Mutated BCR-ABL Generates Immunogenic T-cell Epitopes in CML Patients


Abstract

Purpose: Characterization of an approach to identify leukemia neoantigens arising in the context of drug resistance.

Experimental Design: We assessed whether leukemia neoantigens could be generated from drug-resistant mutations in BCR-ABL after imatinib relapse in patients with chronic myelogenous leukemia (CML).

Results: We computationally predicted that approximately 70 peptides derived from 26 BCR-ABL mutations would bind eight common alleles of MHC class I (IC50 < 1,000 nmol/L). Seven of nine imatinib-resistant CML patients were predicted to generate at least 1 peptide that binds autologous HLA alleles. We predicted and confirmed that an E255K mutation-derived peptide would bind HLA-A3 with high affinity (IC50 = 28 nmol/L), and showed that this peptide is endogenously processed and presented. Polyfunctional E255K-specific CD8+ T cells were detected in two imatinib-resistant HLA-A3+ CML patients concurrent with an effective anti-CML response to further therapy.

Conclusions: Our in vitro studies support the hypothesis that leukemia-driven genetic alterations are targeted by the immune system in association with a clinical response, and suggest the possibility of immunizing relapsed patients with CML against newly acquired tumor neoantigens.

Introduction

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder whose pathognomonic feature is translocation between chromosomes 9 and 22 to form the fusion protein BCR-ABL. This gene fusion results in constitutive activation of the ABL kinase whose oncogenic properties have been well described (1, 2). For decades, enduring curative therapy for young patients with CML has been achieved with allotransplantation (allo-HSCT; although at significant risk of toxicities), and these results have provided clear demonstration of the potency of donor immunity in eradicating leukemia through graft-versus-leukemia (GvL) effects (3). The well-tolerated BCR-ABL targeting tyrosine kinase inhibitor (TKI) imatinib, however, has emerged as front-line therapy because of its U.S. Food and Drug Administration approval in 2001 (4). More potent second-generation TKIs, dasatinib and nilotinib, have also been developed and approved for treatment of CML with prior imatinib exposure (5, 6). All 3 TKIs efficiently inhibit the catalytic activity of BCR-ABL by binding to the ATP-binding pocket of the ABL kinase domain (7).

Although imatinib effectively controls CML with relatively minor toxicity, relapse and drug resistance remain as important problems, with annual progression rates of 3.3% to 7.5% during the first 3 years of treatment (8, 9). The most common mechanism of drug resistance is the emergence of various BCR-ABL kinase domain mutations (10), with detection of BCR-ABL mutations varying from 35% to 89% of patients (11–13). Most mutations localize to the p-loop (or ATP binding site) of the fusion protein, followed by the imatinib binding site, catalytic domain, or activation loop, and have been reported to range from 25 to more than 70 distinct mutations (10). Nearly two-thirds of all reported mutations localize to 7 amino acids: G250 (10%), Y253 (11%), E255 (10.5%), T315 (13.5%), M351 (10%), F359 (6.5%), and H396 (5%; refs. 10, 14, 15). Resistance to second-generation TKIs has been similarly observed, with each drug generating its own spectrum of BCR-ABL mutations (16–18).
**Translational Relevance**

Tyrosine kinase inhibitors are currently front-line therapy for chronic myelogenous leukemia (CML), but relapse from drug resistance remains an important problem. Because BCR-ABL mutations arise in the setting of drug resistance, we sought to identify potential neoantigens derived from mutated BCR-ABL that could be targeted for the treatment of this immune-responsive malignancy. We identified polyfunctional E255K-specific CD8+ T cells from two imatinib-resistant HLA-A3+ CML patients arising in the setting of effective anti-CML response to further therapy. Our studies show that leukemia-driven genetic alterations can be targeted by the immune system in association with a clinical response. Our studies support the approach of immunizing patients with relocalized CML against newly acquired tumor neoantigens as a promising antileukemia treatment strategy.

Therapeutic alternatives that complement the potent cytolytic effects of TKIs and address drug resistance would substantively advance the treatment of patients with CML. In particular, an immunotherapeutic approach is promising for CML, due to its exquisite sensitivity to immune modulation (19). BCR-ABL has been suggested as an ideal immunologic target because it is essential for leukemogenesis and its expression is specific to CML. However, chronic expression of this cancer-driving leukemia protein may result in selective host deletion of high-avidity T cells directed against BCR-ABL-expressing cells, as has been described for another CML-associated antigen PR-1 (20), or in reduced antitumor functionality. Consistent with this idea, T cells with specificity for BCR-ABL have been detected in patients with CML, but they show poor cytolytic activity (21). Conversely, as tumors evolve, such as with drug resistance, newly acquired genetic alterations may generate novel peptides that can stimulate new CTL clones, although immunosuppression exerted by leukemia cells may prevent these from controlling the expansion of leukemia cells in vivo (22, 23). Fortunately, however, new potent adjuvants and checkpoint blockade inhibitors are now available that have the potential to circumvent these obstacles and will likely be incorporated in future immunotherapies (24–27).

