POLE Proofreading Mutations Elicit an Antitumor Immune Response in Endometrial Cancer


Abstract

Purpose: Recent studies have shown that 7% to 12% of endometrial cancers are ultramutated due to somatic mutation in the proofreading exonuclease domain of the DNA replicase POLE. Interestingly, these tumors have an excellent prognosis. In view of the emerging data linking mutation burden, immune response, and clinical outcome in cancer, we investigated whether POLE-mutant endometrial cancers showed evidence of increased immunogenicity.

Experimental Design: We examined immune infiltration and activation according to tumor POLE proofreading mutation in a molecularly defined endometrial cancer cohort including 47 POLE-mutant tumors. We sought to confirm our results by analysis of RNAsseq data from the TCGA endometrial cancer series and used the same series to examine whether differences in immune infiltration could be explained by an enrichment of immunogenic neoepitopes in POLE-mutant endometrial cancers.

Results: Compared with other endometrial cancers, POLE mutants displayed an enhanced cytotoxic T-cell response, evidenced by increased numbers of CD8+ tumor-infiltrating lymphocytes and CD8A expression, enrichment for a tumor-infiltrating T-cell gene signature, and strong upregulation of the T-cell cytotoxic differentiation and effector markers T-bet, Eomes, IFNG, PRF, and granzyme B. This was accompanied by upregulation of T-cell exhaustion markers, consistent with chronic antigen exposure. In silico analysis confirmed that POLE-mutant cancers are predicted to display more antigenic neoepitopes than other endometrial cancers, providing a potential explanation for our findings.

Conclusions: Ultramutated POLE proofreading-mutant endometrial cancers are characterized by a robust intratumoral T-cell response, which correlates with, and may be caused by an enrichment of antigenic neoepitopes. Our study provides a plausible mechanism for the excellent prognosis of these cancers.

Introduction

Endometrial cancer is the commonest gynecologic malignancy in the Western world, and affects approximately 150,000 women each year in Europe and the United States combined (1). Endometrial cancers have traditionally been classified into endometrioid and nonendometrioid tumors according to clinical and histopathological criteria. However, recent work has shown that this dualistic model can be improved upon by a molecular classification into subgroups that more accurately reflect underlying tumor biology and clinical outcome (2, 3).

One interesting subgroup is the 7% to 12% of endometrial cancers with somatic mutation in the proofreading exonuclease domain of the DNA replicase POLE (2, 4–6). Polymerase proofreading is essential for ensuring fidelity of DNA replication (7), and in keeping with its dysfunction, POLE proofreading-mutant cancers have a frequency of base substitution (8). We and others have recently shown that, despite the association with high grade, POLE-mutant endometrial cancers display other distinctive features, including a characteristic mutation signature, with a preponderance of C>A transversions and bias for particular amino acid substitutions, and strong associations with endometrioid histology, high grade, and microsatellite stability (10). We and others have recently shown that, despite the association with high grade, POLE-mutant endometrial cancers have an excellent prognosis (5, 6, 11). However, the reasons for this were unclear.

Although the ability of the immune system to suppress malignant disease has long been recognized (12), the last few years have seen a remarkable increase in our understanding of...
the complex and dynamic interplay between cancers and the host immune response. For example, preclinical and translational studies have confirmed that tumor missense mutations can lead to presentation of antigenic neoepitopes by MHC class I molecules, resulting in activation of T-cell–mediated cytotoxicity (13–16). Consequently, mutations that cause strongly antigenic epitopes are likely to undergo negative selection in developing tumors (13, 14). Cancers also demonstrate multiple alternative mechanisms of immune escape, including downregulation of HLA class I expression, and upregulation of immunosuppressive molecules, including PD1/PD-L1, TIM3, LAG3, and TIGIT, in a phenomenon referred to as adaptive immune resistance (17, 18). Despite this, it is clear that in many cancers, the immune system retains a degree of control over tumor growth—illustrated by the association between increased density of tumor-infiltrating lymphocytes (TIL), particularly CD8+ cytotoxic T cells, and favorable outcome in multiple cancer types, including endometrial cancer (19–22). Interestingly, a recent study has shown that CD8+ cell infiltration correlates strongly with the number of predicted antigenic mutations in tumors (23), suggesting that the immunogenicity of cancers is determined at least partly by their mutational burden (24). These data are consistent with the observations that hypermutated microsatellite unstable (MSI) endometrial and colorectal cancers typically display greater TIL density than other tumors (25, 26).

