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Definition of TCR Epitopes for CTL-Mediated Attack of Cutaneous T Cell Lymphoma

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Therapeutic vaccination against cutaneous T cell lymphoma (CTCL) requires the characterization of cancer cell-specific CTL epitopes. Despite reported evidence for tumor-reactive cytotoxicity in CTCL patients, the nature of the recognized determinants remains elusive. The clonotypic TCR of CTCL cells is a promising candidate tumor-specific Ag. In this study, we report that the clonotypic and framework regions of the TCRs expressed in the malignant T cell clones of six CTCL patients contain multiple peptides with anchor residues fitting the patients’ MHC class I molecules. We demonstrate that TCR peptide-specific T cells from the blood of healthy donors and patients can be induced to become cytotoxic effectors after repeated stimulation with 6 of 11 selected peptides with experimentally proven affinity for HLA-A*0201. Importantly, 4 of these 6 CTL lines reproducibly recognize and lyse autologous primary CTCL cells in MHC class I/CD8-dependent fashion. These tumoricidal CTL lines are directed against epitopes from V, hypervariable, and C regions of TCRα. We therefore conclude that recombined as well as V framework regions of the tumor cell TCRs contain predictable epitopes for CTL-mediated attack of CTCL cells. Our data further suggest that such peptides represent valuable tools for future anti-CTCL vaccination approaches.

Cutaneous T cell lymphomas (CTCLs) form a heterogeneous group of lymphoproliferative disorders with poorly understood etiology. Mycosis fungoides and its leukemic variant, Sézary syndrome, represent the vast majority of all CTCLs. Mycosis fungoides is characterized by an accumulation of clonal CD3+CD4+CD7− T cells with memory phenotype in the skin resulting in patches, plaques, and tumors (1, 2). Disease progression is associated with the loss of T cell epidermotropism and, as the result, the spreading of the tumor cells throughout the body (3). Despite the general relevance of this pattern of disease progression, marked variations between individual patients have been noted with regard to onset, time course, and extent of systemic spread. Indirect evidence exists that the occurrence of T cell-mediated antitumor immunity correlates with stable, rather than rapidly progressing disease (4–7). Eventual treatment responses in CTCL patients may also depend on the presence of CD8+ T cells, which expand with induced disease remission (4). MHC class I-restricted, tumor-reactive CTLs can be grown in vitro from the blood and skin lesions of CTCL patients (5–7). However, no conclusive evidence about the peptide epitopes recognized by these CTLs exists. In depth understanding of the in vivo displayed CTL epitopes will be crucial for the design of strategies aiming at boosting or eliciting protective cell-mediated tumor immunity against CTCL. Candidate epitopes may include members of the tumor/testis family of cancer-related Ags recently identified by the SEREX technology (8), a nonidentified tumor Ag that shares T cell recognition properties with a recently developed mimotope peptide (9), and the TCR of the tumor cell (10).

The mechanism of somatic gene rearrangement produces functional T and B cell receptors (BCRs). Thus, each clonal descendant of a malignant B or T cell is characterized by the expression of these unique proteins as well as peptide sequences derived from there. Immunogenic epitopes for CTL in the idiotypic and framework regions of lymphoma BCRs have been identified and successfully used as immunogens for the elicitation of cytotoxic T cell responses in the form of full protein Ag (11), Id-pulsed dendritic cells (12, 13), synthetic peptides (14), or DNA encoding the idotypic protein (15) or artificial minigenes (16). In analogy to the BCR in B cell lymphoma, the TCR of a malignant T cell clone contains tumor-specific sequences that could be used for immunization.

TCR-directed cytotoxic immune responses were in fact described. Anti-idiotypic CD8+ T cells were induced by vaccination with apoptotic T cells and were shown to control myelin basic protein-reactive Th cell clones in multiple sclerosis (17, 18) and experimental allergic encephalomyelitis (19). Previously, a peptide with partial sequence homology to an idiotypic TCRβ region was eluted from MHC class I complexes expressed by CTCL cells (10). Whether T cells specific for the corresponding natural peptide can kill CTCL cells remains to be resolved. To date, the potency of anti-idiotypic T cells to control T cell neoplasms was investigated.

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3 Abbreviations used in this paper: CTCL, cutaneous T cell lymphoma; βm, β2-microglobulin; BCR, B cell receptor; EGFP, enhanced green fluorescent protein; HBc, hepatitis B virus core protein; HV, hypervariable region; MFI, mean fluorescence intensity; moDC, monocyte-derived dendritic cell.

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only in rodents that were inoculated with high passage tumor cell lines. In these studies, anti-TCR immunity against a murine thymoma cell line was induced with recombinant TCR protein (20, 21) and TCR-encoding adenovirus constructs (22). Additionally, the observation of proteasomal TCRα degradation after TCR misassembly (23, 24) and after Ag-induced TCR triggering (25) suggests that certain TCR peptides can end up bound to MHC class I molecules. This, however, constitutes only one prerequisite for T cell-mediated cytotoxicity. The reliable induction of protective cytoxic anti-TCR immune responses could be complicated for several reasons. In contrast to B cells, T cells enter the thymus, and consequently the development of central tolerance to many TCR-derived epitopes appears possible. Furthermore, most T cell lymphomas lack costimulatory signals and may induce peripheral tolerance in TCR-specific T cells.

We combined molecular, cell biologic, and bioinformatic techniques for the identification of candidate tumor-specific TCR epitopes and used these epitopes to study whether MHC/peptide-directed cytotoxic T cell responses can be elicited in CTCL patients and healthy control individuals. Our results confirm and extend the previous observation that TCR Id-reactive CD8+ T cells can be isolated from CTCL patients (10). Beyond that, we demonstrate that besides truly clonotypic regions, framework regions of the tumor cell TCRs also contain peptides that are recognized by autologous CTLs. Importantly, we show that a subset of the predicted peptides is also displayed by tumor cells, as evidenced by peptide-specific lysis of tumor cells by in vitro generated CTL lines. Thus, our present study supports the potential of TCR peptide-based immunotherapy in patients suffering from CTCL.

