Surveillance of the tumor mutanome by T cells during progression from primary to recurrent ovarian cancer

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STATEMENT OF 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 three ovarian cancer patients undergoing standard treatment. Of 79 mutations tested, we identified a CD8+ T cell response to a point mutation in the hydroxysteroid dehydrogenase-like protein 1 (HSDL1) 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 future.
ABSTRACT

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 ovarian cancer patients undergoing standard treatment.

Experimental Design: Tumor-associated T cells from three ovarian cancer patients were assessed by ELISPOT for recognition of non-synonymous 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 HSDL1L25V was detected in one patient. In the primary tumor, the HSDL1L25V 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 HSDL1L25V–specific CD8+ T cell response. At second recurrence, the HSDL1L25V 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, 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.
INTRODUCTION

There is longstanding 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-beta2 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 anti-tumor responses in many cancer patients.

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 pre-existing CD8+ TIL show survival benefit from anthracycline-based chemotherapy, whereas patients lacking CD8+ TIL do 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 clinical responses in patients with a variety of solid tumors (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 immunological 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 three patients who had responded well to therapeutic TIL infusions (14). A large proportion of T cells in the therapeutic TIL product were found to recognize MHC class I epitopes derived from 2-3 somatic mutations from each patient. Similar results were obtained for a melanoma patient 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 1-3 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 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 three HGSC patients (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 the present study, we investigated the hypothesis that acquired mutations might trigger responses by tumor-associated T cells, potentially resulting in immunological selection against tumor subclones harboring such mutations.
METHODS AND MATERIALS

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 high-grade serous carcinoma (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% fetal bovine Serum (FBS), 40% complete media (RPMI 1640 containing 10% FBS, 25 mM HEPES, 2 mM L-glutamine, 50µM beta-mercaptoethanol and 1 mM 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, Tucson, AZ) with primary antibodies to CD3 (Clone SP7, Spring Biosciences, catalogue #M3074), CD8 (Clone SP16, Spring Biosciences, catalogue #M3162), CD4 (Clone SP35, Spring Biosciences catalogue #M3354), CD20 (Spring Biosciences catalogue #M3354), CD20 (Spring Biosciences catalogue #E2560), MHC class I (clone EMR8-5, MBL catalogue #D226-3, Woburn, MA), and MHC class II (clone CR3/43, Affinity Bioreagents catalogue # MAI-25914). Bound antibodies were detected using a biotinylated secondary antibody (Jackson Immunoresearch, West Grove, PA) and a DABMap kit (Ventana) followed by counterstaining with hematoxylin (Ventana). Lymphocyte densities and MHC intensity were scored by visual inspection using semi-quantitative 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 semi-quantitatively 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 1x10^6 cells ml in the presence of either high dose human 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-PE MicroBeads (Miltenyi Biotec). CD8+ T cells were expanded *in vitro* using the rapid expansion protocol (REP) (32). Cultures were split every 3-4 days with addition of fresh cytokines, and additional REPs were performed as needed. Prior to ELISPOT, cells were cultured in resting media consisting of complete media containing 10 ng/ml IL-7 (Peprotech) and 1 IU/ml IL-2 for 3 days.

**T cell cloning**

An 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<sup>125V</sup> reactivity were positively selected through magnetic bead separation and serially diluted down to one cell per well in 96-well tissue culture plates. Cultures were stimulated to proliferate by adding irradiated feeder cells (a pool of three 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 2-3 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 Vβ 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 (34) (Supplemental Tables 1-4). All peptides predicted to bind MHC with an affinity $\leq 50$ nM were selected for screening.

Additionally, for those mutations that did not yield peptides with binding scores of $\leq 50$ nM, the minimal peptide with the highest predicted MHC binding affinity was selected. In addition to predicted minimal peptides, for each mutation we designed three overlapping 15-mer peptides with a 12-residue overlap such that all possible 8-, 9-, 10- and 11-mer peptides containing the point mutation were represented, as well as many MHC class II binding peptides. Initial screens used crude peptides, whereas all subsequent experiments involving HSDL1 used peptides with $>90\%$ purity. Peptides were commercially synthesized (Genscript) and reconstituted in dimethyl sulfoxide (Sigma-Aldrich).