During our previous studies (4–6), we noted that POLE-mutant endometrial cancers frequently displayed strikingly high TIL density, often accompanied by a Crohn-like reaction. Similar observations have recently been reported following pathologic review of POLE-mutant TCGA endometrial cancers (27). We hypothesized that this may represent infiltration by cytotoxic T lymphocytes, which could in turn contribute to the favorable prognosis of these tumors. We also speculated that this might relate to an increase in antigenic neoepitopes in POLE-mutant endometrial cancers secondary to ultramutation. We tested this using a molecularly defined cohort of endometrial cancers, including 47 POLE-mutant tumors, and the recently published TCGA series (2).

**Translational Relevance**

The recently described subgroups of tumors with mutations in the proofreading domain of the DNA polymerase POLE are notable for both their striking mutation burden and, in the case of endometrial cancer, their excellent prognosis. The demonstration that POLE proofreading-mutant endometrial cancers display an enhanced antitumor T-cell response may explain the favorable outcome of these tumors, and suggests that similar investigation in POLE proofreading-mutant cancers of other types may be informative. Furthermore, as both tumor mutation burden and T-cell infiltration have been shown to predict response to immune checkpoint inhibitors, our results also raise the possibility that these drugs may benefit patients with recurrent POLE proofreading-mutant endometrial cancers, and patients with POLE-mutant tumors of other histologies, for whom clinical outcomes are less certain.

**Materials and Methods**

**Patients and tumors**

Tumors were selected from the PORTEC-1 and PORTEC-2 studies (n = 57; refs. 28, 29) and endometrial cancer series from the Leiden University Medical Centre (LUMC, Leiden, the Netherlands; n = 67) and the University Medical Centre Groningen (UMCG, Groningen, the Netherlands; n = 26), to provide similar numbers of low- (grade 1/2) and high-grade (grade 3) tumors of the common molecular subtypes: POLE wild-type, MSS; POLE wild-type, MSI; and POLE (proofreading) mutant (Supplementary Table S1). All cases were of endometrioid histology (EEC), and all POLE-mutant tumors were MSS. Of 373 cases reported in the TCGA series, 245 had paired whole-exome and RNA-seq data and were informative for this analysis (2). Of these, 197 were EECs, four mixed EEC/NEECs, and 44 serous (NEEC) cancers. Ethical approval for tumor molecular analysis was granted at LUMC, UMCG, and by Oxfordshire Research Ethics Committee B (Approval No. 05/Q1605/66).

**POLE mutation and microsatellite instability status**

DNA was extracted from FFPE blocks and sequencing of POLE hotspot exons 9 and 13 (which contain around 90% of pathogenic proofreading mutations) was performed in tumors from the PORTEC, LUMC, and UMCG series as previously reported (4, 5). All mutations were confirmed in at least duplicate PCR reactions. Details of whole-exome sequencing performed by the TCGA have been previously reported (2). Pathogenic POLE proofreading mutations were defined as somatic variants within the exonuclease domain associated with ultramutation and a frequency of C>A transversions of ≥20% (10).

MSI status was determined in the PORTEC, LUMC, and UMCG cases by a five-marker panel of microsatellites as reported previously (30), with exception of four cases in which this failed, where status was determined by MLH1, MSH2, MSH6, and PMS2 IHC (6). Determination of MSI in the TCGA series was by a seven-marker panel, with MSI-H defined as alteration at ≥3 markers (2). Classification of POLE-mutant TCGA endometrial cancers was also informed by results of recent analysis of mononucleotide repeats in 48 genes (10). In our analysis, only MSI-H cases were classified as MSI—we excluded one TCGA case for which MSI status could not be determined. The single tumor that displayed both POLE proofreading mutation and MSI by these criteria was assigned to the POLE-mutant group on the basis of its characteristic mutation signature, in accordance with a recent report (10).

**Histologic assessment**

Tumors were evaluated for the presence/absence of TILs and for Crohn-like reaction by a gynecologic pathologist (T. Bosse) blinded to other clinicopathological data.

