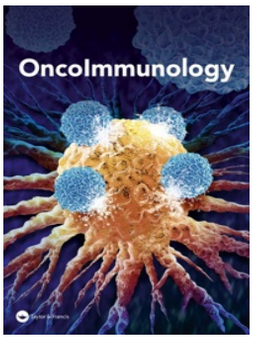

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Identification of TTLL8, POTEE, and PKMYT1 as immunogenic cancer-associated antigens and potential immunotherapy targets in ovarian cancer

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ABSTRACT

Most high-grade serous ovarian cancers (OC) do not respond to current immunotherapies. To identify potential new actionable tumor antigens in OC, we performed immunopeptidomics on a human OC cell line expressing the HLA-A02:01 haplotype, which is commonly expressed across many racial and ethnic groups. From this dataset, we identified TTLL8, POTEE, and PKMYT1 peptides as candidate tumor antigens with low expression in normal tissues and upregulated expression in OC. Using tissue microarrays, we assessed the protein expression of TTLL8 and POTEE and their association with patient outcomes in a large cohort of OC patients. TTLL8 was found to be expressed in 56.7% of OC and was associated with a worse overall prognosis. POTEE was expressed in 97.2% of OC patients and had no significant association with survival. In patient TILs, increases in cytokine production and tetramer-positive populations identified antigen-specific CD8 T cell responses, which were dependent on antigen presentation by HLA class I. Antigen-specific T cells triggered cancer cell killing of antigen-pulsed OC cells. These findings suggest that TTLL8, POTEE, and PKMYT1 are potential targets for the development of antigen-targeted immunotherapy in OC.

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Cancer immunology; immunotherapy; ovarian cancer; tumor antigen

Introduction

Ovarian cancer (OC) is the fourth most common gynecological malignancy. The most frequently diagnosed subtype, high-grade serous ovarian cancer (OC), is also the deadliest, with a mortality rate of 70%.[1] Despite this, there has been little improvement in patient treatment outcomes, which have remained unchanged for decades. Recent breakthroughs in cancer immunotherapies that instruct a patient's immune system to specifically target their tumors have quickly become standard of care options for many patients. Despite these promising results, immune checkpoint inhibitor therapy has only proven successful in a subset of patients, and patients with OC have not responded favorably.[2–5] Other immunotherapies, including adoptive cell therapy and cancer vaccines, also continue to be ineffective treatment options for OC.

The failure of immunotherapy in OC is often attributed to the relatively low mutation burden present in OC tumors, affording low numbers of neoantigens for the immune system to target.[6,7] OC is thus regarded as a non-immunogenic cancer. Nevertheless, multiple publications over decades have shown a positive association between an increased number of tumor-infiltrating T cells and prolonged survival in OC.[8–10] Furthermore, earlier work from our lab[11] as well as others,[12–14] has demonstrated that clinically relevant tumor antigens do exist in OC. Taken together, these studies indicate that the immune system is strongly involved in this disease's biology and that patients could benefit from new immunotherapeutic strategies.

Cancer/testes (CT) antigens are a class of tumor-specific antigens (TSAs) with an expression commonly restricted to normal, immune-privileged tissues such as testes and placenta and with aberrant expression in tumor cells. This means they can be targeted for their immunogenicity without the risk of off-tumor effects on normal tissues. There currently is considerable interest in identifying tumor antigens that can serve as targets for engineered T cell-based therapies.

In this study, we have used immunopeptidomics to identify new CT antigens in ovarian cancer that may have clinical utility. We have demonstrated that antigen-specific T cells exist within the T cell repertoire of ovarian cancer patients and that *ex vivo* activation of antigen-specific T cells can target cancer cells in a co-culture system. These findings suggest that TTLL8, POTEE, and PKMYT1 hold promise as targets for the development of antigen-specific immunotherapy for OC.

Materials and methods

Immunopeptidomics

HLA peptide purification, mass spectrometric analysis, and data analysis were performed as previously described.[11,15]

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Briefly, we used 1×10^8 OVCAR-5 cells, which were lysed using a buffer containing 0.25% sodium deoxycholate, 0.2 mm iodoacetamide, 1 mm EDTA, protease inhibitors, 1 mm PMSF, and 1% octyl-β-D glucopyranoside in PBS. HLA class I immunoprecipitation was performed using the W6/32 monoclonal antibody clone conjugated to Protein-A Sepharose beads.

Proteomic data analysis

The MS/MS data was analyzed using Proteome Discoverer v2.4 (Thermo Fisher Scientific). Peptide length was restricted to 7–25 amino acids with a maximum peptide mass of 1500 Da and a maximum charge state of 3. Peptide spectra were searched against the Human UniProtKB database combined with a common contaminant database. Results were filtered to retain peptides with a false discovery rate (FDR) <5%.

Peptide affinity analysis

Duplicate peptides were filtered from the dataset before affinity analysis. MHCMotifDecon 1.2[16] was used to deconvolute the peptide data and map the peptide repertoire for each HLA allele. Default settings for class I peptides (length 8-14aa) were used for affinity analysis. NetMHCpan version 4.1 and NetMHCcons 1.1[17] was used to generate consensus affinity values for each peptide GTEx Gene expression data was obtained from The Human Protein Atlas,[18] GTEx Portal,[19] and TCGA TARGET GTEx through the UCSC Xena Browser (https://xenabrowser.net/).[20]

Patient samples

Informed consent was obtained from women diagnosed with advanced-stage ovarian cancer prior to surgery. Ascites, tumor, and blood samples were collected during standard procedures at Mayo Clinic, Phoenix, AZ. This study was approved by the IRB 18-010082 of Mayo Clinic.

Ascites cell isolation

Ascites fluid was collected by centrifugation. The supernatant was removed, and red blood cell lysis was performed using BDPharmLyse (BD Biosciences). The cells were cryopreserved in a solution of 90% FBS and 10% DMSO.