Materials and Methods

Patients

Heparinized blood (n = 32) and lesional skin (n = 5) from CTCL patients and blood of healthy HLA-A*0201 donors (n = 6) were obtained after informed consent. Diagnosis of mycosis fungoides or Sézary syndrome was based on histology, immunohistology, and laboratory findings, according to the European Organization for Research and Treatment of Cancer classification for cutaneous lymphomas (3). None of the patients had evidence of acute or persistent hepatitis B virus infection.

Cells and cell lines

PBMCs and T cells were prepared, as described (26). Briefly, heparinized blood was diluted 1:2 and sedimented over Ficoll-Paque PLUS (Pharmacia, Uppsala, Sweden) at 400 × g for 30 min at room temperature. Erythrocytes were removed by osmotic shock in a buffer containing 0.15 M NH4Cl, 1 mM KHCO3, and 0.1 mM EDTA, followed by thorough washing in cold PBS (Life Technologies, Paisley, Scotland). T cells were enriched by immunomagnetic depletion (MACS; Miltenyi Biotec, France), followed by immunomagnetic depletion (MACS; Miltenyi Biotec, Bergisch-Gladbach, Germany). The resulting cell population contained routinely >95% CD3+ T cells. Biopsies (6 mm punch) were minced in RPMI 1640 (Life Technologies). Cells were squeezed out by manually applying mechanical pressure. Debris was removed by filtering the resulting cell suspension through a 40-μm cell strainer (BD Labware Europe, Le Pont De Claix, France). Cells were washed twice in RPMI 1640 and analyzed. The viable cell populations contained between 17.2 and 96.8% of CD7+ and CD8+ cells from blood or lesional T lymphocytes. Immature monocyte-derived dendritic cells (moDCs) were generated, as previously described (27). Briefly, monocytes were purified by panning PBMC in 50-ml plastic bottles for 90 min at 37°C, followed by thorough washing and removal of the nonadherent cells. The adherent cell fraction was incubated for 7 days in X-VIVO 15 medium (BioWhittaker Europe, Verviers, Belgium) containing GM-CSF (800 U/ml; Novartis, Basel, Switzerland) and IL-4 (1000 U/ml; Genzyme Diagnostics, Cambridge, U.K.). EBV-B cells were generated, as described (28). HLA-A*0201 T2 cells (29) were purchased from American Type Culture Collection (Manassas, VA).

Flow cytometry analysis

For three-color immunolabeling, 5 × 104 cells were resuspended in 50 μl containing fluorochrome-conjugated mAbs and incubated for 30 min on ice. Following two washes in PBS, at least 1 × 104 cells were analyzed on a FACScan flow cytometer (BD Immunocytometry Systems, San Jose, CA). Anti-CD3 (Leu-4) PerCP, anti-CD4 (Leu-3a) PerCP, anti-CD7 (Leu-9) FITC, or PE (BD Biosciences, San Jose, CA), and a panel of anti-TCR Abs composed of anti-TCRαv2 (F1) FITC, anti-TCRαv12.1 (6D6) FITC, anti-TCRβ (8F10) FITC, anti-TCRβ5.1 (Immu222) FITC, anti-TCRβ5.3 (W112) FITC, anti-TCRβ6.7 (OT145) FITC, anti-TCRβ13.1 + 13.3 (BA13) FITC (all from Serotec, Oxford, U.K.), anti-TCRβ (BL17.3) PE, anti-TCRβ2 (MPD25) FITC, anti-TCRβ5.2 (36213) FITC, anti-TCRβ7 (ZOE) FITC, anti-TCRβ8 (56C5.2) FITC, anti-TCRβ9 (FIN9) PE, anti-TCRβ11 (C21) FITC, anti-TCRβ12 (VER2.32.1) FITC, anti-TCRβ13.1 (Immu213) FITC, anti-TCRβ14 (Immu214) FITC, anti-TCRβ15 (Immu215) FITC, anti-TCRβ17 (E17.5F3) FITC, anti-TCRβ18 (BA62.6) PE, anti-TCRβ20 (ELL.1.4) FITC, anti-TCRβ21.3 (IG125) FITC, anti-TCRβ22 (Immu546) FITC, and anti-TCRβ23 (AF23) PE (all from Immunotech) were used to screen for expanded T cell populations.

Molecular identification of the TCR of malignant clones

A total of 1 × 106 purified T cells from blood or lesional skin was lysed, and mRNA was isolated using oligot(IgG)-bound magnetic beads (Roche Diagnostics GmbH, Mannheim, Germany). cDNA was generated by reverse transcription (first strand cDNA synthesis kit for RT-PCR; Roche) and amplified with individual sense primers in the V regions (Vα1–22, Vβ1–24) and C region-specific (Cu, Cβ) antisense primers (TCR typing amplimer kit; Clontech, Palo Alto, CA) in 30 cycles with an annealing temperature of 50°C. Southern blot analysis of all TCRα and TCRβ amplimers using α-32P-dATP-labeled C region-specific probes (Ca, 5'- GTACACGGCAGGGTCAGGGTTCTGGATAT-3'; Cβ, 5'- CTTTTGGGTGTGGGAGATCTCTGCTCTGGA-3') was performed, as described previously (30).

For TCR GeneScan analysis, the antisense primers were 3' terminally ligated to FAM (VBC Genomics, Vienna, Austria). Analysis was performed on an ABI PRISM 377 DNA Sequencer (PE Biosystems, Warrington, U.K.) supported by the GeneScan 3.1 software (PE Biosystems). The TCR GeneScan protocol allows the detection of T cells of defined hypervariable (HV) region lengths and defined V region usage above a threshold representation of 0.5–1% (data not shown). For sequencing of TCRs, V region-specific PCR-derived amplimers were ligated into pCR2.1 (Invitrogen, Carlsbad, CA), subcloned in competent INVaF-Top 10 Escherichia coli (both from Invitrogen), and resolved by dye terminator cycle sequencing (PE Biosystems).

