Purpose: Cancers accumulate mutations over time, each of which brings the potential for recognition by the immune system. We evaluated T-cell recognition of the tumor mutanome in patients with ovarian cancer undergoing standard treatment.

Experimental Design: Tumor-associated T cells from 3 patients with ovarian cancer were assessed by ELISPOT for recognition of nonsynonymous mutations identified by whole exome sequencing of autologous tumor. The relative levels of mutations and responding T cells were monitored in serial tumor samples collected at primary surgery and first and second recurrence.

Results: The vast majority of mutations (78/79) were not recognized by tumor-associated T cells; however, a highly specific CD8⁺ T-cell response to the mutation hydroxysteroid dehydrogenase–like protein 1 (HSDL1)¹²⁵V was detected in one patient. In the primary tumor, the HSDL1¹²⁵V mutation had low prevalence and expression, and a corresponding T-cell response was undetectable. At first recurrence, there was a striking increase in the abundance of the mutation and corresponding MHC class I epitope, and this was accompanied by the emergence of the HSDL1¹²⁵V-specific CD8⁺ T-cell response. At second recurrence, the HSDL1¹²⁵V mutation and epitope continued to be expressed; however, the corresponding T-cell response was no longer detectable.

Conclusion: The immune system can respond to the evolving ovarian cancer genome. However, the T-cell response detected here was rare, was transient, and ultimately failed to prevent disease progression.

These findings reveal the limitations of spontaneous tumor immunity in the setting of standard treatments and suggest a high degree of ignorance of tumor mutations that could potentially be reversed by immunotherapy. Clin Cancer Res; 1–10. ©2013 AACR.

Introduction
There is long-standing interest in the concept of immune surveillance of cancer. For example, in murine models, several lines of evidence indicate that the immune system can recognize nascent tumors and prevent their outgrowth (1). In a chemical carcinogenesis model, host T cells were shown to prevent tumor development through recognition of a single somatic point mutation in the spectrin-β2 gene (2). However, equivalent evidence of primary immune surveillance in humans is lacking, apart from T-cell-mediated control of virus-induced cancers (3). More obvious in humans is the influence of the immune system on cancer progression and clinical outcomes. In particular, the presence of CD8⁺ tumor-infiltrating lymphocytes (TIL) is strongly associated with favorable prognosis in virtually every solid human cancer studied (4). Other TIL subsets, including CD20⁺ B cells, further contribute to this effect (5–7). Thus, the immune system can mount seemingly protective antitumor responses in many patients with cancer.

In addition to spontaneous immune responses, there is increasing evidence that tumor immunity is enhanced by certain cancer treatments, including hormone, radiation, and chemotherapy (8). This is thought to occur by the process of immunogenic cell death, in which dying tumor cells release tumor-specific antigens and danger-associated molecules such as calreticulin, HMGB1, and ATP, leading to enhanced presentation of tumor antigens to the immune system (8). For example, we recently showed in estrogen receptor–negative breast cancer that patients with preexisting CD8⁺ TIL show survival benefit from anthracycline-based chemotherapy, whereas patients lacking CD8⁺ TIL do...
Clinical Cancer Research

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Translational Relevance

Cancers progress through the accumulation of somatic mutations. To investigate how the immune system responds to the tumor genome over time, we evaluated T-cell responses to mutations identified by whole exome sequencing of serial tumor samples from 3 ovarian cancer patients undergoing standard treatment. Of 79 mutations tested, we identified a CD8\(^+\) T-cell response to a point mutation in hydroxysteroid dehydrogenase-like protein 1 in one patient. This T-cell response was undetectable at diagnosis but arose during first remission in step with increased expression of the mutation. At second recurrence, the mutation continued to be expressed by tumor cells, but the T-cell response disappeared. Thus, spontaneous T-cell responses to tumor mutations are rare and transient in the context of standard treatment of ovarian cancer. The fact that many tumor mutations go unrecognized opens the possibility for immunotherapeutic targeting in the future.

not (9); this suggests that anthracyclines work in part by enhancing tumor immunity. To build on such effects, many groups are pursuing the development of immunomodulatory agents that further stimulate tumor immunity. Most notably, antibodies that block the negative regulatory molecules CTLA-4 and PD-1 on T cells have produced striking responses (10). Further enhancement of tumor immunity can potentially be achieved using therapeutic cancer vaccines or adoptive transfer of in vitro expanded TIL (11).