In vitro tumor-infiltrating lymphocyte (TIL) culture

TILs from OC patients expressing the HLA-A02:01 haplotype were expanded with peptide antigens *in vitro*. 1E6 TILs per mL were cultured in RPMI 1640, 10% FBS, 50 µM of 2-ME (63689-25 ML-F, Millipore Sigma), 5 ng of IL-2 (200–02, PeproTech), 2.5 ng of IL-7 (581904, BioLegend), 2.5 ng of IL-15 (21–8153-U010, Tonbo Biosciences) and 2 µg/mL of peptide. Media and cytokines were replenished every 3 days during culture. After 7–20 days, cells were restimulated with peptide for subsequent analyses.

Tetramer generation and staining

Tetramer exchange was performed using the MBL QuickSwitch™ Quant tetramer kit, employing a proprietary peptide exchange factor. Exchange efficiency was quantified using magnetic capture beads to capture tetramers and FITC-labeled antibody for detecting the exiting peptide, with decreased FITC signal indicating successful exchange. PE-labeled MHC class-I tetramers carrying PKMYT1 (HLDVKPANI), PKMYT1–2 (GLSSELRSV), POTEE (SGDGVTHTV), TTLL8 (ALLAPLRGA), CMV (NLVPMVATV), and HIV (SLYNTVATL) peptides were generated using the QuickSwitch™ Quant HLA-A *02:01 Tetramer Kit (TB-7300-K1, MBL International) following the accompanying instructions. Tetramer exchange efficiency was quantified using magnetic capture beads to capture tetramers and FITC-labeled antibody for detecting the exiting peptide, with decreased FITC signal indicating successful exchange. The cells were incubated for tetramer staining with PE-labeled MHC class-I tetramers and surface markers (CD3, CD4, CD8).

Flow cytometry

Expanded TILs were restimulated with peptides in the presence of GolgiPlug and incubated for 6 hours. The cells were stained with live/dead stain and antibodies for surface markers. Cells were fixed, permeabilized with Cytofix/Cytoperm™ buffer (BD Biosciences), and stained with antibodies for intracellular targets. OVCAR-5 cells were seeded in a 96 well-plate for co-culture experiments and loaded with corresponding peptides. For blocking experiments, OVCAR-5 cells were peptide-pulsed and pre-treated with W6/32 antibody 10 µg/mL (#311428, Biolegend) or IgG control (#400166, Biolegend) for one hour before adding TILs at 3:1 effector-to-target (E:T) ratio. Following 8 hours of co-culture, the cells were collected for staining. A positive response was defined as at least a 2-fold increase over the unstimulated sample from the same donor. In addition, we have confirmed that a minimum of 20–30 cytokine-producing cells were present per positive response and ensured that a of 50,000 events per condition were acquired and analyzed using FlowJo software (version 10.8.0), except for Patient 3's unstimulated condition where 30,000 events were analyzed due to limited sample availability. The detailed gating strategy showing parent and daughter populations with applied gates is presented in Supplementary Figure S3A.

Tumor cell killing assay

The T cell killing assay was performed following co-culture of the peptide-stimulated T cells with peptide-pulsed, GFP-labeled OVCAR-5 cells for 48 hours and 5 days. For IFNγ pretreatment experiments, GFP-labeled OVCAR-5 cells were cultured with recombinant human IFNγ 20 ng/ml (21–8319-U020, Tonbo Biosciences) for 24 hours prior to co-culture with patient-derived TILs at 10:1 E:T ratio. Cell confluency of GFP-labeled OVCAR-5 cells in co-culture was assessed and images were acquired using the IncuCyte S3 system (Sartorius).

Data availability

All data supporting this study's findings are available within the article and its supplemental information files or from the Lead Contact upon reasonable request.

Results

Identification of HLA-A02:01-restricted peptide antigens in ovarian cancer

The goal of our study was to identify new tumor antigen targets presented through the HLA-A02:01 allele, which is the dominant allele expressed across several racial and ethnic groups.[21] We performed immunopeptidomics with the HLA-A02:01-positive OVCAR-5 cell line (Figure 1a, schematic) and identified 10,197 peptides corresponding to 5,604 unique proteins (Supplementary Table S1). Most peptides were nine amino acids in length (Figure 1b), as expected of class I restricted peptides. Next, we used MHCMotifDecon to map each peptide to the known HLA alleles expressed in OVCAR-5. Most of the peptides were predicted to bind to HLA-A02:01, and the experimentally derived HLA motifs matched the known sequence motifs for the dominant alleles, A01:01 and A02:01 (Figure 1c,d, Supplementary Figure. S1). We next used NetMHCcons to generate consensus affinity values for each peptide. The top 2% of the predicted binders to HLA-A02:01 all fell well below the 500 nM cutoff, which is commonly used to predict the high-affinity binding of the peptide to the HLA (Figure 1e). These data indicate that our immunopeptidomics pipeline was sufficient to identify high-confidence HLA-A02:01-restricted peptides.

Next, we sought to identify potential CT antigens within our protein list. To do this, we compared our protein list to a previously published list of 1105 putative CT antigens,[22] many of which have yet to be fully characterized. We identified 25 unique proteins, represented by 34 peptides, that overlapped between the lists. Since many of these proteins have yet to be validated as CT antigens, we next analyzed the expression profiles of each mRNA transcript in the GTEx Portal (www.gtexportal.org) to assess the expression of the proteins across normal tissues. 21/25 of the antigen transcripts demonstrated tissue-enriched expression within the testes (Supplementary Figure. S2A). Three of the transcripts (SAMD7, CT45A7, and HNRNPCL4) were not detected in any normal tissue, and one transcript (C11orf71) was widely expressed across all tissues (Supplementary Figure. S2A).