Peptide prediction

SYPFEITHI (http://syfeithi.de/) was used to predict MHC class I affinities of TCR peptides.

MHC class I peptide-binding studies

Reconstitution assay. Peptides were from MWG-Biotech (Ebersberg, Germany). Acid elution of peptides from MHC class I on T2 cells was performed, as described (31). Briefly, 2 × 106 cells were incubated for 90 s on ice in a buffer containing 131 mM citric acid and 66 mM NaHPO4, adjusted to a pH of 3.3 with 1 N NaOH, washed twice, and resuspended in IMDM (BioWhittaker Europe) at a concentration of 1 × 106 cells/ml.

T2 cells (100 μl) were incubated with 1 μg/ml β2-microglobulin (β2m; Sigma-Aldrich, St. Louis, MO) and peptide (0.03–10 μg/ml) for 4 h at 26°C. Samples were tested for W6/32-PE (Immunotech) immunoreactivity and reconstitution of W6/32 immunoreactivity was calculated as: [(MFI acid-treated cells/peptide/μg/ml) – (MFI acid-treated cells/μg/ml)]/ (MFI acid-treated cells/μg/ml) × 100.

Competition assay. Acid-stripped T2 cells (100 μl) were reconstituted with β2m and various concentrations of TCR peptides in the presence or absence of a FITC-labeled hepatitis B virus core protein (HBC)-derived peptide (0.5 μg/ml) (32). Staining was performed with PBS, and analyzed by FACS. The competition of TCR-derived peptides with the binding of HBe FITC to HLA-A*0201 was calculated as: [(MFI acid-treated cells/μg/ml/μg/HBe FITC) – (MFI acid-treated cells/peptide/μg/ml/μg/HBe FITC)]/ (MFI acid-treated cells/μg/ml/μg/HBe FITC) × 100.

Generation of peptide-specific CTL lines

T2 cells (2 × 106) were stimulated with 0.5 μg/ml peptides or TAP1 deficient MoDCs and B cells for 5 days in X-VIVO 15 medium (BioWhittaker Europe, Verviers, Belgium) containing GM-CSF (800 U/ml; Novartis, Basel, Switzerland) and IL-4 (1000 U/ml; Genzyme Diagnostics, Cambridge, U.K.). EBV-B cells were generated, as described (28). HLA-A*0201 T2 cells (29) were purchased from American Type Culture Collection (Manassas, VA).
1 μg/ml β2-m for 4 h and washing, cells were incubated with HLA-A*0201 T cells (1:1) in RPMI 1640 containing 10% FCS (Life Technologies), 100 U/ml human rIL-2 (Stratham Biotech, Hanover, Germany), 5 ng/ml human rIL-7 (PromoCell GmbH, Heidelberg, Germany), and 5 ng/ml human rIL-12 (R&D Systems, Abingdon, U.K.). T cells were restimulated with peptide-pulsed moDCs or T2 cells in weekly intervals. After two rounds of restimulation, routinely >95% of the cells were CD3+ T cells and 73–94% expressed CD8. If NK cells were detected, they were removed by immunomagnetic depletion. T cell lines were tested after each round of stimulation for specific cytotoxicity in Eu³⁶ release assays using peptide-pulsed target cells (see below). The frequency of peptide-specific T cells was determined using dimeric HLA-A2:Ig fusion proteins (BD Biosciences) following the manufacturer’s directions.

Transfection

A full-length TCRα transcript of the malignant T cell clone of patient 1 (Vα21-Jα51-Cα) was amplified using the primers 5'-ATGGCCGATGCCTCCTGGGG-3' and 5'-TTGCGATCTCATCGTGA-3' (30 cycles, annealing temperature 50°C) and inserted into pCMs-EGFP (enhanced green fluorescent protein) (Clontech). For transfection, 1 × 10⁶ cells were electroporated with 50 μg of plasmid with or without insert (Gene Pulser; Bio-Rad Life Sciences, Hercules, CA). The instrument was set to 390 V/m and 960 μF; the gap size of the cuvette was 0.4 mm. After electroporation, cells were incubated for 10 min at room temperature, washed twice with RPMI, and incubated for 3 days. Transfection efficacy on day 3 was up to 70% (mean 40%) for control-transfected and up to 65% (mean 25%) for TCR-transfected EBV-B cells. Immunoblotting with the anti-TCR α mAb 3AB (Serotec) was performed to confirm protein expression (33). EGFP-expressing transfectants were purified using a FACStar™ cell sorter (BD Biosciences).

Eu³⁶-labeled target cells were prepared as described (34). Briefly, 5 × 10⁶ cells were incubated on ice for 15 min in 1 ml labeling buffer (0.125 mM EuCl₃, 0.625 mM Na₂N-bis-(2-hydroxyethyl)amine)-ethyl)glycine. After addition of 20 μl of 0.1 M CaCl₂, cells were washed three times in a buffer containing 10 mM D⁻ glucone and 2 mM CaCl₂ and incubated for 1 h in serum-free IMDM. Labeled viable cells were isolated over 50% Percoll (Pharmacia).

A total of 2 × 10⁵ target cells was incubated for 4 h with CTLs at various E:T ratios. A total of 20 μl of the supernatant was mixed with 200 μl Enhancement Solution (Wallec Oy, Turku, Finland). Eu³⁶ release was quantified by measuring time-resolved fluorescence on a 1234 DELFIA Fluorometer (Wallec Oy). Specific target cell lysis was calculated as: (experimental release - spontaneous release)/(maximum release - spontaneous release) × 100.

Maximum release was induced by incubation with 2% Triton X-100 (Sigma-Aldrich).