Herein, we present a strategy to evaluate the immunogenicity of peptides generated from mutated BCR-ABL. This analysis is facilitated by the increasing accuracy of algorithms that predict binding capacity of peptides to a wide range of HLA alleles (28). We detected that T-cell responses against processed mutated BCR-ABL peptides develop in association with tumor regression after allo-HSCT or second line TKIs. Although the concept of tumor neoantigens as a target for immunotherapeutics has been long studied (29), our studies are the first to show that mutations that develop in the setting of drug resistance can be an abundant source of tumor-specific antigens. Our results provide a proof-of-concept for a systematic approach to discover immunogenic tumor antigens arising from evolving genetic alterations, with potential for developing novel immunotherapies.

**Materials and Methods**

**Patient samples**

Heparinized blood was obtained from patients and normal donors enrolled on clinical research protocols at the Dana-Farber Harvard Cancer Center (DFHCC; Boston, MA) approved by the DFHCC Human Subjects Protection Committee. Peripheral blood [peripheral blood mononuclear cells (PBMC)] from normal donors and patients was isolated by Ficoll/Hypaque density-gradient centrifugation, cryopreserved with 10% dimethyl sulfoxide (DMSO), and stored in vapor-phase liquid nitrogen until the time of analysis. HLA typing was done by either molecular studies or serotyping (Tissue Typing Laboratory, Brigham and Women’s Hospital, Boston, MA).

**Prediction of peptide binding to HLA alleles**

To discover peptide targets, MHC-binding affinity was predicted across all possible 9 and 10 mer peptides encoded by BCR-ABL mutations by using MHC–peptide binding prediction algorithms that have been independently verified as highly accurate (28, 31). The NetMHC algorithm met this criterion for 9 mer prediction of HLA-A*02:01, *03:01, *11:01, *14:02, *07:02, *08:01, and *15:01; and for 10 mer prediction of HLA-A*01:01, *02:01, and B*07:02 (32). Alternatively, we used the IEDB SMM (IEDB) algorithm, which met the criterion for high accuracy for HLA-A*02:01, *03:01, *11:01, B*07:02, *08:01, and *15:01 (28). An IC50 value of less than 50 nmol/L was considered a predicted strong binder, an IC50 of more than 50 and less than 500 nmol/L was considered an intermediate binder, and IC50 between 500 and 1,000 nmol/L was considered a weak binder.

**Experimental binding of peptides**

Experimental confirmation of peptide–MHC prediction of a subset of peptides was done using a competitive MHC allele binding assay to test binding to several common HLA alleles (A*02:01, A*03:01, A*11:01, B*07:02, and B*15:01). This assay measures the ability of peptide ligands to inhibit the binding of a high-affinity radiolabeled peptide to purified MHC molecules, and has been described in detail elsewhere (33). Briefly, purified MHC molecules, test peptides, and a radiolabeled probe peptide were incubated at...
room temperature in the presence of human β2-microglobulin and a cocktail of protease inhibitors. After a 2-day incubation, binding of the radiolabeled peptide to the corresponding MHC class I molecule was determined by capturing MHC/peptide complexes on W6/32 antibody (anti–HLA A, B, and C) or B123.2 (anti–HLA B, C, and some A) coated plates, and measuring bound cpm using a microscintillation counter. For competition assays, the concentration of peptide yielding 50% inhibition of the radio labeled peptide binding was calculated. Peptides were typically tested at 6 different concentrations covering a 100,000-fold dose range, and in 3 or more independent assays. Under the conditions used, where [label] < [MHC] and IC₅₀ ≥ [MHC], the measured IC₅₀ values are reasonable approximations of the true Kᵦ values (34, 35).

Sources of antigen

Peptides were synthesized to more than 95% purity (confirmed by high-performance liquid chromatography; New England Peptide). Peptides were reconstituted in DMSO (10 mg/mL) and stored at −20°C until use. A minigene composed of a sequence of 227 bp encompassing the E255K mutation was PCR-cloned into the HindIII and EcoRI restriction enzyme sites of the expression vector pcDNA3.1 using the following primers: 5'-taagctttcaCTGTATGTTGGTC; 3'-primer: atagatcttgcatggattggttgtCTACTCTTCCACCCTCTAGTC. To express the E255K mutation in target cells of T-cell assays, 10 μg of the minigene plasmid was introduced into 2 million K562 cells (American Type Culture Collection) by Amaxa nucleofection (Solution V, Program T16; Lonza Inc.). Expression of HLA-A3 was confirmed by flow cytometry analysis using an anti–HLA-A3 mAb (One Lambda), followed by FITC-conjugated F(ab’)₂ goat antimouse IgM (Zymed). Cells were incubated in Dulbecco’s modified Eagle medium (DMEM; Cellgro), supplemented with 10% FBS (Cellgro), 1% HEPES buffer (Cellgro), and 1% L-glutamine (Cellgro). The nucleofected cells were harvested at least 2 days later for immune assays.