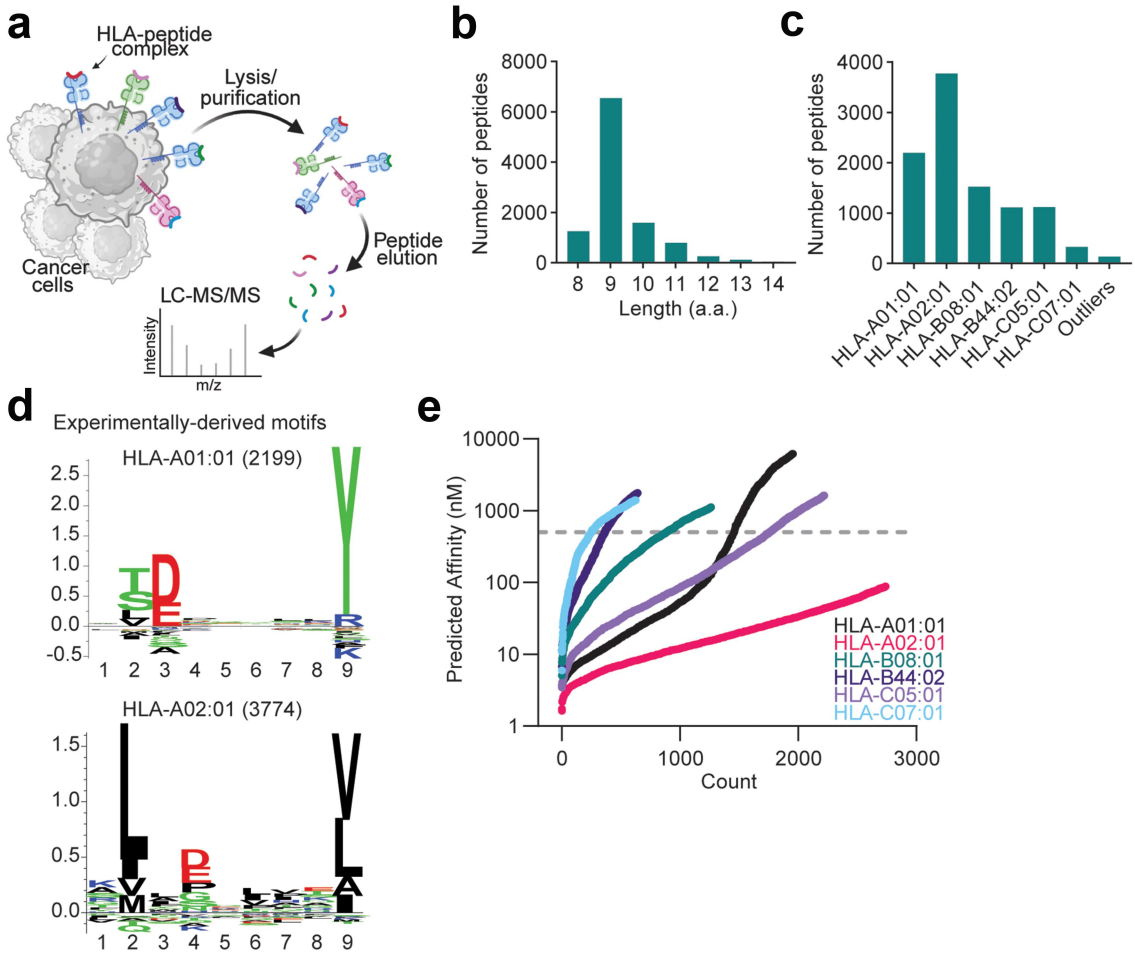

Figure 1. Immunopeptidomics analysis of the HLA-A02:01 OVCAR-5 cell line. (a) Schematic overview of the immunopeptidomics approach. OVCAR-5 cells were collected and lysed, followed by the immunoprecipitation and purification of HLA-peptide complexes. HLA-bound peptides were eluted and identified by LC-MS/MS. Created with BioRender.com. (b) Distribution of identified peptide length in HLA-A02:01-positive OVCAR-5 cell line. (c) Deconvolution of peptide data according to the predicted binding to each HLA allele using MHCMotifDecon. (d) Experimentally derived HLA motifs in OVCAR-5 using MHCMotifDecon. (e) Assessment of peptide binding affinity using NetMHCcons . Peptides were filtered to include only the top 2% HLA binders.

Since the primary focus of our study was to identify HLA-A02:01-binding antigens, we next prioritized the peptides based on the predicted binding affinity to HLA-A02:01 using NetMHCcons (Figure 2a). Of the 34 peptides, 18 peptides from 16 unique proteins had a predicted binding affinity of less than 5,000 nM to HLA-A02:01 (Figure 2a). We next assessed each of