Despite the clear association between TIL and clinical outcomes, very little is known about the underlying antigens recognized by TIL. A recent study of melanoma—the best understood human cancer from an immunologic perspective—revealed that “known” antigens account for only a small percentage of TIL responses (12). This and other findings have fueled speculation that the majority of TIL might instead recognize the products of somatic mutations, collectively referred to as the “mutanome.” Indeed, there have been many anecdotal reports of TIL recognizing somatic mutations in melanoma and other cancers (13). This concept received further support from a recent study in which whole exome sequencing was performed on melanoma samples from 3 patients who had responded well to therapeutic TIL infusions (14). A large proportion of TIL cells in the therapeutic TIL product were found to recognize MHC class I epitopes derived from 2 to 3 somatic mutations from each patient. Similar results were obtained for a patient with melanoma treated with CTLA-4 blockade (15). Thus, TIL can recognize mutant gene products, and these specificities may underlie successful immunotherapy. However, melanoma is a highly immunogenic cancer, and immunotherapy a specialized form of treatment. It is unclear whether these concepts also apply to epithelial cancers treated with conventional therapies.

High-grade serous ovarian cancer (HGSC) is a challenging disease with a 5-year survival rate of only 40% (16). A large majority of patients respond well to primary treatment with surgery and platinum- and taxane-based chemotherapy; however, most relapse within 1 to 3 years and ultimately succumb to their disease. Despite these unfortunate statistics, the presence of CD8\(^+\) TIL is strongly associated with survival in HGSC, as with other cancers (17). CD8\(^+\) and CD4\(^+\) TIL in ovarian cancer have an activated cell surface phenotype (18, 19), show oligoclonal T-cell receptor (TCR) repertoires (20–23), and can recognize and kill autologous tumor tissue in vitro (22, 24–30). However, the underlying antigens remain poorly defined (24). Moreover, little is known about the fate of tumor-specific TIL as patients progress from primary to recurrent disease. A better understanding of immune activation and subsequent failure could open new frontiers in cancer immunotherapy for HGSC and other malignancies.

We recently performed whole exome sequencing of matched primary and recurrent tumor samples from 3 patients with HGSC (31). By comparing samples collected at primary surgery, first recurrence, and second recurrence, we showed that the HGSC mutanome evolves over time, likely reflecting the growth dynamics of different tumor cell subpopulations, as well as the acquisition of new mutations during chemotherapy. In this study, we investigated the hypothesis that acquired mutations might trigger responses by tumor-associated T cells, potentially resulting in immunologic selection against tumor subclones harboring such mutations.

Materials and Methods

Patients, biospecimens, and clinical data

Participant samples and clinical data were collected with informed written consent through a prospective study in partnership with the BC Cancer Agency’s Tumour Tissue Repository. Ethics approval was granted by the Research Ethics Board of the BC Cancer Agency and the University of British Columbia. Patients had a diagnosis of HGSC and underwent standard treatment consisting of surgery followed by carboplatin-based chemotherapy with or without paclitaxel. Further clinical details can be found in our previous publication (31).

Malignant ascites samples were collected during primary surgery and palliative paracentesis. Ascites cells were isolated by centrifugation and cryopreserved in 50% FBS, 40% complete media (RPMI 1640 containing 10% FBS, 25 mmol/L HEPES, 2 mmol/L L-glutamine, 50 μmol/L β-mercaptoethanol, and 1 mmol/L sodium pyruvate) and 10% dimethyl sulfoxide (Sigma-Aldrich). Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll density centrifugation. PBMC and tumor cell preparations were stored in the vapor phase of liquid nitrogen.