Where indicated, CTL were preincubated with 6 μg/ml anti-CD4 (VIT-4), anti-CD8 (VIT-8), or control mAbs for 2 h, CTL1 cells with anti-MHC class I (W6/32) or isotype control (mAbs kindly provided by Dr. O. Maджic, Institute of Immunology, University of Vienna Medical School). Abs stayed present through the whole assay. Cold target inhibition was performed by coincubating effector and Eu³⁶-labeled target cells in the presence of nonlabeled T cells pulsed with 10 μg/ml TCR or HBc peptide and 1 μg/ml β2-m. The percentage of specific cold target inhibition was calculated as: 100 – (percentage of lysis of tumor cells in the presence of specific competitors/percentage of lysis of tumor cells in the presence of nonspecific competitors) × 100.

Statistical significance

STATISTICA 5.0 was used for Mann-Whitney U test and Spearman Rank coefficient test.

Results

Identification of clonally expanded T cells and their TCRs

We obtained skin biopsies and peripheral blood from 5 CTCL patients. In 27 additional CTCL patients, we had access to peripheral blood cells only. To identify the TCR of the clonal T cells of our patients, we prepared cDNA from skin and blood mononuclear cell suspensions and performed a TCR V region-specific RT-PCR analysis with individual primers for 22 Vα and 24 Vβ elements vs consensus primers that bind nonpolymorphic sequences in the C regions of the TCRα and TCRβ chains. Typically, 1–7 prominently expressed V region-defined TCR transcripts were identified in skin and blood samples by Southern blotting (Fig. 1A). These transcripts were further subjected to TCR GeneScan analysis. In all skin samples, TCR GeneScanning revealed uniformly sized transcripts for one of the various Vα- and Vβ-defined TCR amplicons (Fig. 1B). The other prominently expressed TCRs in the RT-PCR contained cDNA species of different length, and thus were of multiclonal origin. To identify the sequences of the clonal TCRs and to control the results of the TCR GeneScan analysis, the most abundant amplicons obtained in the RT-PCR assay were ligated into a cloning plasmid, and multiple subclones (7–15 per amplicon) were sequenced. A single predominant TCRα cDNA sequence and a single predominant TCRβ cDNA sequence were identified in each of the five skin samples (Fig. 1C, Table I). In three of these five patients, the skin-derived clonal TCR sequences were also identified in the peripheral blood. In the remaining two patients, no evidence for TCR clonality was noted in the peripheral blood, even when we tried to amplify TCR transcripts containing idiotypic regions of the clonal TCR chains identified in the skin (detection limit >1 Id-expressing cell/1000 cells; data not shown).

In the blood of one patient from whom no skin biopsy was available, a clonal TCR was identified (Table I). In the remaining patients, the disease was restricted to the skin or the level of the CTCL cell dissemination was below our level of detection. We used V region-specific mAbs to directly demonstrate that T cells that express the V region of the identified TCR display the typical CD7⁺ CD4⁻ CD8⁺ immunophenotype of CTCL cells (Fig. 1D).

Identification of TCR peptides with binding affinity for patients’ MHC class I molecules

We next asked whether the identified clonal TCRα and TCRβ chains contain peptides that can bind the MHC class I molecules expressed by the patients. Furthermore, we analyzed whether the number and quality of MHC-fitting TCR peptides are different in the TCRs of CTCL clones and V region-matched normal T cells from the same donors. We deduced the protein sequences of the identified CTCL and subjected them to SYFPEITHI (35), an anchor position-based prediction model for MHC class I-binding peptides. Peptides for one to four MHC class I alleles/patient (mean 2.2/patient) could be detected using this algorithm.

The search for TCR peptides with a significant SYFPEITHI score (i.e., >10) resulted in a total of 3175 peptides. Of these, 869 were HLA-A*0201-binding moieties from two HLA-A*0201+ CTCL patients. Only the 2% top-scoring peptides have a substantial affinity for HLA-A*0201 that was defined (35), an anchor position-based prediction model for MHC class I-binding peptides. Peptides for one to four MHC class I alleles/patient (mean 2.2/patient) could be calculated using this algorithm.

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 Eleven high-scoring peptides from the V, HV, and C regions of TCRs and TCRβ were used for the experimental verification of calculated HLA-A*0201-binding properties. BLAST searches confirmed that all selected peptides were TCR-specific. First, we checked the ability of TCR peptides to form ternary MHC class I
complexes with nonoccupied MHC class I on T2 cells in the presence of free \( \text{Hb} \). Second, we measured the ability of the peptides to compete with MHC class I binding of a fluorochrome-labeled reference HBc peptide on T2 cells (32). As shown in Fig. 2B, MHC class I binding of TCR peptides and their competitor activity are interrelated functions (Spearman rank coefficient: 0.52, \( p < 0.07 \)). Thus, all predicted TCR peptides bound MHC class I. TCR peptides, their key features, and results of both assays are compiled in Table II.

To gain evidence whether the quality of MHC class I-binding peptides differs between TCRs from normal and malignant T cells, we compared the mean SYFPEITHI scores of predicted peptides from the HV regions of the TCRs of CTCL cells (6α-, 6β-chains) and those of V region-matched TCRs of autologous normal T cells (26α-, 38β-chains). No statistical difference in the density and the mean calculated binding ability of predicted MHC class I-fitting peptides from TCRs of CTCL cells and normal T cells was noted (data not shown). Thus, it appears unlikely that CTCL clones are subject to immune selection based on the MHC class I-binding properties of idiotypic TCR sequences.