**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 ug/mL. Wells containing anti-CD3/anti-CD28 coated beads (bead-to-cell ratio of 1:1) or CEF viral antigen peptide pools (10 ug/mL) (Anaspec) served as positive controls. Spots were enumerated using an automated plate reader (AID GmbH). We defined responses using Empirical Response (ER) methods: positive wells were required to contain a minimum of 10 spots/2 x 10^5 cells and have at least three-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 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 2x10^5 B-LCL pulsed with the CYMEAVAL minimal peptide (10 ug/mL).

Clone 1 (10^5 cells/well) was assessed by IFN-γ ELISPOT for recognition of ascites tumor samples (10^5 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 ug/mL for 2h) and used as a positive control.

**Polymerase Chain Reactions**

To measure the relative abundance of the Clone 1 TCRβ 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 (TCAGTGATAAGGAGAAGTGCG) and CDR3 clone-specific primer (AGTACTGGGTCCTACGCGG) were used to amplify a 150bp region of the TCRβ of clone 1. Actin transcript was amplified as a reference (forward primer CGTCTTCCCCTCCATCGGT; reverse primer TTCTCCATGTCGTCCAGTTG).

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 1:10^5 T cells.

**RESULTS**

**Patient characteristics and mutational profiles**

All three 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 Supplemental Fig. 1). Moreover, tumors from all three 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 non-responsive to second line treatment, whereas Patient 3 again achieved clinical remission, albeit for a shorter interval than the first remission. All three patients received a third cycle of chemotherapy, after which they succumbed to their disease. Additional clinical details have been published (31).

As previously reported, we performed whole exome sequencing on ascites tumor samples from these three patients (31). Recognizing the considerable spatial and temporal heterogeneity of mutational profiles in prior studies of HGSC (31, 37, 38), we elected to sequence tumor cells from the ascites compartment rather than solid tumor, reasoning that ascites would contain cells from multiple tumor regions. To address temporal heterogeneity, we sequenced tumor cells from three 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).

Screening for T cell responses to tumor mutations

We investigated whether the three 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 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 (Supplemental Tables 1-3) 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 non-synonymous mutations except those that were present in germline (e.g., BRCA1), or resulted in non-translated genes (e.g. SPATS2MIV), 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 (Supplemental Fig. 2). As positive controls, TAL lines responded to peptides from common viral antigens (CEF peptides) to which the patients had previously been found to respond. As an additional positive control, TAL lines responded strongly to stimulation with anti-CD3/anti-CD28 coated beads (data not shown). To mitigate the concern that mutation-specific T cells might have been lost during IL-2 expansion, we generated additional T cell lines from ascites samples using an alternate expansion method involving anti-CD3/anti-CD28 coated beads. In addition, we tested TAL directly *ex vivo* in bulk ascites samples. As before, we failed to detect T cell responses to any of the mutant peptides, whereas T cell responses were seen to the CEF peptides and anti-CD3/anti-CD28 coated beads (data not shown).

In contrast to the first two 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 (HSDL1^{L25V}) (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 37 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 HSDL1^{L25V}.

**Characterizing the T cell response to HSDL1^{L25V}**

By assessing magnetically sorted CD4^+ and CD8^+ T cell populations, we determined that the response to HSDL1^{L25V} was mediated exclusively by CD8^+ T cells (data not shown). A CD8^+ T cell clone recognizing HSDL1^{L25V} (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 TCRβ sequence and both a productive and non-productive TCRα 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 HSDL1^{L25V} point mutation, the 8 amino acid sequence CYMEAVAL was defined as the minimal epitope (Supplemental Fig. 3). 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 (Supplemental Fig. 4). This interaction was also predicted by the NetMHCpan-2.4 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 (Supplemental Table 3). 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) (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 three time points (Fig. 4B). However, the two recurrent tumor samples elicited a far stronger response than the primary tumor sample. In contrast, all three 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 appears 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 three 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 two 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 rapid expansion protocol (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 to 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, HSDL1\textsuperscript{L25V}–specific T cells were only detected at first recurrence, despite the fact that the HSDL1\textsuperscript{L25V} gene and CYMEAVAL epitope were abundant at both first and second recurrence.