Immunohistochemistry

Immunohistochemistry was performed using a Ventana Discovery XT autostainer (Ventana) with primary antibodies to CD3 (Clone SP7; Spring Biosciences, Cat. No.
M3074), CD8 (Clone SP16; Spring Biosciences, Cat. No. M3162), CD4 (Clone SP35; Spring Biosciences, Cat. No. M3354), CD20 (Spring Biosciences, Cat. No. E2560), MHC class I (clone EmR8-5; MBL, Cat. No. D226-3), and MHC class II (clone CR3/43, Affinity Bioreagents, Cat. No. MAI-25914). Bound antibodies were detected using a biotinylated secondary antibody (Jackson Immunoresearch) and a DABMap Kit (Ventana) followed by counterstaining with hematoxylin (Ventana). Lymphocyte densities and MHC intensity were scored by visual inspection using semiquantitative scales described in Table 1. MHC class I and class II were scored by visual inspection; the intensity of expression by tumor epithelium was scored semiquantitatively by comparison to positive stromal cells in the same or neighboring tissue cores.

**In vitro T-cell line generation**

Bulk ascites or tumor cells were thawed and incubated in complete media in 6-well plates at a concentration of 1 × 10^6 cells/mL in the presence of either high-dose human interleukin (IL)-2 (6000 IU/mL; National Cancer Institute) or anti-CD3/anti-CD28–coated beads (Dynabeads Human T-Activator CD3/CD28; Life Technologies) plus low-dose IL-2 (300 IU/mL). For CD8-enriched lines, bulk ascites cells or tumor cells were thawed and incubated in complete media in 6-well plates at a concentration of 1 × 10^6 cells/mL in the presence of either high-dose human interleukin (IL)-2 (6000 IU/mL; National Cancer Institute) or anti-CD3/anti-CD28–coated beads (Dynabeads Human T-Activator CD3/CD28; Life Technologies) plus low-dose IL-2 (300 IU/mL). For CD8-enriched lines, bulk ascites cells were labeled with PE-conjugated anti-human CD8 antibody (BD Biosciences), and magnetic separation was performed using anti-CD8-coated beads (Dynal). CD8^+ T cells were expanded in vitro using the rapid expansion protocol (REP; ref. 32). Cultures were split every 3 to 4 days. 10 ng/mL IL-7 (Peprotech) and 1 IU/mL IL-2 for 3 days.

**T-cell cloning**

An hydroxysteroid dehydrogenase–like protein 1 (HSDL1)-specific CD8^+ T-cell clone (clone 1) was generated by limiting dilution cloning. In brief, CD8^+ T cells from an IL-2–expanded cell line exhibiting HSDL1^L25V^ reactivity were positively selected through magnetic bead separation and serially diluted down to 1 cell per well in 96-well tissue culture plates. Cultures were stimulated to proliferate by adding irradiated feeder cells (a pool of 3 irradiated allogeneic human PBMC), 30 ng/mL anti-human CD3 (eBioscience), and IL-2 (300 IU/mL). Complete media containing IL-2 was replaced every 2 to 3 days.

**Flow cytometry**

Bulk ascites cells were assessed directly ex vivo by staining with antibodies to CD4 or CD8 in combination with an anti-TCR Vß repertoire panel (IOTEST BetaMark; Beckman Coulter) and analyzed with a Becton Dickinson FACScalibur.

**Epitope prediction and peptide design**

Whole exome sequencing results were previously published (31). HLA typing was performed as previously described (33) and analyzed using IMGT/HLA database version 3.3.0. Peptide/MHC binding scores for all possible 8-, 9-, 10-, and 11-amino acid in length peptides containing the mutated residue relative to each patient’s MHC class I alleles were generated using the epitope prediction software NetMHCpan-2.4 (ref. 34; Supplementary Tables S1–S4). All peptides predicted to bind MHC with an affinity ≤50 nmol/L were selected for screening. In addition, for those mutations that did not yield peptides with binding scores of ≤50 nmol/L, the minimal peptide with the highest predicted MHC binding affinity was selected. In addition to predicted minimal peptides, for each mutation, we designed 3 overlapping 15-mer peptides with a 12-residue overlap such that minimal peptides, for each mutation, we designed 3 overlapping 15-mer peptides were added to wells at a final concentration of 10 µg/mL. Wells containing anti-CD3/anti-CD28 coated beads (bead-to-cell ratio of 1:1) or anti-CD3/anti-CD28 coated beads (bead-to-cell ratio of 1:1) or human cytomegalovirus, Epstein-Barr virus, and influenza virus (CEF) peptides (10 µg/mL; AnaSpec) served as positive controls. Spots were enumerated using an automated plate reader (AID GmbH). We defined responses using empirical response methods: positive wells were required to contain a minimum of 10 spots/2 × 10^4 cells and have at least 3-fold more spots than negative control wells. These criteria have been shown to yield very low false positive rates (36).