TCR peptide-specific CTLs can be generated from the peripheral blood of CTCL patients and healthy donors

We next asked whether TCR peptides with proven HLA-A*0201-binding ability can be used to generate TCRα and TCRβ peptide-specific CTLs from the peripheral blood. Peripheral blood T cells were purified from healthy HLA-A*0201+ donors and the two HLA-A*0201+ CTCL patients who displayed circulating tumor
patients and control subjects and assayed for cytotoxic activity with peptide/HLA-A2 complexes. Inhibition (% inhibition) assay significantly reduced target cell killing by patient and control CTLs as seen in Fig. 4B. Interestingly, C region-specific CTLs could be reproducibly generated against one of two Ce peptides (Ce1), but not against two Cβ peptides (Cβ1, Cβ2). Moreover, when we titrated the peptide concentration used for pulsing of target cells, we observed that patient and control CTLs gave a similar dose-response profile (data not shown). This together with the results shown above demonstrates that TCR-specific CTLs of similar specificity and avidity can be elicited in CTCL patients and healthy controls.

**TCR peptides are recognized in a CD8/MHC class I-restricted fashion and are generated by the proteolytic machinery of the cell**

We next asked whether TCR-specific CTLs lyse peptide-pulsed targets in a CD8-dependent/MHC class I-restricted proteolysis-dependent fashion. In selected experiments, direct evidence for TCR peptide/HLA-A2 specificity of CTL TCRs was obtained in MHC class I dimer-binding studies. As exemplified by a Vα13/2-specific CTL line, a significant portion of CD8+ T cells bound HLA-A2/TCR peptide dimers, but did not react with MHC class I dimers associated with irrelevant peptide (Fig. 3B). Inclusion of anti-CD8 and anti-MHC class I mAbs, but not anti-CD4 mAbs during the 4-h Eu3+ release assay significantly reduced target cell killing by various TCR peptide-specific CTLs (Fig. 4A, data not shown). Depletion of CD4+ T cells from bulk CTL lines lowered the E:T ratio required to obtain substantial target cell destruction (Fig. 4B). The inability of three of three TCR-specific CTL lines to lyse MHC class I-deficient Daudi cells demonstrated the absence of NK cell or NK cell-like cytotoxic activity (data not shown). To investigate whether the induced expression of TCR chains in target cells allows for the generation and presentation of the peptides used for the generation of CTL lines, we transfected EBV-B cells with constructs containing a full-length TCRα chain of the CTCL cells of patient 1 (Vα21-Jα51-Cα) or with empty vector as control (Fig. 4C). The expression of the TCRα chain was confirmed in Western blotting experiments (Fig. 4D). FACS-sorted transfectants were used as targets for V and C region-specific autologous CTL lines. As seen in Fig. 4E, both CTL lines efficiently lysed TCR-transfected, but not mock-transfected EBV cells. These data suggest that TCR peptide-specific cytotoxicity is mediated by CD8+ T cells.

**Table 1. Phenotype, frequency, and TCR usage of the malignant T cell clones**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Phenotype</th>
<th>o.p.</th>
<th>Frequency (skin)</th>
<th>Frequency (blood)</th>
<th>TCRα</th>
<th>TCRβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD3+CD4+CD7+β13+</td>
<td>9</td>
<td>95–99%</td>
<td>55–99%</td>
<td>Vα21-ARTSYDV1FPGTSLV1P-Cα</td>
<td>Vβ17-MVCRGNNKLFRGSGTQLSVL-β1</td>
</tr>
<tr>
<td>2</td>
<td>CD3+CD4+CD7+β17+</td>
<td>10</td>
<td>13%</td>
<td>38–52%</td>
<td>Vα6-PRGNSNLPVGKTISSLV1A-Cα</td>
<td>Vβ17-LFGQGTLYPVFGTGLTTLV-β2</td>
</tr>
<tr>
<td>3</td>
<td>CD3+CD4+CD7+β12+</td>
<td>55</td>
<td>80–88%</td>
<td>&lt;1–71%</td>
<td>Vα4-LRQVRETQGERTLQTVNFD1-Cα</td>
<td>Vβ17-DSQGAGNTGEFLWVRRLTVL-β2</td>
</tr>
<tr>
<td>4</td>
<td>CD3+CD4+CD7+β13.1+</td>
<td>50</td>
<td>n.a.</td>
<td>&lt;1–79%</td>
<td>Vα13-LGAGGQYFGTGLTSLV1P-Cα</td>
<td>Vβ13-PWGQIFYFGTSGTTLTVV-β3</td>
</tr>
<tr>
<td>5</td>
<td>CD3+CD4+CD7+ββ2+</td>
<td>17</td>
<td>39%</td>
<td>&lt;1%</td>
<td>Vα3-ATDLGQGFLSELYLWELGKLQVF1-Cα</td>
<td>Vβ2-CSFRAQNGEYFGTRTLTV-β2</td>
</tr>
<tr>
<td>6</td>
<td>CD3+CD4+CD7+ββ5.1+</td>
<td>13</td>
<td>30–97%</td>
<td>&lt;1%</td>
<td>Vα19-ASTSWGKFQFQGATQVVT-Cα</td>
<td>Vβ5-LWGGQEQYFGTGLTTLTV-β2</td>
</tr>
</tbody>
</table>

* Immunophenotype of the malignant clone as determined by FACS analysis.
* Observation period with skin and blood sampling in months.
* Frequency of malignant cells among CD3+ lymphocytes in single cell suspensions from lesional skin. n.a., No biopsy available.
* Frequency of malignant cells among CD3+CD4+ lymphocytes in the patients' blood. Asterisks indicate that the malignant clone could not be detected by HV region-specific PCR.
* TCRα (e) and TCRβ (f) chains on the malignant T cell clones of the patients. The HV regions are shown in single letter amino acid code; the flanking V and C regions are indicated.

**FIGURE 2.** Distribution and MHC class I-binding properties of TCR peptides. A, Potential high affinity HLA-A*0201-binding peptides are concentrated in the V and HV regions of the TCR. MHC class I-binding peptides were predicted in the TCRα and TCRβ chains of six patients. The top-scoring 2% of the peptides were grouped according to their position on the TCR. Mean peptide densities in the V, HV, and C regions are shown for the peptides binding to all computable MHC class I alleles of all six patients (open bars) and for the HLA-A*0201-binding peptides from two HLA-A*0201+ patients (filled bars). For comparison, peptide densities in HV regions from V region-matched normal T cells from the respective patients are shown. SEM is indicated. B, Correlation between the ability of TCR peptides to reconstitute and to inhibit the formation of ternary HLA-A*0201 complexes. Inhibition (y-axis) and reconstitution (x-axis) values were obtained with peptide concentrations of 10 μg/ml. The linear regression between both sets of data is depicted.
cells. Endogenous peptide generation and loading in TCR-expressing cells produce the Ags recognized by the CTLs.