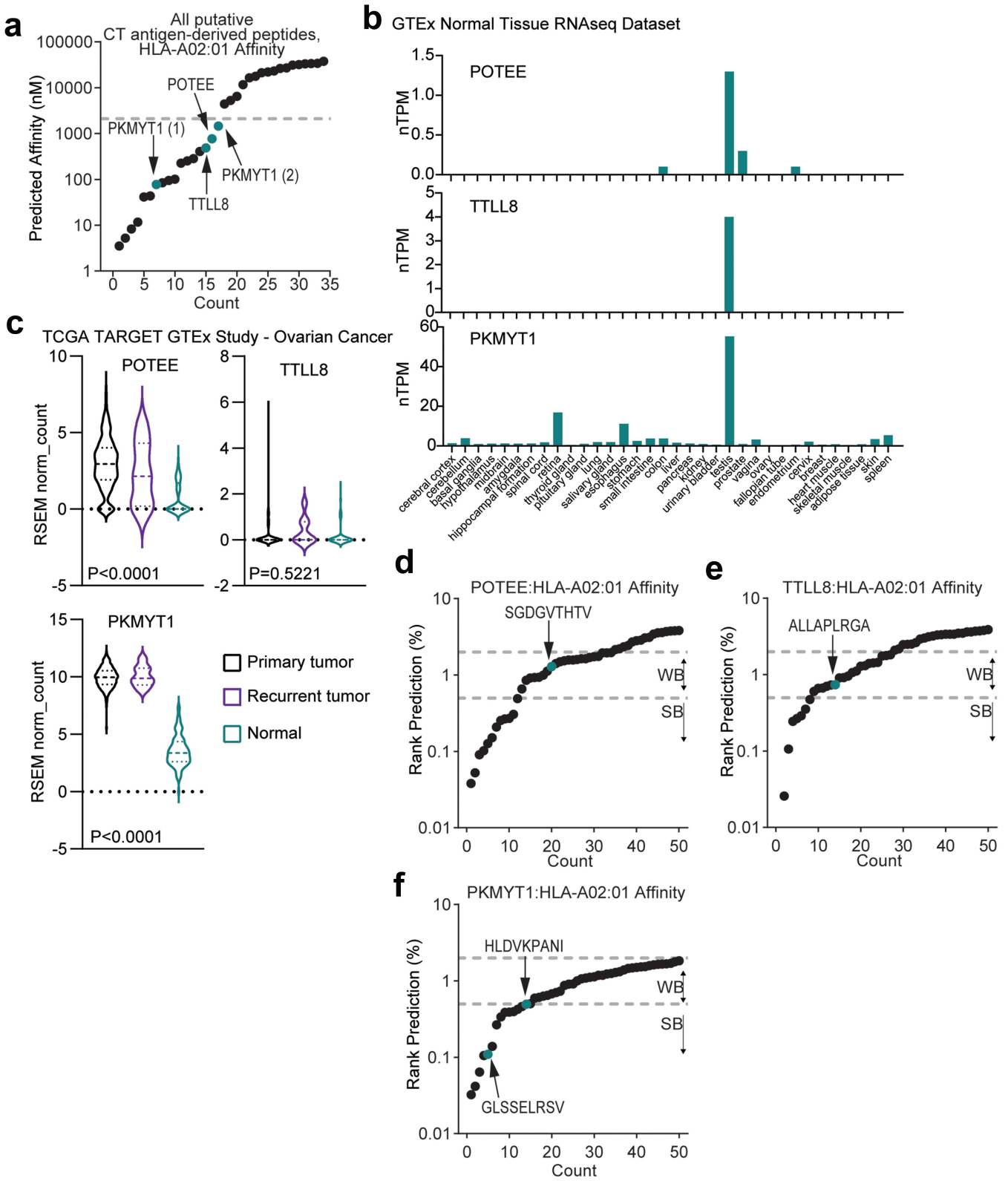

Figure 2. Immunopeptidomics identifies putative CT antigens in ovarian cancer. (a) The predicted binding affinities of CT peptides were evaluated for their binding affinity to HLA-A02:01 using NetMHCcons. TTLL8, PKMYT1, PKMYT1–2, and POTEE peptides are indicated. The dashed line indicates 5,000 nM. (b) mRNA expression of POTEE, TTLL8, and PKMYT1 across normal tissues in the GTEx Portal (www.Gtexportal.org). (c) Data from the TCGA TARGET GTEx study was used to compare mRNA expression of POTEE, TTLL8, and PKMYT1 in primary tumors, recurrent tumors, and normal ovaries. Data was downloaded using the UCSC Xena Browser ([20]). The rank prediction of all possible (d) POTEE, (e) TTLL8, and (f) PKMYT1 peptides from each whole protein. The rank represents the relative binding strength of peptides to HLA-A02:01 and was analyzed using NetMHCpan for each protein. Peptides identified by immunopeptidomics are indicated.

these prioritized protein targets for its mRNA and protein level expression in human OC using the UCSC Xena Browser and the Human Protein Atlas dataset, respectively. Of the 16 potential binders, 8 targets had mRNA expression in ovarian cancer that was significantly elevated over normal control samples, while two targets were significantly elevated in the normal control as compared to the tumor (Supplementary Figure. S2B). However, protein expression data was available for only 12/16 targets in the Human Protein Atlas. Among these, 6 targets showed validated protein expression in human ovarian cancer, with three proteins expressed in more than 50% of the tumors examined (Supplementary Figure. S2C). Of these, POTEE demonstrated low mRNA expression across all normal tissues except for testes (Figure 2b) and high mRNA and protein expression in OC tumors (Supplementary Figure. S2C) with the mRNA significantly increased in the tumors as compared to normal tissue (Figure 2c). TTLL8 likewise demonstrated tissue-enriched expression in normal testes (Figure 2b) and was expressed at the protein level in greater than half of ovarian tumors (Supplementary Figure. S2C). However, this expression was divergent from the mRNA expression in the TCGA dataset, which only showed TTLL8 mRNA expression in a limited number of patients (Figure 2c). PKMYT1 protein expression in OC was not yet determined in the Human Protein Atlas (Supplementary Figure. S2C), though it's expression in ovarian cancer has been previously described.[23] The mRNA expression was testes-enriched and significantly increased in OC compared to normal tissue (Figure 2b,c). Assessment of the affinity of all possible peptide binders from each protein to HLA-A02:01 demonstrated that the peptides identified using immunopeptidomics from POTEE (Figure 2d) and TTLL8 (Figure 2e) were predicted to be weak binders. In contrast, the two identified peptides from PKMYT1 were predicted to be strong binders (Figure 2f). Taken together, this data indicates that POTEE, TTLL8, and PKMYT1 exhibit favorable tissue-restricted expression, are prevalent in a high percentage of ovarian cancers, and cancer cells can present endogenously processed peptides from these proteins that bind to HLA-A02:01. Consequently, these proteins were selected for further investigation as putative OC antigens.