To determine HLA restriction of the HSDL1 reactive clone from patient 3, B-lymphoblastoid cell lines (B-LCL) matched at 0 to 3 HLA alleles and corresponding to all 6 HLA class I alleles from patient 3 were obtained from an HLA restriction of the HSDL1 reactive clone from patient 3, B-lymphoblastoid cell lines (B-LCL) matched at 0 to 3 HLA alleles and corresponding to all 6 HLA class I alleles from patient 3 were obtained from an

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**Table 1. Immunohistochemistry scores for TIL and MHC**

<table>
<thead>
<tr>
<th>Patient 1</th>
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<td>CD3</td>
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**NOTE:** TIL were scored according to the number of intraepithelial cells: +++, ≥20; ++, 6–19; +, 1–5; or −, 0. MHC expression by tumor epithelium was scored semiquantitatively by comparison to positive stromal cells in the same or neighboring tissue cores: ++++, very high; ++, high; +, moderate; +, weak; −, negative.

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**IFN-γ ELISPOT**

Standard IFN-γ ELISPOT assays were performed as previously described (35). Predicted minimal peptides and overlapping 15-mer peptides were added to wells at a final concentration of 10 µg/mL. Wells containing anti-CD3/anti-CD28 coated beads (bead-to-cell ratio of 1:1) or human cytomegalovirus, Epstein-Barr virus, and influenza virus (CEF) peptides (10 µg/mL; AnaSpec) served as positive controls. Spots were enumerated using an automated plate reader (AID GmbH). We defined responses using empirical response methods: positive wells were required to contain a minimum of 10 spots/2 × 10^4 cells and have at least 3-fold more spots than negative control wells. These criteria have been shown to yield very low false positive rates (36).
in-house B-LCL bank or the Fred Hutchinson Cancer Research Center International Histocompatibility Working Group Cell and Gene Bank (Seattle, WA). HLA restriction was determined by IFN-γ ELISPOT using 9,000 cells/well of clone 1 incubated with 2 × 10⁵ B-LCL pulsed with the CYMEAVAL minimal peptide (10 μg/mL).

Clone 1 (10⁵ cells/well) was assessed by IFN-γ ELISPOT for recognition of ascites tumor samples (10⁵ cells/well), which had been depleted of CD45+ cells by magnetic bead separation (Miltenyi Biotec). An autologous CD4+ T-cell line served as a negative control target. CD45- ascites and the CD4+ T-cell line were pulsed with CYMEAVAL peptide (10 μg/mL for 2 hours) and used as a positive control.

**PCRs**

To measure the relative abundance of the clone 1 TCR-β transcript, RNA from ascites samples and IL-2 expanded T-cell lines was isolated using the AllPrep DNA/RNA Isolation Kit (Qiagen) and converted to cDNA using a qScript cDNA Synthesis Kit (Quanta Biosciences). A TRVB6-6-specific forward primer (TCACTGATAAAGGAGAAPCCTCCCC) and CDR3 clone-specific primer (AGTACTGGGTCTC-TAACGGCC) were used to amplify a 150 bp region of the TCR-β of clone 1. Actin transcript was amplified as a reference (forward primer CGTCTTCCCCTCCATCGTG; reverse primer TTCTCCATGTCGTCCCAGTTG). Amplification of target genes was detected using perfeCTa green supermix (Quanta Biosciences) and analyzed with a MyiQ thermocycler (Bio-Rad). By serial dilution of clone 1 into irrelevant polyclonal CD8+ T cells, the sensitivity of detection was determined to be approximately 1:10⁵ T cells.

**Results**

**Patient characteristics and mutational profiles**

All 3 HGSC cases showed evidence of spontaneous tumor immunity as indicated by the presence of intraepithelial TIL expressing CD3, CD8, and in some cases CD4 and CD20 (Table 1 and Supplementary Fig. S1). Moreover, tumors from all 3 patients expressed MHC class I and variable levels of MHC class II. Patients underwent standard treatment consisting of cytoreductive surgery followed by platinum-based chemotherapy with or without taxanes (Fig. 1). Patients 1 and 2 showed partial responses to primary treatment, whereas patient 3 achieved an initial clinical remission. All patients experienced progressive or recurrent disease, at which time they received additional chemotherapy. Patients 1 and 2 were generally nonresponsive to second line treatment, whereas patient 3 again achieved clinical remission, albeit for a shorter interval than the first remission. All 3 patients received a third cycle of chemotherapy, after which they succumbed to their disease. Additional clinical details have been published (31).