**TCR peptide-specific CTL lines lyse primary CTCL cells**

We next purified CTCL cells from our HLA-A2\(^+\) patients by immunomagnetic depletion to assess the possible lytic activity of TCR-specific CTLs for tumor cells. Skin-derived tumor cells of patient 1 were lysed reproducibly by all three CTL lines that recognize epitopes on the TCR\(\alpha\) chain, but not by HBc-specific CTLs (Fig. 5A). In control experiments, the TCR\(\alpha\) chain-specific CTL lines and control HBc-specific CTLs were equally effective in killing peptide-pulsed targets (Fig. 5B). Similarly, tumor cells of patient 4 were killed by TCR-specific CTLs. In this patient, the V\(\alpha\)131-specific CTL line almost completely eradicated the CTCL cells at a E:T ratio of 33:1 (Fig. 5C). The other TCRV (V\(\alpha\)132, V\(\beta\)13)-specific CTL lines, HBc-specific control CTLs, and three additional CTL lines without peptide-specific cytolytic activity failed to lyse the purified tumor cells (data not shown). Thus, it appears that certain V and HV region peptides, in particular the V\(\alpha\)131 peptide, and C region peptides are constantly generated and presented by MHC class I of CTCL cells. In line with this assumption stands the notion that inhibitors of proteasome function as well as anti-CD8 and anti-MHC class I, but not anti-CD4 mAbs reduce the killing of CTCL cells by V\(\alpha\)131-specific CTLs (Fig. 5D, data not shown). Moreover, the V\(\alpha\)131 peptide has a proteasomal cleavage site at the C terminus and lacks disrupting internal cleavage sites, as revealed by the public domain algorithm MAPProC (36) (data not shown). To further ask whether peptides identical with the TCR peptides used for the elicitation of the CTL lines are displayed by the tumor cells, cold target competition experiments were performed. The addition of TCR peptide-bound cold targets resulted in a peptide-specific and dose-dependent reduction of tumor cell destruction by peptide-specific CTLs (Fig. 5E). These findings confirm that CTCL cells generate and present peptides that are identical or very similar to those predicted and used for the priming of CTLs.

**Discussion**

Advanced CTCL, like other lymphoproliferative malignancies, continues to pose serious clinical management problems, because only a small fraction of patients responds to standard radio- and/or chemotherapy treatments. One possible alternative to these broad cytotoxic measures is therapeutic vaccination. Such a strategy may involve immunotherapy directed against the Ag receptor of malignant T or B cells. To date, more information on the immunogenicity of Ag receptors expressed by cancer cells is available in B cell neoplasias than in T cell lymphoma/leukemia. Relevant immunogenic CTL epitopes have been mapped to the HV, the V, and even the C regions of lymphoma BCRs (14). Indeed, clinical vaccination studies with idiotypic BCR encouraging induce remissions in B cell lymphoma patients (13, 37).

Data on the immunogenicity of lymphoma TCRs are, however, scarce at the moment. Lack of immunogenicity, i.e., the absence of protective immunity against a thymoma inoculum, was observed after viral gene transfer of thymoma-derived full-length TCR in a murine study (22). Models that are based on the gene transfer of TCRs in which the murine C region was exchanged for the human homolog or on immunization with TCR protein plus adjuvant sufficiently produce CD8\(^+\) T cell-dependent tumor immunity (21, 22). Thus, TCRs can be powerful immunogenic Ags in lymphoma, provided that the relevant TCR epitopes are presented in an altered form, possibly in the context of danger signals (21). Nevertheless, we are still missing broad information concerning the relevant TCR epitopes. Such peptides must be generated by the proteolytic machinery of the tumor cells and recognized by CTLs of patients. This knowledge will be crucial for the design of tumor-specific vaccines.

In this study, we have used a series of TCR peptides that we identified bioinformatically in the \(\alpha\)- and \(\beta\)-chains of TCRs from CTCL patients and loaded them onto HLA-A2 molecules of dendritic cells. These cells and HLA-A2\(^+\) T2 cells were used to generate CTLs from

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**Table II. TCR-derived peptides used in this study**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Origin (c)</th>
<th>Sequence (a)</th>
<th>Condition (b)</th>
<th>CTL Lines (all) (c)</th>
<th>CTL Lines (patients) (c)</th>
<th>Competition (c)</th>
<th>Reconstitution (c)</th>
<th>Score (c)</th>
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<td>V(\alpha)13 (63)</td>
<td>HBc</td>
<td>FLPSFceFPSV</td>
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<td>20% (2/10)</td>
<td>25% (2/10)</td>
<td>20% (2/10)</td>
<td>25% (2/10)</td>
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<tr>
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<tr>
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<td>25% (2/10)</td>
<td>20% (2/10)</td>
<td>25% (2/10)</td>
<td>20% (2/10)</td>
</tr>
</tbody>
</table>

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\(a\) Position of the peptide in the TCR (FR, V framework region; CDR, idiotypic region; C, C region; HV, HV region).

\(b\) Numbers in the subscripts indicate the patient from which the parental TCR of the peptide originates; "c" indicates that the peptide is derived from a nonpolymorphic sequence in the TCR C region.

\(c\) Overall success rate for the elicitation of peptide-specific MHC class I-restricted CTL lines. The numerator of the fraction in parentheses gives the number of independently generated T cell lines with specific cytotoxicity against the respective peptide. The number of attempts to generate such lines is given in the denominator.