Generation of antigen-presenting cells

Autologous dendritic cells (DC) were generated from immunomagnetically isolated CD14⁺ cells (Miltenyi Biotec) that were cultured in RPMI (Cellgro) supplemented with 3% FBS, 1% penicillin–streptomycin (Cellgro), 1% L-glutamine, and 1% HEPES buffer in the presence of 120 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF; Genzyme) and 70 ng/mL IL-4 (R&D Systems). On days 3 and 5, additional GM-CSF and interleukin-4 (IL-4) were added. On day 6, cells were matured for 48 hours with 10 ng/mL TNF-α (Genzyme) and 1 μg/mL prostaglandin E2 (Sigma-Aldrich). CD40-B cells were generated from PBMCs by activation of CD40L-expressing irradiated feeder cells in the presence of IL-4 (R&D Systems) and cyclosporin A (Novartis) as described (36). The MHC class I–deficient immortalized B lymphoblastoid cell line 721.221 was retrovirally infected with a plasmid encoding the full-length A*03:01 (designated B721.221/A3 cells), and maintained in DMEM supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, antibiotics (Invitrogen), and 500 ng/mL of G418 (Cellgro).

Generation of antigen-specific T cells from normal volunteer and patient PBMC

To generate E255K-reactive T cells from a healthy adult volunteer, PBMCs from an HLA-A3⁺ individual were cultured in 24-well plates (10 × 10⁵ cells/well) in complete medium supplemented with 10% human AB serum, 10 ng/mL IL-7, and 20 units/mL IL-2, together with autologous DCs (at 1:100 ratio) that were pulsed for 3 hours with 10 μmol/L peptide. On day 6, CD8⁺ T cells were immunomagnetically isolated from PBMCs (CD8⁺ Microbeads; Miltenyi). CD8⁺ T cells were cocultured with irradiated B721.221/A3 cells (3,200 Gy) pulsed with 10 μmol/L peptide at a ratio of 10:1 in the presence of IL-7 (10 ng/mL) on days 7, 14, 21, and 28, and IL-2 (20 units/mL) on days 8, 15, 22, and 29, and T-cell specificity was tested 1 week after last stimulation. E255K-specific T cells were similarly expanded from cryopreserved patient PBMCs with ELISpot testing after 2 rounds of peptide stimulation.

Detection of antigen-specific T cells

ELISpot assay was done using peptide-pulsed target cells (50,000 cells/well) cocultivated with 50,000 immunomagnetically purified CD8⁺ T cells per well in duplicate on ELISpot plates (Millipore) for 24 hours. IFN-γ secretion was detected using capture and detection antibodies, as directed (Mabtech AB), and imaged (ImmunoSpot Series Analyzer; Cellular Technology). To test T-cell reactivity dependence on class I, ELISpot plates were first coated with APCs cocultivated with class I–blocking antibody (W6/32) for 2 hours at 37°C before the introduction of T cells into the wells. An HLA-A3 tetramer specific for the E255K-B peptide was generated by the NIH Tetramer Facility (Emory University). For tetramer staining, 5 × 10⁶ cells were incubated for 30 minutes at 4°C with 1 μg/mL tetramer, and then incubated with the addition of anti–CD8-FITC antibody (BD Biosciences) for another 30 minutes at 4°C. A minimum of 5,000 events were acquired per sample. Supernatants of cultured CD8⁺ T cells were also collected and analyzed for GM-CSF, TNF-α, and interferon-induced protein (IP-10) using a Luminex multiplex bead-based technology (Milliplex; Millipore) as per the manufacturer’s recommendations. In brief, fluorescent-labeled microspheres were coated with specific cytokine capture antibodies. After incubation with the culture supernatant sample, captured cytokines were detected by a biotinylated detection antibody, followed by a streptavidin–PE conjugate, and median fluorescence intensity was measured (Luminex 200 bead array instrument; Luminex Corporation). On the basis of a standard curve, cytokine levels were calculated in the Bead View Software program (Upstate; Millipore).

Statistical considerations

Comparisons between T-cell responses to APCs pulsed with wild-type and mutated peptide on ELISpot; or between
peptide-pulsed APCs or E255K minigene–transfected APCs were calculated as 2-sided \( P \) values using a 2-sample \( t \) test.