**IHC and cell quantification**

Following deparaffinization, antigen retrieval, and blocking of peroxidase activity, whole slides were incubated overnight at 4°C (CD8, CD3) or room temperature (TIA-1, HLA, FoxP3) with primary antibodies against CD8 (1:50, clone C8/144B, DAKO, Agilent technologies), CD3 (1:25, clone F7.2.38, DAKO), HCA2 and HC10 (both 1:800, kindly provided by Prof. Dr. J. Neefjes, the Netherlands Cancer Institute), FoxP3 (1:100, mAbcam 450, Abcam), and TIA-1 (1:400, clone 2G9A10F5, Beckman Coulter). Sections were subsequently incubated with either anti-mouse...
Envision+ reagent (K4000, DAKO) for 30 minutes (CD8 primary), RAMpo (1:100) and GARpo (1:100) secondary and tertiary antibodies, (CD3 primary), or BrightVision-Poly/HRP (Poly-HRP-GAM/R/R; DPV0110HRP; ImmunoLogic; HCA2, HC10, FoxP3, and TIA-1) before 3,3-diaminobenzidine (DAB) treatment and hematoxylin counterstaining. Slides were then dehydrated and mounted before digitalization (ScanScope, Aperio Technologies, or Ultra Fast Scanner 1.6 RA). Philips and analysis.

CD8+, CD3+, FOXP3+, and TIA-1+ cell numbers were quantified in intraepithelial and intrastromal regions in the center of the tumor (CT) and the invasive margin (IM) as previously reported (19, 22). For each region, the mean number of positive cells in eight high-power fields (HPF; 200 μm × 200 μm) was calculated. For analysis of HLA expression, the percentage of tumor cells with membranous HCA2 and HC10 staining was quantified as previously described (31). In each case, scoring was performed independently by two observers, blinded to other clinicopathological data.

Immunofluorescence
Following deparaffinization, antigen retrieval, and blocking of peroxidase activity, whole slides were stained overnight at 4°C with primary antibody against TIA-1 (1:50, ab2712, Abcam). Sections were subsequently incubated with anti-mouse Envision+ reagent (K4000, DAKO) for 30 minutes and HRP visualized using cyanine 5 tyramide signal amplification (TSA) according to the manufacturer’s instructions (PerkinElmer). Next, whole slides were stained overnight with primary antibody against CD8 (1:25, clone C8/144B, DAKO) and a biotinylated antibody against fibronectin (1:50, ab6584, Abcam). Slides were incubated with GaM-AF555 (1:150 Life Technologies) and streptavidin-dylight488 (1:150, Thermo Scientific), counterstained with DAPI (Life Technologies) and mounted in prolong gold mounting medium (Life Technologies). Immunofluorescent slides were scanned using a TissueFAXs imaging system (TissueGnostics). Processed channels were merged using Adobe Photoshop CS5 (Adobe).

Leukocyte methylation scores
Leukocyte methylation scores (syn1809223; refs. 32, 33) were downloaded from Synapse (https://www.synapse.org/) and annotated according to MSI and POLE status.

TCGA RNAseq data
Details of the TCGA RNAseq analysis have been previously reported (2). RSEM normalized (34) and raw RNAseq count data were downloaded from FireBrowse (http://firebrowse.org/?cohort=UCEC&download_dialog=true) accessed November 11, 2014. After removal of normal tissue controls and technical duplicates, 245 samples with RSEM normalized and 231 samples with raw count data were informative for analysis.

Gene set enrichment analysis
TCGA raw counts were annotated by molecular subtype before normalization and ranking of genes differentially expressed between POLE-mutant (n = 16) and other (n = 205) endometrial cancers using DESeq (35). Gene set enrichment analysis (GSEA; ref. 36) was then performed with the PreRanking setting, using GO Biological Processes and C7 Immunologic Signatures sets from the Molecular Signatures Database (MSigDB) http://www.broadinstitute.org/gsea/msigdb/gene sets.jsp?collection=BP, and a published 200-gene T-cell tumor infiltration gene signature (18).