**Screening for T-cell responses to tumor mutations**

We investigated whether the 3 patients had spontaneous T-cell responses to the mutations identified by whole exome sequencing. Given that the mutational profiles were derived from ascites tumor samples, we assessed tumor-associated lymphocyte (TAL) lines derived from matched ascites samples. In initial experiments, TAL were expanded using reasoning that ascites would contain cells from multiple tumor regions. To address temporal heterogeneity, we sequenced tumor cells from 3 clinical time points, including primary surgery, first recurrence, and second recurrence. Patients 1 and 2 had a total of 22 and 31 mutations, respectively, and their mutational profiles were relatively stable, with only 1 and 6 mutations appearing or disappearing during disease progression. Patient 3 had a total of 40 mutations and a less stable mutational profile, with 11 mutations appearing or disappearing over time. Additional details about mutational profiles have been published (31).
a well-established method involving high-dose IL-2 (32). TAL lines were tested by IFN-γ ELISPOT for recognition of (a) predicted minimal peptides (Supplementary Tables S1–S3) and (b) overlapping 15-mer peptides corresponding to all of the mutations found in the respective patient’s tumor samples. This dual strategy captured the benefits of epitope prediction while additionally providing unbiased coverage of all possible MHC class I epitopes and many class II epitopes (39). We tested all identified nonsynonymous mutations except those that were present in germline (e.g., BRCA1), or resulted in nontranslated genes (e.g., SPATS2MSVT), or resulted in premature stop codons. Moreover, mutations were tested irrespective of their expression level to avoid excluding mutant gene products that might have been downregulated as a result of immune selection.

For patients 1 and 2, the IL-2–expanded TAL lines failed to respond to any of the mutant peptides at any of the time points (Supplementary Fig. S2). As positive controls, TAL lines responded to peptides from common viral antigens (CEF peptides) to which the patients had previously been exposed in vitro. For patients 1 and 2, the IL-2–expanded TAL lines failed to respond to predicted minimal peptides (black bars) and a set of 3 overlapping 15-mer peptides (white bars). Robust responses were seen against both the predicted minimal peptides and overlapping 15-mer sequences encoding the HSDL1L25V mutation. As positive controls, cells were stimulated with a pool of minimal CEF peptides, as well as minimal and overlapping 15-mers corresponding to a single HLA-A2 restriction (3 times background). Data are shown as the mean ± SD from 1 of 2 independent experiments.

Characterizing the T-cell response to HSDL1L25V

By assessing magnetically sorted CD4+ and CD8+ T-cell populations, we determined that the response to HSDL1L25V was mediated exclusively by CD8+ T cells (data not shown). A CD8+ T-cell clone recognizing HSDL1L25V (clone 1) was generated by limiting dilution cloning of CD8+ T cells from an IL-2–expanded TAL line. The TCR from clone 1 was amplified by PCR and sequenced, which revealed a single TCR-β sequence and both a productive and nonproductive TCR-α gene (data not shown). When clone 1 was tested by IFN-γ ELISPOT against a panel of all 8-, 9-, 10-, and 11-mer peptides spanning the HSDL1L25V point mutation, the 8 amino acid sequence CYMEAVAL was defined as the minimal epitope (Supplementary Fig. S3). Using a panel of partially HLA-matched allogeneic B cell lines, we determined that clone 1 recognized CYMEAVAL in the context of HLA-C*14:03 (Supplementary Fig. S4). This interaction was also predicted by the NetMHCpan-2.4 algorithm.