**Results**

**Common imatinib-induced BCR-ABL mutations frequently generate peptides predicted to bind MHC class I alleles**

Twenty-six BCR-ABL mutations have been reported to arise in association with CML relapse after imatinib exposure, with T315I, E255K, and M351T as the most common (21%, 19%, and 15%, respectively; Fig. 1C), and with the remaining mutations occurring at a frequency of 6.8% or less (37). To explore the potential of mutated BCR-ABL to generate novel immunogenic peptides, we applied the validated prediction algorithm NetMHC to the amino acid sequences encompassing these common BCR-ABL mutations. NetMHC has been shown to accurately predict 9 and 10mers binding to 8 common class I HLA alleles: \( \text{A}^\star 02:01, \text{A}^\star 03:01, \text{A}^\star 11:01, \text{B}^\star 07:02, \text{B}^\star 08:01, \text{B}^\star 15:01, \text{A}^\star 01:01, \) and \( \text{A}^\star 24:02 \) (28).

By tiling 9 and 10mers around each mutation, we identified 71 distinct peptides predicted by NetMHC to bind 1 or more common class I HLA alleles with IC\(_{50}\) of less than 1,000 nmol/L for a total of 84 peptide–allele combinations. As shown in Fig. 1A, NetMHC predicted 25 of 84 (30%) as high binders (IC\(_{50}\) < 50 nmol/L), 41 (49%) as intermediate binders (IC\(_{50}\) = 50–500 nmol/L), and 18 (21%) as weak binders (1,000 > IC\(_{50}\) > 500 nmol/L). Predicted high-binding peptides were evenly distributed across the 26 mutations. Of note, E255K produced 3 high and 3 intermediate affinity class I HLA candidate–binding peptides, and M351T produced 2 high and 4 intermediate affinity candidate–binding peptides. In contrast, the highly aggressive T315I mutation yielded only 1 peptide of intermediate binding affinity, predicted to bind HLA-B\(^\star 15:01\).

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**Figure 1.** Predicted class I HLA binding peptides based on common BCR-ABL mutations using the NetMHC prediction server. Twenty-six BCR-ABL mutations, ordered in decreasing frequency from left to right as per Deininger and colleagues (ref. 37; C), were tested by NetMHC for peptides predicted to bind common class I HLA alleles. A, the binding affinities of the predicted high-affinity peptides (IC\(_{50}\) < 1,000 nmol/L) per BCR-ABL mutation are displayed. Strong binders are defined as IC\(_{50}\) < 50 nmol/L (region above the upper dotted line). Intermediate binders are defined as 50 nmol/L < IC\(_{50}\) < 500 nmol/L (between the 2 dotted lines). Weak binders are defined as 500 nmol/L < IC\(_{50}\) < 1,000 nmol/L (in the region below the bottom dotted line). B, fold predicted binding of mutated peptide compared with wild type. Points above the dotted line represent peptides predicted to bind with at least 2-fold greater affinity compared with wild type. C, frequency of predicted binding of mutated peptide compared with wild type.
A priority in the field of immunotherapy is the identification of the targetable tumor-specific epitopes that do not elicit cross-reactivity to normal tissue. We therefore examined whether the predicted mutated peptides had increased binding affinity compared with respective parental peptides. As shown in Fig. 1B, 24 of 84 predicted mutated peptides (29%) showed at least 2-fold higher predicted binding affinity to HLA compared with parental peptides (median 1.2, range 0.02–622). Of the 3 most frequent mutations, 4 of 6 E255K peptides (but none of the T315I or M351T peptides) were predicted to bind with at least 2-fold higher affinity compared with parental peptides.

Similar results were observed using an alternate established algorithm, IEDB, for prediction of peptides derived from mutated BCR-ABL peptides binding to HLA compared with parental peptides. As shown in Fig. 1B, 24 of 84 predicted mutated peptides were predicted to have at least 2-fold greater predicted binding affinity compared with respective parental peptides. In addition, 3 of 4 patients harboring an E255K mutation also expressed HLA-A3 (patients 1–3), and this mutation was predicted to generate 2 promising candidate HLA-A3 binding peptides: E255K-A247–255 (KLGGGQYGK, IEDB IC50 = 113 nmol/L; NetMHC IC50 = 192 nmol/L) and E255K-B255–263 (KVYEGVWK; IEDB IC50 = 29 nmol/L; NetMHC IC50 = 28 nmol/L). Moreover, both peptides were predicted to bind HLA-A’03:01 with at least 10-fold greater affinity than parental peptides. In addition, 3 of 4 patients harboring an E255K mutation expressed HLA-A2 (patients 2–4); E225K was predicted to generate 2 other promising HLA-A2 binding candidate peptides by NetMHC: E225K-A232–240 (GQYGKVYEG; IC50 = 145 nmol/L) and E225K-D251–260 (GQYGKVeEG; IC50 = 16 nmol/L), but not by IEDB.

**Experimental validation of HLA-binding of predicted BCR-ABL peptides**

To validate the HLA-binding predictions, we synthesized fifty-five 9mers and 10mers derived from mutated BCR-ABL and determined their binding capacity IC50 (nmol/L) using a well-established competitive MHC binding assay (33). We focused on peptides predicted to have high binding affinity (IC50 < 100 nmol/L) by NetMHC, for which IEDB binding predictions were also available.