Prediction of antigenic neoepitopes
We created an algorithm to estimate the immunogenicity of individual tumors taking into account the following considerations: (i) to generate a functional neoepitope, a missense mutation must be expressed; (ii) most functional neoepitopes identified to date are predicted to bind MHC class I molecules (IC50 < 500 nmol/L) by NetMHCpan (23, 37, 38); and (iii) the likelihood that a neoepitope is antigenic is reduced if the corresponding wild-type peptide also binds the MHC with similar affinity as T cells to the epitope may be centrally deleted or tolerized (39). Our strategy was similar to others reported recently (15, 23, 38, 40). For each tumor, we calculated all possible 9mers for every missense mutation in expressed genes (defined as nonzero reads from RNAseq) and calculated the binding affinity of the mutant and corresponding wild-type peptide for HLA-A*02:01 (as a single model example HLA allele) using NetMHCpan 2.8 (37). In the event that several peptides had an IC50 < 500 nmol/L, the strongest binder was used for analysis. We defined antigenic mutations as neoepitopes predicted to bind MHC molecules (IC50 < 500 nmol/L) for which the corresponding wild-type peptide was not predicted to bind MHC (IC50 > 500 nmol/L).

Statistical analysis
We used the nonparametric Mann–Whitney test for all comparisons of continuous data and Spearman rho to analyze correlation between variables. Categorical variables were compared using the Fisher exact test. All statistical tests were two sided, with a P value of <0.05 taken to indicate significance. Except where indicated, statistical tests were unadjusted. Statistical analyses were performed using STATA and Prism 6.0 (GraphPad).

Results
POLE proofreading-mutant endometrial cancers show increased lymphocytic infiltrate
Preliminary analysis of hematoxylin and eosin-stained sections suggested that POLE proofreading-mutant endometrial cancers frequently displayed a prominent lymphocytic infiltrate and Crohn-like lymphocytic reaction (Supplementary Fig. S1A and S1B). Formal quantification of this in a set of 150 endometrial cancers comprising approximately equal numbers of POLE proofreading-mutant/microsatellite stable (POLE-mutant), POLE wild-type/MSI, and POLE wild-type/MSI subtypes of low and high grade (Supplementary Table S1), confirmed that TILs were more frequent in POLE-mutant (22/47) than in both MSS (8/54; P = 0.0009, Fisher exact test), and MSI (10/49; P = 0.009) subtypes (Supplementary Fig. S1C). Crohn-like reaction was also significantly more common in POLE-mutant than other tumors (P < 0.001 both comparisons; Supplementary Fig. S1D).

Increased density of intratumoral CD8+ lymphocytes in POLE-mutant endometrial cancers
Mindful of the relationship between cytotoxic T-cell infiltrate and favorable cancer outcome (19–22), and the excellent prognosis of POLE-mutant endometrial cancers (5, 6, 11), we next examined whether POLE mutants showed evidence of increased
T-cell infiltrate in our endometrial cancer cohort. While as anticipated (25), CD8⁺ cell numbers in intraepithelial and intrstromal compartments in the CT and the IM were higher in MSI than MSS endometrial cancers (P < 0.0001, all comparisons, Mann–Whitney test), in POLE-mutant tumors, the density of CD8⁺ infiltrate was frequently striking (Fig. 1A), and significantly exceeded that of both MSS (P<0.0001 for all four regions) and MSI cancers in the CT (median 5.9 vs. 2.6 intraepithelial CD8⁺ cells per high HPF, P = 0.001; 26.0 vs. 13.5 intrastromal CD8⁺ cells, P = 0.002; Fig. 1B). Furthermore, the proportion of tumors with numbers of CD8⁺ cells exceeding the median in all four regions was substantially higher in POLE-mutant (60.0%) than MSS (31.3%, P = 0.007, Fisher exact test) and MSS tumors (7.2%, P < 0.0001). Staining for CD3 and the cytolytic marker TIA-1 in a subset of cases confirmed increased T-cell density in POLE-mutant tumors (Supplementary Fig. S2A and S2B) and suggested that the infiltrate contained lymphocytes capable of cytotoxic activity (Supplementary Fig. S3A and S3B). Coimmunofluorescence confirmed coexpression of TIA-1 in the CD8⁺ lymphocytes comprising the POLE-mutant tumor infiltrate (Fig. 2A–F), further supporting the conclusion that these cells were capable of mediating an antitumor effect.

Interestingly, in light of the correlation previously reported between B- and T-cell subsets at the IM (41), we found that dense CD20 stromal infiltrate in this region was more common in POLE mutants (Supplementary Fig. S4A), while a tendency to increased numbers of FOXP3⁺ cells in both MSI and POLE-mutant tumors (Supplementary Fig. S4B) was also notable, given that this has been associated with favorable cancer prognosis in some studies (41).