Tissue microarray analysis confirms CT antigen protein expression in a large cohort of ovarian cancer patients

Next, we sought to assess the protein expression of TTLL8 and POTEE in a large clinical cohort of OC tumors. PKMYT1 was not included in this analysis as other groups have previously published these findings and found that PKMYT1 is a predictive marker of poor prognosis in several cancer types.[23–27] We first validated commercially available antibodies for immunohistochemistry (Figure 3a). TTLL8 and POTEE expression were increased in ovarian tumors compared to the normal tissues tested. TTLL8 displayed diffuse cytoplasmic staining in OC, while POTEE appeared both cytoplasmic and localized to the membrane (Figure 3a). IHC was initially performed on 120 patient samples (360 cores in triplicate). 13 patients were excluded due to lack of evaluable data for both POTEE and TTLL8. Our final analysis included 106 patients for POTEE and 104 patients for TTLL8. The majority (85%) of the patients had high-grade serous histology and had late-stage disease (84.2%) (Supplementary Table S2–4). TTLL8 was expressed in 56.7% of tumors, while POTEE was expressed in 97.2%. We next assessed whether the expression of the antigens had any effect on patient survival. TTLL8 expression (score 1, 2, or 3) was notably associated with poorer overall survival (Unadjusted HR 1.8, $p = 0.01$). However, this correlation was not significant when adjusted for stage and debulking (HR 1.5, $p = 0.12$) (Figure 3b). While a trend was observed regarding the correlation between TTLL8 expression and progression-free survival, it did not reach significance (Unadjusted HR 1.4, $p = 0.25$; Adjusted for stage and debulking HR 1.1, $p = 0.64$) (Figure 3c). In contrast, POTEE was found to be expressed at some level in nearly all tumors with only 3/106 tumors scoring negative. Therefore, we compared those tumors with 0 or 1 (a little) expression levels with those that scored 2 or 3 (a lot). However, we found that POTEE expression had no prognostic significance (OS: Unadjusted HR 1.0, $p = 0.85$; Adjusted for stage and debulking HR 1.2, $p = 0.45$; PFS: Unadjusted HR 0.6, $p = 0.08$; Adjusted for stage and debulking HR 0.8, $p = 0.48$) (Figure 3d,e). Since many CT antigens are known to be regulated by methylation, we next assessed whether the DNMT inhibitor, decitabine (DAC), could increase the expression of the antigens in OC cell lines. DAC treatment significantly increased the expression of POTEE at the mRNA level (Supplementary Figure. S2d) but had no effect on TTLL8 or PKMYT1 expression (data not shown).

Patient TILs respond to CT antigen stimulation

Next, we determined whether TTLL8, POTEE, and PKMYT1 could elicit antigen-specific CD8 T cell responses by analyzing cytokine production and tetramer-positive populations in patient samples. To accomplish this goal, we identified four ascites samples within our biobank collected from patients with a confirmed histologic diagnosis of high-grade serous OC undergoing surgery at the Mayo Clinic. Ascites-derived T cells were used for the functional assays conducted. We confirmed that the identified patients were positive for HLA-A02:01 and then stimulated these samples with each peptide and low-dose IL-2, IL-7, and IL-15. After 7–20 days, the samples were restimulated, and intracellular interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα) were evaluated. Overall, the CT peptides were found to induce CD8 T cell activation as assessed by an increase in IFNγ and TNFα over the control peptides (Figure 4a,b). Positive responses were observed in 4/4 of patients tested for TTLL8 and 3/4 tested for POTEE and PKMYT1 peptides. Next, to show that patient-derived T cells expressed T cell receptors that were specific for the CT antigen peptide/HLA-A02:01 complex, we generated custom HLA-A02:01 tetramers containing each CT peptide (Supplementary Figure. S4a). Staining of the expanded TIL cultures from Patient 1 with tetramers revealed the presence of antigen-specific T cells that target the CT antigens TTLL8, POTEE, and PKMYT1 bound to HLA-A02:01 (Figure 4e,f).