As shown in Fig. 2, 7 of 9 imatinib-resistant patients with CML could potentially generate at least 1 mutated BCR-ABL–derived peptide with predicted binding affinity to autologous HLA alleles of IC50 < 1,000 nmol/L (median number of predicted binders/patient was 2, range 0–5). None of the 3 patients with the T315I mutation (patients 1, 4, and 8) expressed HLA-B15 (the only allele predicted to strongly bind a T315I-derived peptide). However, CML cells from 3 of 4 patients harboring an E255K mutation also expressed HLA-A3 (patients 1–3), and this mutation was predicted to generate 2 promising candidate HLA-A3 binding peptides: E255K-A247–255 (KLGGGQYGK, IEDB IC50 = 113 nmol/L; NetMHC IC50 = 192 nmol/L) and E255K-B255–263 (KVYEGVWK; IEDB IC50 = 29 nmol/L; NetMHC IC50 = 28 nmol/L). Moreover, both peptides were predicted to bind HLA-A’03:01 with at least 10-fold greater affinity than parental peptides. In addition, 3 of 4 patients harboring an E255K mutation expressed HLA-A2 (patients 2–4); E225K was predicted to generate 2 other promising HLA-A2 binding candidate peptides by NetMHC: E225K-A232–240 (GQYGKVYEG; IC50 = 145 nmol/L) and E225K-D251–260 (GQYGKVeEG; IC50 = 16 nmol/L), but not by IEDB.

**Imatinib-resistant CML patients can potentially express immunogenic mutated BCR-ABL epitopes**

To establish the frequency that immunogenic peptides arising from mutated BCR-ABL can potentially be generated from patients with CML, we analyzed 9 imatinib-resistant patients with CML, whose HLA typing and BCR-ABL mutation status were known. All patients had been exposed to imatinib for a median of 6 months (range 1–3) before developing drug resistance (Table 1). All leukemia samples harbored a median of 1 BCR-ABL mutation (range 1–3). In total, 20 distinct HLA-A and HLA-B alleles were represented among 9 patients, and 8 distinct mutations were detected, 5 of which were unique to particular patients. The 3 most common HLA alleles were A1, A2, and B7, represented in 4, 5, and 4 patients, respectively.

**Table 1. Patient characteristics of 9 patients with known mutations in BCR-ABL following the development of imatinib resistance**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/sex</th>
<th>Months on Imatinib until resistance</th>
<th>HLA-A and -B alleles</th>
<th>BCR-ABL mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64/M</td>
<td>32</td>
<td>A3, A11, B7, B52</td>
<td>L150M, E255K, T315I</td>
</tr>
<tr>
<td>2</td>
<td>44/M</td>
<td>6</td>
<td>A2, A3, B7, B49</td>
<td>E255K</td>
</tr>
<tr>
<td>3</td>
<td>59/M</td>
<td>2</td>
<td>A2, A3, B7, B44</td>
<td>E255K</td>
</tr>
<tr>
<td>4</td>
<td>53/M</td>
<td>6</td>
<td>A2, A33, B37, B14</td>
<td>E255K, T315I</td>
</tr>
<tr>
<td>5</td>
<td>70/M</td>
<td>1</td>
<td>A1, A2, B8, B38</td>
<td>M244V, E281K, T315A</td>
</tr>
<tr>
<td>6</td>
<td>61/M</td>
<td>14</td>
<td>A2, A2, B40, B51</td>
<td>G250E</td>
</tr>
<tr>
<td>7</td>
<td>48/F</td>
<td>2</td>
<td>A1, A30, B18, B57</td>
<td>V379I</td>
</tr>
<tr>
<td>8</td>
<td>58/F</td>
<td>7</td>
<td>A1, A31, B7, B57</td>
<td>T315I</td>
</tr>
<tr>
<td>9</td>
<td>64/F</td>
<td>33</td>
<td>A1, A24, B8, B57</td>
<td>E281K, G254R</td>
</tr>
</tbody>
</table>