\(d\) The number of attempts to generate such lines is given in the denominator.

\(e\) Cytotoxicity against the respective peptide. The number of attempts to generate such lines is given in the denominator.

\(f\) Cytotoxicity against the respective peptide. The number of attempts to generate such lines is given in the denominator.

\(g\) The number of attempts to generate such lines is given in the denominator.

\(h\) The number of attempts to generate such lines is given in the denominator.
the blood of patients suffering from advanced CTCL. With this protocol, we bypassed the apparent poor immunogenicity of tumor cell TCRs and could reliably induce robust CTL responses against certain TCR epitopes. CD8+ T cells were the main TCR peptide-specific effectors, because: 1) TCR peptide-specific cytolytic activity was strongly enriched in the CD8+ T cell compartment of the T cell lines; 2) anti-CD8 and anti-MHC class I mAbs, but not anti-CD4 mAbs, inhibited peptide-specific cytolytic activity; and 3) a significant portion of

FIGURE 3. TCR peptide-specific CTLs can be elicited from peripheral blood T cells of healthy donors and CTCL patients. A, T cells of patient (pat.) 4 and of a healthy HLA-A2+ donor were stimulated three times in weekly intervals with peptide-pulsed APCs and tested in Eu3+ release assays for cytotoxicity. The two sets of CTL lines were handled in parallel. A total of $2 \times 10^5$ EBV-B cells each loaded with TCR peptides/β2m (filled bars), HBc peptide/β2m (gray bars), or β2m alone (open bars) were used as targets. HBc peptide-pulsed target cells were used as control for TCR peptide-primed CTLs, Cβ1 peptide-pulsed EBV-B cells for HBc peptide-primed CTLs. The E:T ratio was 33:1. Asterisks indicate CTL lines that lysed targets loaded with the relevant peptide significantly better than targets pulsed with control peptide and targets without exogenous peptide. Peptide-specific CTL generated from the patient and the healthy donor presented quantitatively and qualitatively similar results. The experiments were performed in triplicate; error bars represent SEM. B, Vα13/2 peptide-reactive CTLs obtained from patient 4 were incubated with HLA-A2:Ig dimers conjugated with either Vα13/2 or HBc peptide, washed, and counterstained with anti-CD8 mAb. The stained cells were analyzed by flow cytometry. CD8+ cells were gated and analyzed for their binding of peptide-conjugated dimers (filled histograms). Dimer without peptide was used as negative control (open histograms). The percentages of CD8+ T cells binding peptide-conjugated dimers are given in the panels.
CD8⁺ T cells within the lines bound MHC class I complexes containing the relevant TCR peptides, but not MHC class I complexes containing control peptides.

Our data clearly show that CTL responses directed against TCR epitopes of the malignant clone can be generated or at least amplified in lymphoma patients. It is conceivable that only CTL responses that are directed against epitopes not displayed by the malignant cells can be elicited in vitro, because T cells that recognize tumor-displayed epitopes might be anergic in vivo. In this case, one must expect that the repertoire of TCR-specific CTLs is different in healthy controls and patients. To explore this possibility, we raised a total of 58 T cell lines from HLA-A2⁺ healthy individuals and CTCL patients against various portions of the TCRs. Importantly, patients and controls could generate cytolytic T cell responses against the same set of TCR peptides. Among 11 TCR peptides tested, 6 peptides reproducibly allowed for the elicitation of TCR-specific CTL activity, while 5 were only poorly or not immunogenic. In contrast to previous results from T cell vaccination studies in multiple sclerosis (17), our work clearly proves that immunogenic TCR peptides are not restricted to the HV regions. Immunogenic TCR epitopes were scattered over the entire TCR and included peptides in the V, HV, as well as the C regions of the TCR. In fact, this broad scattering is reminiscent of the CTL epitope distribution on BCRs of lymphoma cells (14).

TCR peptides that were nonpermissive or permissive for CTL induction had similar calculated MHC class I-binding affinities, and were also equally effective in binding to MHC class I and competing for MHC class I binding with control peptides. Thus, the ability to induce CTL responses is not a function of the physicochemical properties of the peptides, but rather reflects the existence vs the lack of a significant peptide-specific TCR precursor population. Moreover, TCR peptide-specific CTLs from patients and controls displayed similar killing efficacies when we titrated the effector cell number and the TCR peptide loading of targets (data not shown). This excludes significant differences in TCR avidity between TCR-specific CTLs from controls and patients. It appears that TCR-specific CTLs are neither systemically deleted nor silenced in an irreversible manner in CTCL patients.

An apparent challenge to vaccination with MHC class I-binding peptides is to select those epitopes that are generated and displayed by the tumor cells themselves. Calculation models for proteasomal cleavage are not yet advanced enough to reliably predict the display of a given peptide. Moreover, alterations in the proteolytic machinery of lymphoma cells may further contribute to a poor predictability of TCR peptide display. To date, direct evidence for proteasomal degradation and MHC class I-dependent display of TCR epitopes is sparse. Huppa and Ploegh (24) showed that transfected TCRα is rapidly proteasomally degraded, and Berger et al. (10) have purified a MHC class I-bound nonapeptide from CTCL cells that is in five positions identical with a peptide sequence in the β-chain of the parental TCR. Our study has obtained novel persuasive evidence that TCRs expressed in transfectants and, more importantly, in primary lymphoma cells directly isolated from patients indeed display TCR peptides in the context of MHC class I. Our arguments derive from the results that: 1) full-length TCR-transfected target cells, but not empty vector-transfected control cells, are lysed by TCR peptide-specific CTLs; 2) TCR lysis of TCR-expressing targets is inhibited by compounds that specifically interfere with proteasomal degradation (data not shown); and, most importantly, 3) a subset of TCR peptide-specific CTLs lysed freshly isolated tumor cells from the skin and the blood of two CTCL patients. This lysis most likely involves recognition of the peptide used for CTL priming in the context of MHC class I on the surface of the