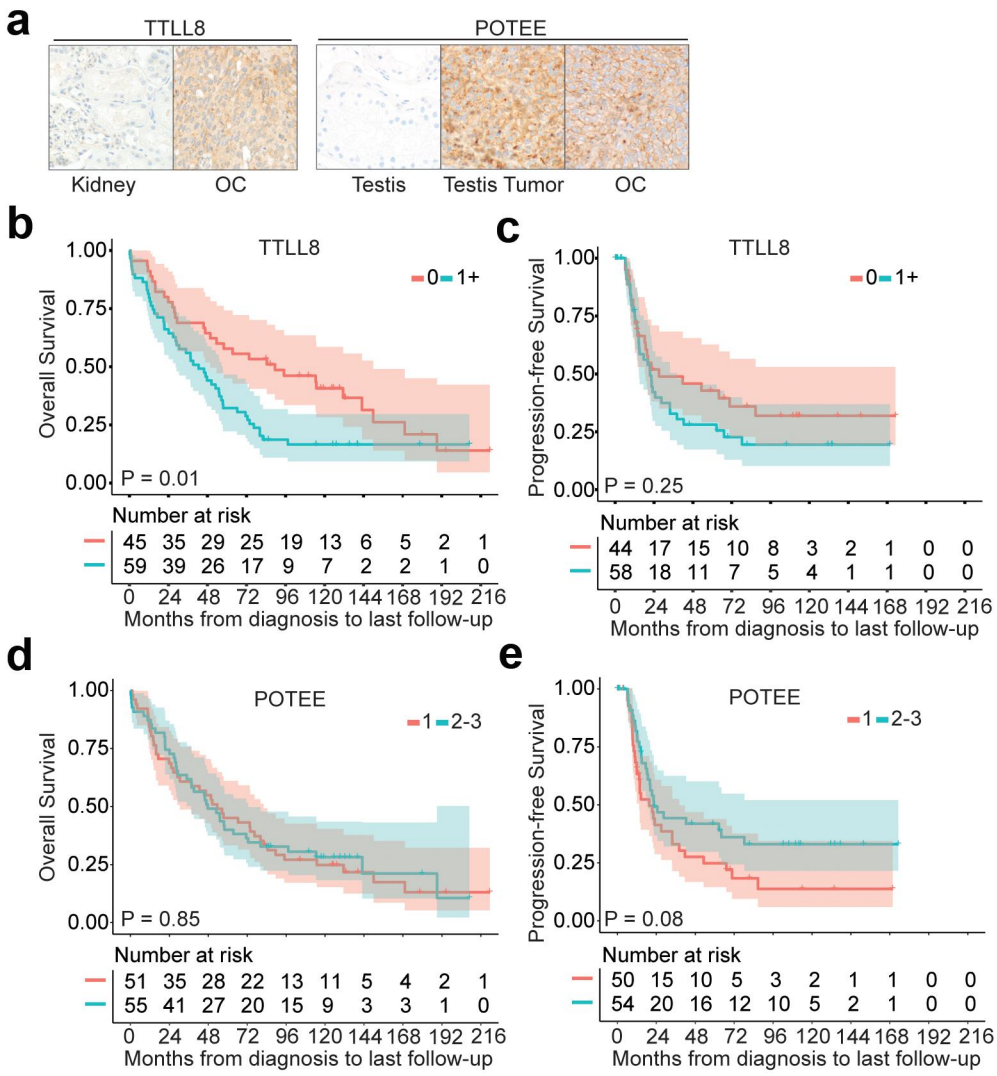

Figure 3. TTLL8 and POTEE are frequently expressed in clinical ovarian cancer. (a) Representative images of immunohistochemistry-based evaluation of TTLL8 and POTEE protein expression in indicated tissues. (b, c) Kaplan-Meier curves showing overall survival (b) and progression-free survival (c) comparing patients with no TTLL8 expression (score: 0) versus patients with TTLL8 expression (scores: 1, 2, or 3) in their tumors. (d, e) Kaplan-Meier curves showing overall survival (d) and progression-free survival (e) comparing patients with no or little POTEE expression (score: 0 or 1) versus patients with high POTEE expression (scores: 2 or 3) in their tumors.

CT antigen stimulation induces T cell-directed tumor cell killing

Next, we analyzed the ability of antigen-primed TILs to target and kill OC cells *in vitro*. HLA-A02:01-positive OVCAR-5 cells were pulsed with each peptide, and then pre-activated TILs from three patients were co-cultured with the target cancer cells in multiple E:T ratios. Overall, the TILs were found to target and kill the peptide-pulsed OVCAR-5 cells in a dose-dependent manner (Figure 5a,b and Supplementary Figure. S4B, C). Consistent with the cytokine production observed in Figure 4a, OVCAR-5 cells pulsed with the TTLL8 peptide were killed more effectively by the activated T cells from patient 1 than those stimulated with other peptides. Each patient sample exhibited enhanced cytotoxicity when compared to the non-stimulated cells. Pretreatment of non-peptide pulsed OVCAR-5 cells with IFNγ to enhance antigen presentation significantly enhanced TIL-mediated cytotoxicity across all three patient samples (Supplementary Figure. S4D). We next sought to determine whether T cell activation depended on HLA class I presentation of the peptides. To accomplish this, we treated TTLL8-pulsed OVCAR-5 cells with a pan-class I HLA blocking antibody, W6/32, prior to the co-culture with TTLL8-activated TILs and then analyzed cytokine production from the TILs. Treatment of the cancer cells with W6/32 significantly abrogated the increased production of cytokines from TILs that resulted from the co-culture compared to IgG-treated cells (Figure 5c).

Discussion

Our primary objective of this study was to use immunopeptidomics to discover novel antigens in ovarian cancer. Various immunotherapies have shown efficacy in targeting specific tumor-associated markers, yet their success in treating OC has been restricted. Nevertheless, studies consistently indicate that infiltrating T cells in ovarian tumors enhances the survival rates of OC patients, implying the existence of OC-specific tumor antigens that have yet to be elucidated. This study identified immunogenic peptides from the antigens TTLL8,