As shown in Fig. 2, 7 of 9 imatinib-resistant patients with CML could potentially generate at least 1 mutated BCR-ABL–derived peptide with predicted binding affinity to autologous HLA alleles of IC50 < 1,000 nmol/L (median number of predicted binders/patient was 2, range 0–5). None of the 3 patients with the T315I mutation (patients 1, 4, and 8) expressed HLA-B15 (the only allele predicted to strongly bind a T315I-derived peptide). However, CML cells from 3 of 4 patients harboring an E255K mutation also expressed HLA-A3 (patients 1–3), and this mutation was predicted to generate 2 promising candidate HLA-A3 binding peptides: E255K-A247–255 (KLGGGQYGK, IEDB IC50 = 113 nmol/L; NetMHC IC50 = 192 nmol/L) and E255K-B255–263 (KVYEGVWK; IEDB IC50 = 29 nmol/L; NetMHC IC50 = 28 nmol/L). Moreover, both peptides were predicted to bind HLA-A’03:01 with at least 10-fold greater affinity than parental peptides. In addition, 3 of 4 patients harboring an E255K mutation expressed HLA-A2 (patients 2–4); E225K was predicted to generate 2 other promising HLA-A2 binding candidate peptides by NetMHC: E225K-A232–240 (GQYGKVYEG; IC50 = 145 nmol/L) and E225K-D251–260 (GQYGKVeEG; IC50 = 16 nmol/L), but not by IEDB.
< 100 nmol/L, and 21 (66%) with IC50 between 100 and 1,000 nmol/L. The only peptide with high binding per NetMHC, but weak predicted binding per IEDB (IC50 > 1,000 nmol/L) showed experimental IC50 > 1,000 nmol/L. The tested peptides and corresponding IC50 results are listed in Supplementary Table S1.

We subsequently focused our attention on peptides E255K-A and E255K-B. Each was predicted to strongly bind to HLA-A*03:01 with at least 10-fold greater affinity than their parental peptides by both NetMHC and IEDB. Both were experimentally confirmed to bind HLA-A3 with high affinity (IC50 scores of 208 and 17 nmol/L, respectively; Table 2). In addition, E255K-B also bound other HLA-A3 superfamily members HLA-A*11:01, HLA-A*30:01, HLA-A*31:01, and HLA-A*68:01 with high to moderate affinity (IC50: 39–603 nmol/L).

Mutated BCR-ABL peptide E255K-B is immunogenic

T-cell lines were generated against the E255K-B peptide but not E255K-A from PBMC of a normal HLA-A3+ volunteer after 4 rounds of weekly stimulation of T cells against mutated peptide-pulsed autologous APCs. To assess the specificity of T-cell response, we generated HLA-A3–expressing APCs by transfecting a plasmid encoding HLA-A3 into K562 cells (Fig. 4A right). As shown in Fig. 4B, the expanded T-cell lines against E255K-B showed greater IFN-γ–ELISpot reactivity against the mutated peptide (2330 ± 325 SFC/million cells; P = 0.045) than the parental peptide (1270 ± 42 SFC/million cells). Moreover, T-cell reactivity against HLA-A3–expressing APCs that were transfected with a minigene (Fig. 4A left) encompassing 227 base pairs surrounding the E255K mutation (1,900 ± 85 SFC/million cells) was increased compared with unpulsed APCs (710 ± 70 SFC/million cells) or to APCs pulsed with wild-type peptide (P = 0.011). Furthermore, confirming that E255K-B is a processed and surface-displayed peptide, we detected an expanded and discrete population of E255K-B reactive CD8+ T cells within E255K-B stimulated T-cell lines (at a
frequency of 0.64%) compared with control PBMC populations from normal adult volunteers (frequency <0.01%), using an E255K/A3-specific tetramer (Fig. 4C). These data together support E255K-B as an endogenously processed and presented peptide epitope in the context of HLA-A3.

Mutated BCR-ABL peptide can elicit memory responses from imatinib-resistant patients

We next examined the potential of E255K-B for stimulating T-cell responses in patients with CML. We focused on patients 2 and 3, from whom cryopreserved PBMCs were available. PBMCs from patient samples were stimulated twice with E255K-B pulsed APCs, and the expanded CD8\(^+\) T cells were subsequently tested for the evidence of memory T-cell responses by ELISpot, tetramer, and cytokine secretion assays. Although patients 2 and 3 underwent different therapy subsequent to imatinib resistance, both patients showed that T-cell immunity to E255K-B can develop in vivo in the setting of effective CML cytoreduction.

The results of the T-cell studies from patients 2 and 3 are shown in Figs. 5 and 6, respectively. Patient 2 developed rising BCR-ABL PCR levels while on imatinib and dasatinib, and subsequently initiated treatment with nilotinib, to which a major cytogenetic response was achieved. PBMCs collected at the time of nilotinib exposure were stimulated against E255K-B peptide-pulsed A3\(^+\) expressing APCs, and tested for reactivity by ELISpot assay. Compared with wild-type E255K-B peptide (390 \pm 14 SFC/million cells),
increased IFN-γ secretion was detected against both the mutated E255K-B peptide (1,050 ± 71 SFC/million cells; P = 0.006) and APCs expressing the E255K minigene (590 ± 14 SFC/million cells; P = 0.005; Figs. 5A and B). T-cell responses were abrogated by blocking with the class I MHC blocking antibody w6/32, confirming the class I restriction of these T-cell responses. B, graphical representation of ELISpot results. C, T cells reactive to the mutated peptide are polyfunctional, with secretion of GM-CSF, IP-10, and TNF-α, measured by Luminex assay. Cytokine secretion results were depicted as fold over control cells [background cytokine secretion by T cells lines exposed to the APCs (K562-A3) alone].