In contrast to the first 2 patients, TAL lines from patient 3 showed a clear response to predicted minimal and 15-mer peptides corresponding to a point mutation in the hydroxysteroid dehydrogenase-like protein 1 gene (HSCL1L25V; Fig. 2). This T-cell response was detected in TAL lines generated with high-dose IL-2 (Fig. 2) or anti-CD3/anti-CD28 beads, but it was not detected directly ex vivo in bulk ascites samples (data not shown). Moreover, the T-cell response was only detected in TAL lines from the first recurrence sample. We failed to see T-cell responses to peptides corresponding to any of the other mutations from patient 3, despite clear responses to the positive controls (Fig. 2 and data not shown). No mutation-specific responses were detected using anti-CD3/anti-CD28–expanded T cells from the primary solid tumor (data not shown). Thus, we focused our analysis on the T-cell response to HSDL1L25V.
algorithm in that CYMEAVAL had the strongest predicted HLA binding score of all candidate epitopes encoding HSDL1L25V across all 6 HLA alleles for this patient (Supplementary Table S3). Notably, clone 1 demonstrated absolute specificity for mutated HSDL1 when assessed using either minimal peptides or 15-mer peptides comprising the wild-type HSDL1 sequence (Fig. 3).

Recognition of autologous tumor by the HSDL1L25V-specific CD8+ T-cell clone

Based on read counts from the whole exome sequencing data, the relative frequency of the HSDL1L25V allele increased from 3.5% in the primary sample to 55.0% and 60.2% in the first and second recurrent samples (Fig. 4A; ref. 31). Thus, there was an increase in the number of cells harboring the HSDL1L25V point mutation during the progression from primary to recurrent disease. To assess the corresponding expression and presentation of the CYMEAVAL epitope over time, clone 1 was tested by IFN-γ ELISPOT for recognition of serial tumor samples. Clone 1 responded to tumor samples from all 3 time points (Fig. 4B). However, the 2 recurrent tumor samples elicited a far stronger response than the primary tumor sample. In contrast, all 3 tumor samples elicited strong responses from clone 1 when pulsed with CYMEAVAL peptide, indicating they were all conducive to T-cell stimulation when the epitope was not limiting (data not shown). Thus, it seems that the abundance of the CYMEAVAL epitope increased significantly between the time of primary surgery and first recurrence and was maintained at second recurrence.

We next examined the activity and abundance of HSDL1L25V-specific T cells in tumor samples from the 3 time points. By IFN-γ ELISPOT, HSDL1L25V-specific T-cell responses were only seen with in vitro expanded TAL lines derived from the first recurrence (Fig. 4C). To further investigate whether HSDL1L25V-specific T cells might be present at the other 2 time points, we performed additional TAL expansions. Knowing that clone 1 was CD8+, we used magnetic beads to enrich CD8+ T cells from ex vivo ascites and then expanded them using a REP (32). Despite using highly purified CD8+ lines, we again only detected an HSDL1L25V-specific T-cell response in the line derived from the first recurrence (data not shown). Intriguingly, this response was greatly diminished compared with that previously seen with the high-dose IL-2–derived TAL line, indicating that clone 1 expanded preferentially under the high-dose IL-2 condition. In summary, using several expansion methods, HSDL1L25V-specific T cells were only detected at first recurrence, despite the fact that the HSDL1L25V gene and CYMEAVAL epitope were abundant at both first and second recurrences.

To quantify HSDL1L25V-specific T cells independent of their ability to make IFN-γ, we considered measuring their abundance directly with an MHC class I tetramer. However, tetramer reagents for HLA-C*14:03 are not currently available. Moreover, flow cytometry with an antibody to the Vβ region used by clone 1 (Vβ 13.6) indicated that clone 1 represented at most 0.5% of CD8+ TAL at any time point (Supplementary Fig. S4A), indicating that there would be insufficient events for robust analysis with the available biospecimens. Instead, we designed clonotype-specific primers and measured TCR-β chain levels by quantitative PCR of genomic DNA and cDNA. By titrating known numbers of clone 1 T cells into a polyclonal CD8+ T-cell preparation, we found that the limit of detection of the PCR assay was approximately 1.10^5 cells (data not shown). By this assay, the presence of clone 1 paralleled that seen by ELISPOT in that (i) clone 1 was not detected directly ex vivo (i.e., in nonexpanded ascites samples), and (ii) it was detected in IL-2- or anti-CD3/anti-CD28 bead-expanded TAL lines from the first recurrence but not the primary sample or second recurrence (Fig. 4D). The PCR assay further revealed that clone 1 was not detectable in primary solid tumor. Thus, it seems that clone 1 arose during the first remission in step with the increasing abundance of the HSDL1L25V epitope but disappeared during the second remission despite continued expression of the epitope.