Cytotoxic T-cell infiltration and activation in POLE-mutant endometrial cancers in TCGA series

We sought to confirm our results using the TCGA endometrial cancer series (2), in which the improved clinical outcome of POLE-mutant tumors was first suggested (Fig. 3A). Of 244 informative tumors in this study, 157 were MSS, 69 MSI, and 18 POLE-proofreading mutant (the single tumor with both POLE proofreading mutation and MSI was categorized as POLE mutant according to its mutation spectrum, in keeping with a recent report; ref. 10). Forty-three of the MSS tumors were NEECs, while all MSI and POLE-mutant endometrial cancers cases were EECs (Supplementary Table S1).

We first examined leukocyte methylation scores, which estimate the proportion of a heterogeneous tumor sample that consists of leukocytes (32, 33). After confirming that scores correlated strongly with CD8A expression (P = 0.65, P < 0.0001), we noted that, following exclusion of MSI and POLE-mutant tumors, both leukocyte methylation scores and CD8A expression did not differ between EECs and NEECs (P = 0.52 and P = 0.16 respectively, Mann–Whitney test). We therefore included tumors of both histologies in the MSS cohort in all subsequent analyses. Leukocyte methylation scores were similar in MSI and MSS tumors (median 15.5% vs. 14.2%, P = 0.2), in contrast with a significant increase in POLE mutants (median 23.3%; P = 0.006 vs. MSS, P = 0.07 vs. MSI; Fig. 3B), concordant with our previous results. Given the biologic differences between NEECs and EECs, we formally confirmed that these results were essentially unaltered following exclusion of the former from the MSS group (median 14.7%, P = 0.008 vs. POLE-mutant endometrial cancers).
We proceeded to explore whether infiltration of POLE-mutant endometrial cancers by cytotoxic T cells was manifest as immune expression signatures and/or increased expression of key immunologic genes. Agnostic pathway analysis of TCGA RNAseq data by GSEA demonstrated significant enrichment of immune-related pathways in POLE-mutant endometrial cancers compared with other tumors, including Immune Response [normalized enrichment score (NES) 3.77, q < 0.0001] and Immune System Process (NES 3.77, q < 0.0001). GSEA also confirmed that POLE-mutant cancers showed striking enrichment of a recently reported, highly specific 200 gene signature corresponding to tumor T-cell infiltration (ref. 18; Fig. 3C).

Focused analysis of genes involved in T-cell–mediated cytotoxicity confirmed that, compared with MSS tumors, MSI endometrial cancers had higher expression of CD8A (2.1-fold, P = 0.0005) and IFNY (2.1 fold, P = 0.0006) though expression of the cytotoxic differentiation and activation markers T-bet (TBX21), Eomes, perforin, and granzymes B,H,K and M was either essentially unchanged (<1.1-fold) or not significantly increased. In contrast, and once again consistent with our previous results, POLE mutants demonstrated substantial upregulation of CD8A (3.0-fold vs. all MSS tumors, P = 0.002; 3.2-fold vs. MSS EECs only, P = 0.004), accompanied by significant increases in T-bet (1.9-fold, P = 0.006), Eomes (2.3-fold, P = 0.008), IFNG (3.6-fold, P = 0.0003), PRF (2.5-fold, P = 0.001), granzymes B,H,K, and M (1.6- to 2.3-fold, P = 0.002–0.02), and the IFNG-induced cytokines CXCL9 (4.3-fold, P < 0.0001) and CXCL10 (3.5-fold, P = 0.002; Fig. 3D and Supplementary Fig. S5). Upregulation of most of these genes in tumors has been shown to predict good prognosis (19, 41). POLE mutants also demonstrated striking upregulation of the T-follicular helper genes CXCL13 (7.0-fold, P = 0.0001) and CXC5 (3.9-fold, P = 0.0004), which have recently been shown to strongly predict favorable outcome in colorectal cancer (41). Notably, despite limited numbers, in several cases, expression of cytotoxic markers and cytokines exemplifying effector status in POLE mutants significantly exceeded that in MSI tumors (e.g., PRF, P = 0.02; GZMH P = 0.04; CXCL19/10 both P = 0.03; Fig. 3D and Supplementary Fig. S5). Collectively, these data not only corroborated our previous finding that POLE-mutant tumors had greater T-lymphocyte infiltration than other endometrial cancers, but also strengthened the conclusion that these lymphocytes were capable of exerting antitumor activity.