In another example, patient 3 developed a partial response to dasatinib for the treatment of imatinib-resistant disease, and subsequently underwent myeloablative allogeneic HSCT. PBMCs collected before and after allo-HSCT were tested for reactivity to E255K-B. As shown in Fig. 6, reactivity was expanded in the setting of donor-derived engraftment after HSCT, concurrent with attainment of molecular remission. Increased reactivity by ELISpot (Fig. 6A) was seen in post-HSCT CD8+ T cells tested against APCs pulsed with mutated E255K-B peptide (2,120 ± 340 SFC/million) but not to wild-type E255K peptide (1,140 ± 28 SFC/million; P = 0.067). Increased IFN-γ secretion was also observed against APCs expressing the E255K minigene (1,810 ± 325 SFC/million). Again, reactivity was abrogated in the presence of blocking antibody with 810 ± 156 SFC/million cells detected against E255K-B, and 1,260 ± 113 SFC/million cells to APCs expressing the E255K minigene. From the post-HSCT T cells, an expanded population of E255K-B reactive T cells was detected by tetramer staining (0.83%; Fig. 6B). Finally, posttreatment but not pretreatment T cells from patient 3 were detected to be polyfunctional on the basis of secretion of GM-CSF, IP-10, and TNF-α following stimulation. In contrast, we were unable to elicit E255K-B specific T-cell reactivity from 3 HLA-A3+ patients with CML with imatinib resistance, but who did not harbor the E255K mutation, and had undergone allogeneic HSCT (Fig. 6A). Although polyclonal alloimmune responses are certainly generated after allo-HSCT, our results show the potential for tumor-specific T-cell immunity to also develop in vivo, which may contribute to the GvL responses present after effective HSCT.

Discussion

Gene alterations in malignant cells can drive cancers, lead to disease progression, or generate resistance to drug therapy. These same events, however, have the potential to create novel immunologic targets. As they are unique to cancer cells, mutations that generate novel tumor-specific peptides can be targeted immunologically; this may lead to tumor elimination with minimal toxicity. Our current study provides proof-of-concept of the feasibility of an approach to systematically define potential immunogenic epitopes arising from a mutated tumor protein. Although the immunogenicity of individual examples of tumor neoantigens has been established (29), our studies focusing on mutated BCR-ABL in CML are the first to show that tumor-specific gene alterations, arising commonly as a result of drug resistance during the treatment of CML, can generate immunogenic epitopes. Beginning with a set of known common BCR-ABL mutations, we identified peptides encoded by
mutations that were predicted to bind to high-frequency HLA class I alleles using well-validated prediction algorithms. The majority could be reliably validated through experimental binding studies.

We used the example of a novel predicted HLA-A3\(^+\)-restricted epitope derived from the common E255K mutation to show that mutated BCR-ABL peptides can stimulate CD8\(^+\) T-cell responses that arise in association with effective therapeutic response. Our findings not only provide a rationale for developing tumor-specific immunotherapy for CML, but also support the feasibility of a broadly applicable approach for identifying neoplasm-specific neoantigens.

Our study predicted an unexpectedly large number of peptides encoded by common resistance mutations with potential to tightly bind multiple common HLA class I alleles. Our findings are consistent with recent studies that show that a bioinformatic approach can systematically identify a larger spectrum of immunogenic epitopes than by conventional T-cell cloning–based tumor antigen discovery approaches (38). We used both NetMHC and IEDB, as several comparative analyses have identified them as highly sensitive and specific across many common HLA class I alleles (28). In agreement with Trost and colleagues, we found that the intersecting results from multiple prediction tools more accurately predicted MHC class I peptide binding (39).

Of the 3 candidate peptides that we characterized in cellular assays, only one (E255K-B) was predicted as a high binder by both NetMHC and IEDB, and was determined to be a processed and presented epitope. Together, these findings suggest that a screening strategy using binding prediction criteria can efficiently generate a list of promising tumor neoepitopes. Furthermore, they highlight the increasing reliability of class I prediction servers for peptide binding against an expanded repertoire of HLA class I alleles beyond an examination restricted to HLA-A2 (conventionally, the focus of peptide prediction studies).