To quantify HSDL1\textsuperscript{L25V}–specific T cells independent of their ability to make IFN-\(\gamma\), 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 V\(\beta\) region used by Clone 1 (V\(\beta\) 13.6) indicated that Clone 1 represented at most 0.5% of CD8\(^+\) TAL at any time point (Supplemental Fig. 4A), indicating that there would be insufficient events for robust analysis with the available biospecimens. Instead, we designed clonotype-specific primers and measured TCR \(\beta\) 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 \(1 \times 10^5\) cells (data not shown). By this assay, the presence of Clone 1 paralleled that seen by ELISPOT in that (a) Clone 1 was not detected directly ex vivo (i.e., in non-expanded ascites samples), and (b) 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 appears that Clone 1 arose during the first remission in step with the increasing abundance of the HSDL1\textsuperscript{L25V} epitope but disappeared during the second remission despite continued expression of the epitope.

**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 three patients and three clinical time points, we found a CD8\(^+\) T cell response to the point mutation HSDL1\textsuperscript{L25V} in one 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 appears 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 three patients) appear 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 three 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 three patients, each of whom had shown an objective response to autologous TIL therapy (14). They identified 264 to 574 non-synonymous mutations per tumor, of which 2-3 mutations per patient were specifically recognized by CD8+ T cells from the therapeutic TIL product. Thus, this study too found responses to only 0.3-1.1% 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 melanoma patient (15). Although additional studies are required, this data combined with ours indicates 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 immunological ignorance, in which a potentially visible mutation fails to elicit a T cell response due to 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 immunological 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 two 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 versus 1-6 changes). Indeed, the prevalence of the HSDL1^{L25V} mutation increased from 3.5% to 60% of tumor cells during progression from primary to recurrent disease; the corresponding rise in the abundance of the mutant epitope might have been sufficient to break immunological ignorance or tolerance. Third, Patient 3 experienced the greatest decrease in tumor burden during chemotherapy (Fig. 1) (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 anti-tumor 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-dioxygenase, 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 $\rightarrow$ cytolytic activity $\rightarrow$ proliferation $\rightarrow$ IFN-γ $\rightarrow$ apoptosis (clonal deletion) (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 spectre 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 is available for most cancer patients, 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 highlights the important issue of intratumoral heterogeneity, as the HSDL1L25V mutation at its peak was present in only 60% of 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 15-55 such mutations (37). In summary, our findings with the HSDL1L25V 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.
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REFERENCES


**Table 1.** Immunohistochemistry scores for tumor-infiltrating lymphocytes and MHC. TIL were scored according to the number of intra-epithelial cells: +++ ≥ 20, ++ 6-19, + 1-5, or - 0. MHC expression by tumor epithelium was scored semi-quantitatively by comparison to positive stromal cells in the same or neighboring tissue cores: +++ strong; ++ moderate; + weak; - negative.

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<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>+++</td>
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</tr>
<tr>
<td>CD8</td>
<td>++</td>
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<tr>
<td>CD4</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>CD20</td>
<td>-</td>
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</tr>
<tr>
<td>MHC I</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>MHC II</td>
<td>+++</td>
<td>+</td>
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</tr>
</tbody>
</table>
**FIGURE LEGENDS**

**Figure 1. Clinical course of the three study patients.** All three patients were diagnosed with HGSC and underwent cytoreductive surgery at \( t = 0 \). Tumor burden is indicated by blood CA-125 levels (U/mL) (black dots). Chemotherapy cycles are indicated by horizontal black bars and involved the following drugs: CATX, carboplatin with paclitaxel; CAG, carboplatin with gemcitabine; ETO, etoposide; CA, carboplatin; TX, taxol; GM, gemcitabine; DX, pegylated liposomal doxorubicin. Downward arrows indicate time points at which ascites samples (containing tumor cells and TAL) were collected. The cross symbol indicates the time of death. These and additional clinical data have been previously published (31).