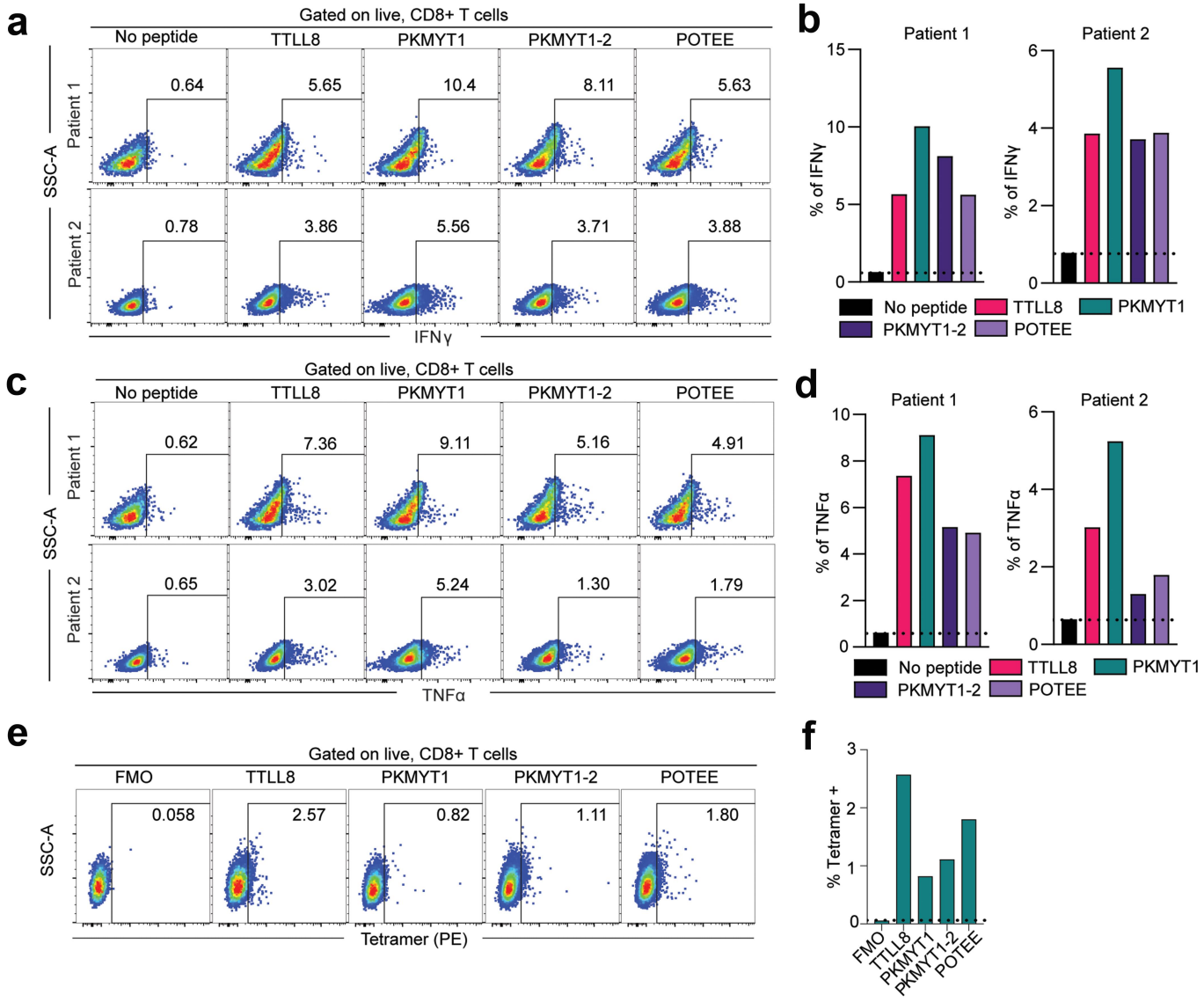

Figure 4. CT antigens activate peptide-specific T cell responses in patient TILs. (a, b) Intracellular cytokine staining (ICS) for IFNγ in CD8 T cells from HLA-A02:01+ patients 1 and 2 and (C, D) ICS for TNFα in CD8 T cells from HLA-A02:01+ patients 1 and 2 with HGSOC. T cells were stimulated with each indicated peptide prior to staining and assessed via flow cytometry. (e, f) Tetramer staining of CD8 T cells from HLA-A02:01+ patient 1. Peptide-expanded T cells were stained with custom pre-labeled tetramers containing TTLL8, PKMYT1, PKMYT1–2, or POTEE peptides.

POTEE, and PKMYT1 in an HLA-matched T cell culture. This study highlights the presence of unique tumor-specific antigens within ovarian tumors and the potential of CT antigens as promising targets for immunotherapy in OC.

CT antigens are a unique class of tumor-associated antigens characterized by tissue-restricted expression to immune-privileged organs, elevated expression in tumor tissues, and the capacity to produce immunogenic antigens.[28] Several members of the CT antigen family have already been identified in ovarian cancer, such as the MAGE genes, NY-ESO-1, SSX, and CT45.[29]

Of the three antigens we focused on in this study, only the expression of POTEE had been previously characterized in ovarian cancer. The POTEE peptide that we identified was previously identified in a large-scale immunopeptidomics study comparing the HLA ligandome of primary ovarian tumors with benign tissue.[30] Another study focused on the analysis of transcript levels of the *POTE* gene family found that they are highly expressed in ovarian cancer tumors, and expression of some of the family members, including POTEE, correlated with worse overall survival.[31] Additionally, it has been shown that POTEE is upregulated in recurrent ovarian cancer as compared to the primary tumor, and it correlated with hypomethylation at the 5' promoter and enhancer regions.[32] These findings were consistent with the findings of two other studies, which revealed unique tumor-promoting functions of POTEE in colorectal cancer.[33,34] While our analysis of POTEE protein expression via IHC in an independent cohort did not reveal a similar correlation with survival, this discrepancy may be attributed to the lack of specificity of the antibody for distinct POTE family members, given their high sequence similarity. The biological function of POTTEE in OC remains unknown. Therefore, further studies are warranted to elucidate whether POTEE plays a similar role in ovarian cancer as has been demonstrated in colorectal cancer.

PKMYT1 has not been previously characterized as a CT antigen. Although it demonstrates tissue-enriched expression