Although BCR-ABL has long been considered a promising immunogen for tumor-specific immunotherapy, clinical studies overall have not been encouraging. This may be related, in part, to the previously unappreciated restricted spectrum of HLA haplotypes that have been confirmed to present BCR-ABL epitopes and thus have the potential to stimulate T-cell responses (HLA-A3, -A11, and -B8 alleles; refs. 40 and 41). Vaccine trials using wild-type BCR-ABL junctional peptides with or without heteroclitic peptides have not definitively shown antitumor responses (42–44). Furthermore, chronic persistence of BCR-ABL\(^+\) tumor cells seems to actively delete high-avidity CML-specific T cells over time (20), and thus many barriers exist to mounting an

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**Figure 6.** The peptide encoded by mutated E255K elicits a memory response in association with disease control after allogeneic HSCT. A, graphical representation of ELISpot results showing IFN-\(\gamma\) secretion in response to E255K-B and the E255K minigene by T cells expanded from patient 3 after allo-HSCT, at molecular remission (left). Increased reactivity against E255K-B and the E255K minigene was not observed in T cells expanded from control patients A–C (HLA-A3\(^+\), but lacking the mutation E255K) after allo-HSCT (right). B, flow cytometric detection of cytotoxic CD8\(^+\) T cells specific for E255K-B in the context of HLA-A3 by tetramer staining in expanded post-allo-HSCT T cells from patient 3. C, post-HSCT E255K-B reactive T cells are polyfunctional, secreting increased GM-CSF, IP-10, and TNF-\(\alpha\) after exposure to E255K-B peptide or the E255K minigene, compared with pre-HSCT T cells. Cytokine secretion was detected by luminex assay. Cytokine secretion results were depicted as fold over control (background cytokine secretion by T-cell lines exposed to the APCs (K562-A3) alone).
effective cytolytic T-cell response against an existing tumor antigen.

In contrast, our findings support the notion that peptides generated from a newly arising tumor mutation are not tolerogenic. We identified the development of CD8+ T-cell responses in 2 examples of therapeutic response. In the first, BCR-ABL mutation-specific T-cell responses developed after institution of a second-generation TKI that effectively cytolysed tumor. Imatinib and the other TKIs have been variably reported to impair or enhance antitumor immunity (45, 46). Independent of the potential immunosuppressive properties of TKIs, recent studies have revealed multiple mechanisms by which tumor cells can directly exert profound local immunosuppressive effects on T-cell function (22, 23). For example, Mumprecht and colleagues detected increased expression of PD-1 in CD8+ T cells in patients with CML that was associated with an exhausted phenotype and with disease progression (22). Thus, for patient 2, effective elimination of tumor cells by nilotinib presumably contributed to restoration of T-cell immunity. In the case of patient 3, E255K-B-specific T-cell responses were detected after allo-transplant, a setting that enables the development of robust T-cell immunity against tumor-associated antigens (47). In both the cases of patients 2 and 3, we could show the limited potential for cross-reactivity to the associated wild-type peptide.

Our toolkit for promoting effective tumor immunity has expanded rapidly in recent years, and now includes potent adjuvants, a variety of potentially efficacious delivery systems, and agents that can overcome checkpoint blockade (48). Thus, a priority is to identify antigens with truly tumor-specific expression so that a focused immune response can be generated without inciting potentially detrimental immune responses against normal tissue. Although the specific application of our studies are limited to a small subset of TKI-treated patients with CML who are both HLA-A3 positive and harbor the E255K mutation, our studies provide a rational basis for developing immunotherapy for any TKI-treated patient with CML. Larger scale studies to test immunogenicity of other imatinib-associated BCR-ABL mutation–related peptides would be anticipated to reveal a wider spectrum of immunogenic peptides that could elicit reactivity from a broader selection of HLA alleles. Hence, one could envision an immunotherapeutic approach that could be personalized on the basis of the individual’s HLA typing and BCR-ABL mutation genotype (49, 50). Our approach is further generally applicable to other mutations related to resistance to other TKIs, which have been described to induce an alternate spectrum of BCR-ABL mutations (see Supplementary Fig. S2 for analysis of potential peptide binders for common mutations related to dasatinib and nilotinib). BCR-ABL mutations “drive” CML relapse, and would be expected to be expressed in all CML cells—even the malignant progenitor cell populations that have been shown to be generally resistant to TKIs. Thus, an attractive future strategy that could potentially provide durable responses with minimal toxicity would be to combine effective cytoreductive molecularly targeting agents together with polyclonal leukemia–targeting immune therapy.

Disclosure of Potential Conflicts of Interest
R. Stone is a consultant/advisory board member of Novartis. No potential conflicts of interest were disclosed by other authors.

Authors’ Contributions
Conception and design: A. Cai, D.B. Keskin, C.J. Wu
Development of methodology: A. Cai, D.B. Keskin, V. Brusic
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Cai, D.B. Keskin, A. Alonso, W. Zhang, N.N. Hammond, V. Nardi, R. Stone, J. Sidney
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): A. Cai, D.B. Keskin, D. DeLuca, A. Alonso, A. Zhang, D. Neuberg, J. Sidney, V. Brusic
Writing, review, and/or revision of the manuscript: A. Cai, D.B. Keskin, D. DeLuca, V. Nardi, R. Stone, D. Neuberg, J. Sidney, V. Brusic, C.J. Wu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Alonso
Study supervision: D.B. Keskin, C.J. Wu

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