![Figure 3.](Image)

**Figure 3.** Clone 1 shows absolute specificity for mutated HSDL1. An HSDL1L25V-specific CD8+ T-cell clone (clone 1) was assessed by IFN-γ ELISPOT for responses to (A) overlapping mutated and wild-type 15-mer peptides spanning the L25V point mutation (1 = HSDL116-29, 2 = HSDL116-20, 3 = HSDL113-36), or (B) a titeration of minimal peptides encoding mutated (CYMEAVAL, closed circles) or wild-type (CYMEALAL, open squares) HSDL1. Responses are shown as the mean ± SD of IFN-γ spot-forming cells per 1 × 10^6 total cells.
Discussion

We systematically assessed the extent to which the mutant genome is recognized by the immune system in the context of standard treatment of HGSC. By studying a panel of 79 mutations from 3 patients and 3 clinical time points, we found a CD8⁺ T-cell response to the point mutation HSDL1L25V in 1 patient. This response was undetectable in the primary ascites and solid tumor samples, but emerged by the time of first recurrence, alongside a marked increase in expression of the mutant epitope by tumor cells. The patient underwent additional chemotherapy and achieved a second remission period, but this was short lived. At second recurrence, the mutant epitope was still abundant in tumor tissue, but the mutation-specific T-cell response was no longer detectable. Thus, it seems that during the first remission period, the immune system of this patient mounted a T-cell response against a mutation expressed by an expanding tumor subclone, but this response ultimately faltered.

Our results provide an example of the unaided immune system responding to the changing tumor mutanome, yet ultimately failing to eliminate mutation-bearing tumor cells. The rare, weak, and transient nature of the response described here highlights the general inadequacy of immune surveillance in the context of standard treatment, which is consistent with the high mortality rate for HGSC. On the positive side, our results leave open the possibility that the mutanome might represent an untapped reservoir of target antigens for immunotherapy.

A key finding of this study is that only a small proportion of tumor mutations (1/79 or 1.3% across 3 patients) seem to be spontaneously recognized by autologous T cells. Our conclusion is based on a comprehensive screening method...
that utilized predicted high-affinity peptides as well as unbiased overlapping 15-mer peptides. In theory, this method should be able to detect responses to all MHC class I epitopes, as well as many MHC class II epitope containing the mutation. Moreover, we interrogated TAL samples from 3 clinical time points, both directly *ex vivo* and after *in vitro* expansion by multiple methods so as to circumvent any immunosuppressive effects of the tumor environment. A low percentage of immunogenic mutations was also recently reported in advanced melanoma. Robbins and colleagues used whole exome sequencing to identify mutations in tumor samples from 3 patients, each of whom had shown an objective response to autologous TIL therapy (14). They identified 264 to 574 nonsynonymous mutations per tumor, of which 2 to 3 mutations per patient were specifically recognized by CD8⁺ T cells from the therapeutic TIL product. Thus, this study too found responses to only 0.3% to 1.1% of mutations, despite selecting patients who had responded well to TIL therapy and hence had demonstrably immunogenic tumors. Similarly, using an HLA tetramer-based assay, Schumacher and colleagues recently reported that only 2 of 448 predicted mutant CD8⁺ T-cell epitopes were recognized by CD8⁺ TIL from a patient with melanoma (15). Although additional studies are required, these data combined with ours indicate that only a minor fraction of point mutations spontaneously trigger T-cell responses. However, it remains possible that other types of mutations such as amplifications, gene fusions, or other large structural rearrangements might prove more immunogenic.

The above conclusion leads to the question of why the remaining 99% of mutations did not trigger a detectable T-cell response in patients. It is possible that additional T-cell responses could have been detected by measuring other markers of T-cell activation or using other assay methods, although IFN-γ ELISPOT is widely used as an indicator of tumor reactivity (40). Apart from detection methods, mutated gene products can be invisible to T cells for elementary reasons such as insufficient expression, lack of a high affinity MHC class I or II epitope, or absence of a corresponding TCR in the patient’s T-cell repertoire. In contrast, mutations that give rise to bona fide epitopes for which a corresponding T cell is present are potentially visible to the immune system. Yet responses to such mutations might still be thwarted by factors such as peripheral tolerance or immune suppression (41). Another possibility is immunologic ignorance, in which a potentially visible mutation fails to elicit a T-cell response because of ineffective priming, competition from higher affinity peptides, physical barriers, or other mechanisms (42, 43). We do not know how many of the mutations studied here were subject to immunologic ignorance, as our stimulation methods were not designed to prime naïve T-cell responses. However, such mutations are worthy of further study, as they represent an attractive class of potential target antigens for immunotherapy (44).