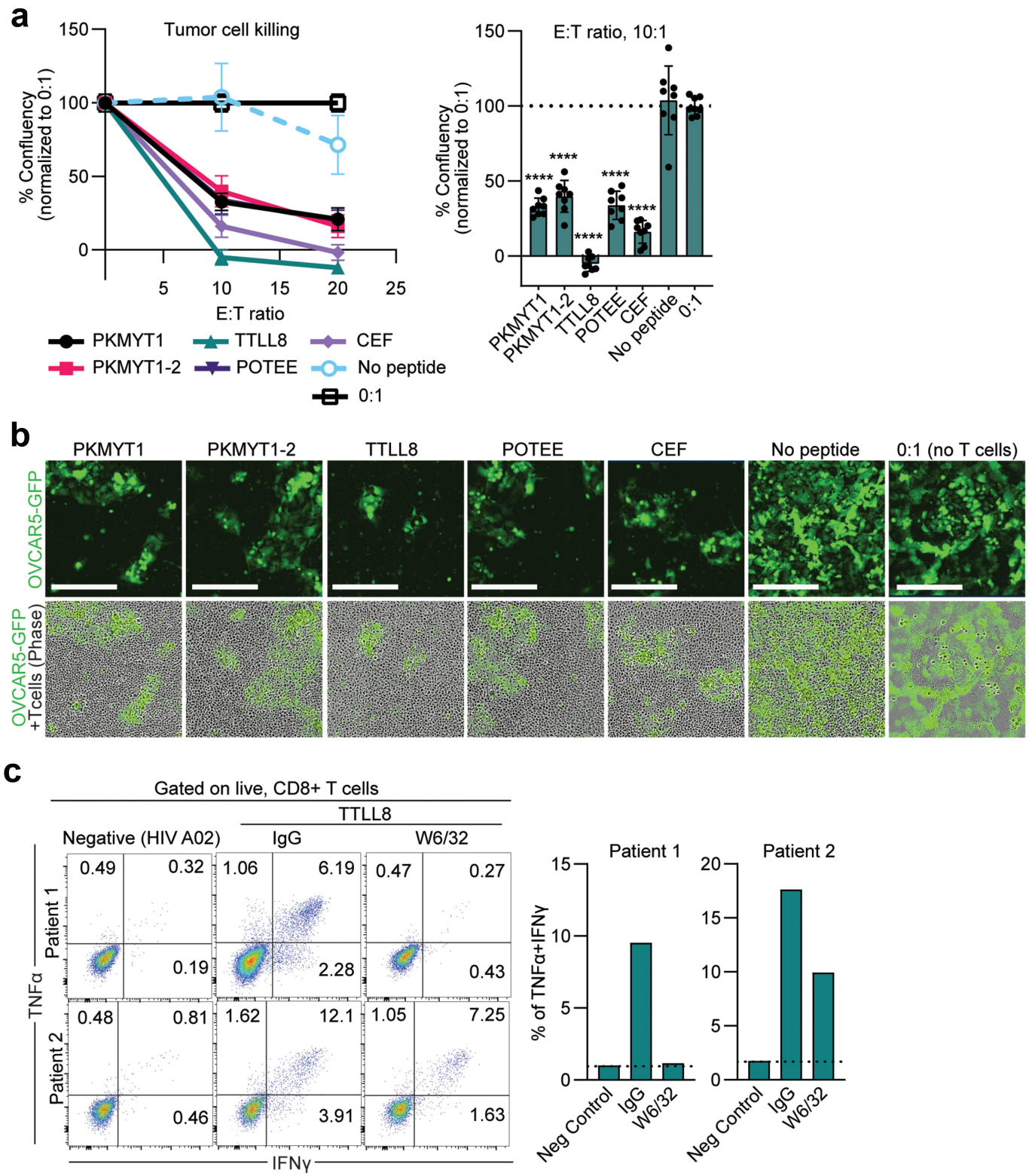

Figure 5. CT antigens activate T cell-mediated killing of ovarian cancer cells. (a) Assessment of peptide-specific T cell cytotoxicity at 96-hour incubation with peptide-pulsed OVCAR-5 cells at the indicated E:T ratios. Data is normalized to OVCAR-5 cells alone at 96-hour. ****$p < 0.0001$. (b) Representative images of the tumor cell killing assay after 96 hours at a 10:1 E:T ratio. The 0:1 ratio represents OVCAR-5 alone without TILs, establishing the baseline confluency. Scale bar: 250 µm. (c) HLA class I blocking assay. ICS for IFNγ and TNFα in CD8 T cells from two HLA-A02:01+ patients with ovarian cancer. CD8 T cells were pre-activated with the TTLL8 peptide and then co-cultured with TTLL8-pulsed OVCAR-5 for 8 hours with W6/32 or IgG control prior to staining. An HLA-A02:01-restricted HIV peptide was used as the negative control.

in the testis, it also exhibits low-to-mid-level RNA expression in some normal tissues. Previous studies have shown that PKMYT1 is upregulated in several types of cancer, including ovarian cancer[23] and this correlates with worse survival.[24–27] Early studies on PKMYT1 identified its central role in regulating cell cycle progression via phosphorylation of Cdc2.[35–37] However, more recent studies have shown that PKMYT1 and the structurally related kinase, Wee1, have redundant functions

in normal cells and that loss of PKMYT1 only becomes lethal in the context of the increased replication stress associated with cancer.[38–40] Notably, PKMYT1 is synthetically lethal, specifically within the context of Cyclin E1-amplified ovarian cancers.[40] Cyclin E1 is amplified in approximately 20% of ovarian cancers and is crucial in chemoresistance.[41,42] This study identified two peptides from PKMYT1 with high affinity for HLA-A02:01 that could elicit T cell activation and T cell-mediated tumor cell killing. Future studies will need to verify whether PKMYT1 can be safely targeted by engineered T cells before such a therapy advances to clinical trials. While numerous "normal" proteins have been targeted via T cell-directed therapies with success and minimal off-tumor toxicities (e.g., CD19, mesothelin, and the folate receptor), the safety of targeting PKMYT1 will require further evaluation.

In contrast to POTEE and PKMYT1, TTLL8 has the least understood role in the context of cancer. TTLL8 is a tubulin glycine ligase and is necessary, along with the related ligase, TTLL3, for initiating the glycyl chain on tubulin, which is crucial to the overall regulation of cytoskeletal function.[43,44] TTLL3 has also been shown to be required for the formation of primary cilia, and loss of TTLL3 in colon epithelial cells resulted in increased cell proliferation and promoted the development of colon cancer in mouse models.[45] In contrast, our study found that increased TTLL8 expression was associated with a worse prognosis. Further studies are needed to elucidate the biological role of TTLL8 in ovarian cancer and understand why its expression may be associated with worse survival outcomes.

In summary, this study has identified and validated three novel ovarian cancer-associated antigens that hold promise for developing antigen-targeted immunotherapies. Given the pressing need for new therapies that can elicit robust anti-tumor immunity in ovarian cancer, these findings offer a promising avenue for future research.

Disclosure statement

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