**Figure 2. T cell responses to tumor-specific mutations in HGSC.** A T cell line was derived from the first recurrence ascites sample of Patient 3 using high dose IL-2 and assessed by IFN-\( \gamma \) ELISPOT for recognition of peptides encoding 37 mutations. For each mutation, the T cell line was exposed to the 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-mers encoding the HSDL1\(^{L25V}\) 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-restricted CEF epitope (CEF11). Responses are shown as the number of IFN-\( \gamma \) spot forming cells per \( 2 \times 10^5 \) total lymphocytes in expanded cultures. The hashed line represents the threshold for positivity (three times background). Data are shown as the mean +/- S.D. from one of two independent experiments.

**Figure 3. Clone 1 shows absolute specificity for mutated HSDL1.** An HSDL1\(^{L25V}\)-specific CD8+ T cell clone (Clone 1) was assessed by IFN-\( \gamma \) ELISPOT for responses to (A) overlapping mutated and wild type 15-mer peptides spanning the L25V point mutation (\( 1 = \text{HSDL1}^{15-29}, 2 = \text{HSDL1}^{18-32}, 3 = \text{HSDL1}^{21-35} \)), or (B) a titration of minimal peptides encoding mutated (CYMEA\text{VAL}, closed circles) or wild type (CYMEAL\text{AL}, open squares) HSDL1. Responses are shown as the mean +/- SD of IFN-\( \gamma \)-spot forming cells per \( 1 \times 10^5 \) total cells.
Figure 4. Dynamics of the HSDL1<sup>L25V</sup> mutation and corresponding CD8+ T cell response during disease progression. (A) To assess the abundance and expression of HSDL1<sup>L25V</sup> in tumor samples over time, the allelic frequencies of mutant versus wild type HSDL1 genes were inferred from whole exome sequencing data of CD45-depleted tumor samples (80-99% CD45-negative by flow cytometry) (31). (B) To determine whether the HSDL1<sup>L25V</sup> epitope was presented by tumor cells, Clone 1 was assessed by IFN-γ ELISPOT for recognition of autologous CD45-depleted ascites tumor samples (96-98% CD45-negative by flow cytometry) from primary and recurrent time points. An autologous CD4+ T cell line served as a negative control in place of tumor cells. Responses are shown as the number of IFN-γ spot forming cells per 2x10<sup>5</sup> total cells as measured by IFN-γ ELISPOT assay. (C) To measure the magnitude of the CD8+ T cell response to HSDL1<sup>L25V</sup> over time, cells from the ascites at each time point were assessed directly <i>ex vivo</i> (white bars) and after expansion with high dose IL-2 (black bars) for responses to the minimal epitope (CYMEAVAL) by IFN-γ ELISPOT. Responses are shown as the number of IFN-γ spot forming cells per 2x10<sup>5</sup> total cells. Similar results were obtained using overlapping 15-mer peptides (data not shown). (D) To directly measure the abundance of Clone 1 in tumor samples, the corresponding clonotype-specific TCRβ sequence was detected by quantitative PCR of cDNA from <i>ex vivo</i> ascites samples and IL-2-expanded T cell lines (from panel C). Data are shown as the mean +/- S.D.
Wick et al., Figure 1

![Graphs showing CA-125 levels over time for three patients, marked with CATX, CAG, and ETO for Patient 1; CA, TX, CM for Patient 2; and CATX, CAG, DX for Patient 3.](image-url)
Wick et al., Figure 2

The figure shows a bar graph with the x-axis labeled as "mutant gene" and the y-axis labeled as "IFNγ spots per 2x10⁶ cells". The graph compares 15-mer peptides and predicted minimal peptides. Each bar represents a different mutant gene, with the height indicating the number of IFNγ spots.
Wick et al., Figure 3

(A) IFN-γ spots per 10^5 cells for HSD1 mutated 15-mers and HSD1 WT 15-mers.

(B) IFN-γ spots per 10^5 cells for CYMEAVL (mutated) and CYMEAL (WT) peptides at different concentrations (ng/mL).
Wick et al., Figure 4

A

B

C

D
Surveillance of the tumor mutanome by T cells during progression from primary to recurrent ovarian cancer

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