Although our sample size was small, we considered several possible reasons why patient 3 mounted a mutation-specific T-cell response whereas the other 2 patients did not. First, patient 3 had more mutations than the other patients, which increases the mathematical probability of having an immunogenic mutation. Second, patient 3 showed the greatest number of changes in the mutanome over time (11 changes vs. 1–6 changes). Indeed, the prevalence of the HSDL1L25V mutation increased from 3.5% to 60% of sequencing reads during progression from primary to recurrent disease; the corresponding increase in the abundance of the mutant epitope might have been sufficient to break immunologic ignorance or tolerance. Third, patient 3 experienced the greatest decrease in tumor burden during chemotherapy (Fig. 1; ref. 31). There is increasing evidence that chemotherapy can induce tumor immunity by causing the release of tumor antigens as well as signaling molecules such as HMGB1, ATP, and calreticulin (8). Reduced tumor burden can also provide relief from tumor-associated immunosuppressive factors. Further work with additional patients will be required to better define the factors that induce spontaneous antitumor immune responses during standard treatments.

Despite being present at first recurrence, clone 1 failed to prevent the outgrowth of antigen-positive tumor at second recurrence, suggesting that a profound impairment of this response occurred. A wide variety of immune suppressive mechanisms could have contributed to this phenomenon, including regulatory T cells, myeloid-derived suppressor cells, indoleamine 2,3-dioxigenase, and PD-L1, each of which has been reported in ovarian cancer and can impair T-cell expansion and function (45–47). In addition, the fact that a large proportion of tumor cells continued to present the mutant epitope at second recurrence suggests that clone 1 may have experienced chronic antigen exposure. This can lead to loss of T-cell functions in a defined sequence: IL-2 production → cytolytic activity → proliferation → IFN-γ → apoptosis (clonal deletion; ref. 48). With IFN-γ ELISPOT, one can detect T cells even at the far end of this continuum, underscoring the appropriateness of this assay. However, at second recurrence, clone 1 was undetectable not only by ELISPOT but also by PCR, which would be consistent with clonal deletion. This raises the specter that other tumor-specific T-cell responses might have experienced a similar fate earlier in tumor progression, contributing to the negative results for other mutations.

Looking forward, our results raise the possibility of targeting the potentially large reservoir of mutant epitopes that are visible to the immune system yet go unrecognized. This might be achieved with immune modulating strategies such as CTLA-4 or PD-1 blockade (10). Notably, a large proportion of CD8⁺ TAL in patient 3 expressed PD-1 by flow cytometry (data not shown), suggesting their activity could potentially have been enhanced by PD-1 blockade. Looking ahead to an era in which mutanome data are available for most patients with cancer, one can envision using mutation-encoding vaccines to focus T-cell responses more specifically toward tumor cells, as recently demonstrated in a mouse model of melanoma (49). Indeed, one can speculate that vaccination against the HSDL1L25V mutation during first remission might have prevented the expansion of the
corresponding tumor subclone in patient 3. However, this example also raises the important issue of intratumoral heterogeneity, as the HSDL1<sup>123V</sup> mutation at its peak was present in only 60% of sequencing reads, suggesting it may not have been present in all tumor cells. Tumor heterogeneity can potentially be addressed by targeting multiple mutations that collectively cover the complete phylogeny of tumor subclones. Alternatively, one could attempt to target mutations that arise early in tumorigenesis and are present in all tumor cells. A recent study of spatial heterogeneity in HGSC revealed that tumors harbor 15 to 55 such mutations (37). In summary, our findings with the HSDL1<sup>123V</sup> mutation illustrate the importance of developing immunotherapeutic strategies that not only overcome immune suppression but also contend with the spatial and temporal heterogeneity of the tumor genome.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions

References

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