**FIGURE 4.** Presentation, recognition, and endogenous generation of TCR peptides. A, TCR peptide-specific lysis is inhibited by anti-CD8, but not by anti-CD4 mAbs. T cells from the blood of a healthy donor were primed with Vα13/1 or Vα21 peptide. CTL lines with specific cytoxicity for Vα13/1 (left panel) or Vα21 (right panel)-pulsed EBV-B cells were incubated with anti-CD4, anti-CD8, or isotype control mAbs, then their ability to lyse target cells pulsed with the respective peptide was assayed. Results are expressed as percent inhibition of target cell lysis by coreceptor blocking Abs as compared with lysis in presence of the isotype control. Zero percent inhibition corresponds to 15% (Vα13/1) or 16% (Vα21) specific lysis of peptide-pulsed EBV-B cells. B, Bulk (squares) or purified (circles, purity >95%) stimulated CD8⁺ T cells were assayed for cytoxicity against Vα21-pulsed (filled symbols) or nonpulsed (open symbols) targets at the E/T ratios indicated on the x-axis of the graph. C, EBV B cells of a healthy donor were transiently transfected with pCMS-EGFP/Vα21-Jo51-Co or with the pCMS-EGFP vector alone. The graph shows green fluorescence from nontransfected cells (open histogram, thin line), control-transfected cells (open histogram, thick line), and TCRα-transfected lines (filled histogram). D, Verification of transgene expression of TCR-transfected cells by immunoblotting with the anti-TCRα mAb 3A8. Bands corresponding to fully glycosylated TCRα (open arrowhead) and to coreglycosylated TCRα chain (filled arrowhead) can be discriminated. Faint bands with higher migration speed may correspond to nonglycosylated TCRα or to short-lived degradation products. E, Vα21-Jo51-Co and control-transfected EBV-B cells were sorted accordingly to their EGFP expression and were used as targets for autologous Vα21 (filled bars) and Cε1 (open bars)-specific CTLs at an E:T ratio of 10:1. For control purposes, the Vα21-specific CTL line was also assayed against Vα21 peptide-pulsed transfectants. SEM are indicated.
tumor cells, because cold target cells that express the relevant peptide-MHC class I complexes inhibit CTL lysis of the tumor cells efficiently and dose dependently.

It can be further concluded from our study that TCRα-derived peptides are to be preferred to TCRβ-derived peptides for the induction of T cell lymphoma-specific immunity. The evidences come from the observations that TCRα peptide-specific CTLs were more easily generated than TCRβ peptide-specific CTLs, and that CTLs that lyse tumor cells were exclusively directed against α-chain epitopes of the lymphoma TCR. This may be related to a bias due to the limited number of peptides and patients and awaits corroboration in larger cohorts. Nevertheless, it has become definitively clear that the epitopes on the TCRs that can be suitable lymphoma tumor Ags are not restricted to peptides from the complementarity-determining 3 regions of the lymphoma TCRs. To date, these issues were not addressed, because no peptide-mapping studies of TCR-reactive CTLs had been performed. Our data clearly show that apart from the truly clonotypic HV regions, the
TCRV regions contain relevant peptides for tumor recognition. The potential value of V region peptides is further supported by the fact that predictable high affinity MHC class I-binding peptides are far more numerous in the TCRV regions than in HV and the TCR regions. Our TCR sample allows the prediction that individual V regions will contain between two and three putative high affinity peptides, while only 0.76 peptides can be statistically expected in HV regions. The further observation that only a proportion of those peptides is actually displayed by lymphoma T cells further limits the feasibility of successful HV-restricted peptide vaccination. The use of V region-derived epitopes would cut back the time- and labor-intensive effort of defining clonotypic Ags for every patient. Shared pools of peptides can be determined for MHC class I-matched patients, with malignant T cell clones expressing the same TCRV regions.

The use of V region-derived peptides as vaccines could be problematic by endangering normal T cells that share V regions with the malignant clone. As previously demonstrated, control of T cells by V region-specific CTLs may be part of physiologic immunoregulatory circuits (19). It has been shown that only activated T cells are controlled by regulatory T cells recognizing TCR-derived determinants (18, 38). Thus, only the few recently activated T cells with the same V region as the target population are at risk of unintended destruction, while resting normal V region-defined T cells are excluded from possible harm.

In line, we also failed to observe destruction of Vε13-expressing nonactivated T cells in bulk T cell cultures primed with Vε13/I peptide-pulsed APCs in our own experiments (unpublished observation). Extensive immunophenotyping of the tumor cells used in this study revealed that CTCL cells express significant amounts of T cell activation Ags such as CD25 and HLA-DR (39) (data not shown). Thus, it is conceivable that TCR epitope display and consecutively successful TCR vaccination are limited to those lymphoma types that display functional features of T cell activation.

To further apply our findings in vaccination approaches against CTCL, studies will be necessary to establish the optimal conditions of Ag delivery for the activation of the patients’ TCR-specific CTLs. A strategy involving defined cytotoxic T cell epitopes is an auspicious alternative to immunization with apoptotic tumor cells or cell lysates, because it minimizes the potential harm of immune responses against unwanted epitopes. Apart from synthetic peptides, artificial minigenes encoding serial defined peptides separated by proteasomal recognition signals are promising immunogens as previously used in B cell lymphoma (16). Using full-length TCR as the immunogen holds the danger of inducing C region-specific responses. We show in this study that such undesirable CTL responses can indeed be elicited. This problem can be evaded by using defined epitopes or, alternatively, by exchanging the C region of the immunizing TCR to a xenogeneic C region (22).

In summary, we deliver the in vitro proof for the feasibility to elicit cytotoxic immune responses against defined MHC class I-binding epitopes from the V and rearranged regions of the TCR of CTCL cells. Although several hurdles still have to be overcome, data provided in this study will hopefully serve as a basis for epitope selection in future anti-CTCL vaccination trials.

Acknowledgments
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References


