HIV peptide – loaded PBMC were lysed by WT1-specific CTL.
the presence of WT1.317-327 peptide, IL-2 and IL-7. As it has been previously shown by other groups that regulatory T cells prevent in vitro expansion of tumor-reactive T cells (39), we next did WT1.317-327–specific CTL generation following the depletion of CD4+CD25+ regulatory T cells. As shown in Fig. 3A, stimulation of regulatory T cell–depleted PBMC from a patient with HLA-A*01–positive melanoma with WT1.317-327 peptide, IL-2, and IL-7 resulted in the induction of 26.5% of WT1.317-327-tetramer+ T cells after 14 days. Without regulatory T cell depletion, no WT1.317-327-tetramer+ T cells could be generated from PBMC. In a second experiment done in this patient, 29.3% of WT1.317-327-tetramer+ T cells could be generated from regulatory T cell–depleted PBMC whereas <3% were found in unfractionated PBMC after 14 days of culture (data not shown). In another patient 7.7% of WT1.317-327-tetramer+ T cells were generated by the stimulation of regulatory T cell–depleted PBMC (Fig. 3B). In this patient, an autologous HLA-A*01–positive melanoma cell line, UKBF-11, with high WT1 expression was available. In a 51chromium release assay WT1.317-327–specific CTL efficiently recognized the autologous HLA-A*01+WT1+ tumor cell line, UKBF-11 (Fig. 3C). Autologous CTL stimulated under the same conditions but in the absence of WT1 peptide (“control CTL”; Fig. 3B, left dot plot) did not lyse UKBF-11; giving evidence for the WT1-specific lysis of UKBF-11 by the autologous WT1-specific CTL line. Further WT1-specific CTL did not lyse the allogeneic HLA class I–positive, but HLA-A*01–negative leukemia cell line TF-1 expressing similar levels of WT1 demonstrating that the presentation of WT1.317-327 peptide in the context of HLA-A*01 is necessary for the recognition of target cells. To further analyze the specificity of the WT1-specific CTL, we did cold-target inhibition assays (Fig. 3D). The lysis of UKBF-11 could be inhibited by the addition of unlabeled WT1 peptide–loaded autologous PBMC (cold target) but not by HIV peptide–loaded PBMC.

Spontaneous T cell responses against WT1.317-327 in patients with solid malignancies. We next tried to detect ex vivo functional T cell responses in four HLA-A*01 patients with lung cancer and the two patients with melanoma. In two of these six patients, T cells specifically secreting IFNγ in response to WT1.317-327 with frequencies of 0.3% and 0.5% of CD3+CD8+ T cells were observed ex vivo (Fig. 4).

Discussion

The search for new T cell epitopes in known tumor antigens using the classical reverse immunology strategy has led to the identification of numerous T cell epitopes, most of them restricted to HLA-A*0201, but this approach has the disadvantage of laborious CTL generation against multiple peptides (40). In this study, we show the feasibility of a modified reverse immunology strategy to more rapidly identify epitopes combining epitope prediction, ex vivo screening for the recognition of candidate epitopes by T cells from patients with spontaneous immune responses, proteasomal digestion pattern analysis to show epitope processing, and the generation of epitope-specific CTL from regulatory T cell–depleted PBMC. Using this procedure, we could identify a first HLA-A*01–binding epitope from the tumor antigen WT1, i.e., WT1.317-327.

Using the prediction model, SYFPEITHI, various epitopes, mostly restricted to HLA-A*0201, could be identified (41–43). However, the SYFPEITHI epitope prediction algorithm, as well as the other available databases for epitope prediction, can only indicate candidate epitopes with a certain likelihood to represent naturally processed epitopes. In order to improve the likelihood of predicting epitopes, we screened candidate epitopes for recognition by T cells from patients likely to display spontaneous immune responses. One of the five candidate epitopes, i.e., WT1.317-327, showed high spontaneous immunogenicity as it was recognized by 5 of 14 patients with hematologic malignancies. To show natural processing of the identified peptide, WT1.317-327, we analyzed the proteasomal cleavage of a precursor peptide. Proteasomal digestion of precursor epitopes has already been used successfully in previous studies to identify HLA-A*0201–restricted epitopes from the tumor antigens PRAME and SSX2 (27, 44). Peptides presented on MHC class I molecules are predominantly generated by proteasomal digestion. The majority of mammalian cells contain constitutive proteasomes, whereas professional antigen-presenting cells or cells exposed to IFNγ express immunoproteasomes, which have different catalytic subunits resulting in a reduced production of several peptide tumor antigens (45, 46). The constitutive proteasome is able to generate both termini of an epitope, whereas the immunoproteasome mostly produces an NH2-terminally extended precursor (27, 28, 47). NH2-termini are subsequently trimmed to their final length by aminopeptidases in the cytoplasm or endoplasmatic reticulum (47). In accordance with these findings, our study shows the generation of a NH2-terminally
extended precursor peptide WT1.313-327 by the immunoproteasome. Although we failed to show the processing of peptide WT1.317-327 by the constitutive proteasome, we observed the cleavage of both COOH- and NH2-terminal amino acids of WT1.317-327, suggesting that the peptide WT1.317-327 could also be generated by the constitutive proteasome. The failure to show peptide WT1.317-327 in our study most likely results from disulfide bridging as the peptide contains cysteine.

Finally, we showed that WT1.317-327 CTL with sufficient avidity to efficiently kill autologous WT1 expressing HLA-A*01+ tumor cells could be generated from the peripheral blood of patients with melanoma. In accordance with recent studies of NY-ESO-specific CD4+ T cell responses in cancer blood of patients with melanoma. In summary, the strategy selected for epitope identification in this study was shown to be a reliable approach for identifying an immunogenic T cell epitope from the tumor antigen WT1. Such epitopes are of great interest for the analysis of T cell responses in tumor patients. Importantly, this epitope broadens the applicability of WT1-based treatment strategies.

References


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