Mechanisms of immune escape in POLE-mutant endometrial cancers

POLE-mutant cancers have significantly better prognosis than other endometrial cancers (5, 6, 11), as evidenced by the absence of recurrences in the TCGA series (2). However, the presentation of all patients in this study with clinically detectable tumors, in some cases with lymphatic spread, indicates that any immune response was, at best, only partially successful in suppressing POLE-mutant endometrial cancer growth. We therefore explored potential mechanisms of immune escape in these tumors.

We first considered the possibility that POLE-mutant endometrial cancers may escape from immune surveillance by loss or inactivation of components required for antigen presentation. Although 31.8% of POLE-mutant endometrial cancers in our study set of 150 endometrial cancers showed loss of HLA class I protein expression by IHC, this was not significantly different to that observed in MSI (28.6%, P = 0.82, Fisher exact test) or MSS (20.0%, P = 0.24) tumors and was not reflected in increased CD8+ cell numbers. Similarly, although we found a tendency to over-representation of POLE mutants among TCGA endometrial cancers with HLA class I gene expression in the lowest quartile, this was also not significantly different from other molecular subtypes (P = 0.07 vs. MSS).

Interestingly, although mutations in MHC pathway components were common in POLE-mutant tumors in the TCGA series, most were of unlikely pathogenicity, with exception of two tumors with potentially functional variants. The first, a β2-microglobulin R117+ mutation also detected in a POLE-mutant colorectal cancer (9), had a variant allele fraction of 0.59 and is likely to affect stability of the MHC complex by disruption of the interaction with the HLA heavy chain (42). The second, an HLA-B S112R substitution with variant allele fraction 0.71, lies
Figure 3.
Clinical outcome and T-cell response according to tumor molecular subtype in TCGA endometrial cancers. A, Kaplan–Meier curves demonstrating recurrence-free survival of POLE wild-type, MSS (n = 147), MSI (n = 63), and POLE proofreading mutant (POLE, n = 18) endometrial cancers in the TCGA series (note that survival data were not available for all cases). Comparison between subgroups was made by the two-sided log-rank test. B, leukocyte methylation scores according to endometrial cancer molecular subtype. Unadjusted comparison between groups was made by the two-sided Mann–Whitney test. C, GSEA of 200-gene tumor-associated T-cell signature (18) in POLE-mutant endometrial cancers compared with other tumors. Raw RNAseq counts were normalized and ranked using DESeq before GSEA analysis with PreRanking. D, heatmap showing expression of immunologic genes according to endometrial cancer molecular subtype. RSEM-normalized RNAseq expression data were log2 transformed, zero centered and assigned unit variance. For each gene, the mean fold change in expression in POLE mutants relative to MSS endometrial cancers was calculated and expression compared between POLE mutants, MSS, and MSI endometrial cancers by the unadjusted, two-sided Mann–Whitney test.
near the P pocket essential for peptide display, and is predicted to be deleterious by both Mutation Assessor and SIFT (Supplementary Table S2). However, the effect of each variant on antigen presentation, in contrast to the prediction of these tools, was analyzed in our study and varied. In general, frameshift mutations were not predicted to be deleterious by both Mutation Assessor and SIFT (see Materials and Methods). Box and whisker plots signify IQR, mean, and median as previously. Comparison between groups was made using unadjusted two-sided Mann-Whitney test; all comparisons, Mann–Whitney test; Fig. 4A, suggests that this upregulation was insufficient to fully suppress the T-cell response in POLE mutants.

**POLE** proofreading-mutant endometrial cancers are likely to display increased numbers of antigenic neopeptides

We hypothesized that the T-cell response in POLE-mutant endometrial cancers might be due to an excess of antigenic neopeptides as a consequence of ulramutation. To quantify this, we analyzed the TCGA endometrial cancer series using a methodology similar to several recent studies (23, 40). Our algorithm was based on three assumptions—first, that a mutation must be in an expressed gene to exert an effect; second, for a neopeptide to act as an antigen, it must bind MHC class I molecules (IC50 ≤ 500 nmol/L); and third, that neopeptides for which the corresponding wild-type peptide also binds MHC molecules are less likely to be immunogenic due to central deletion or tolerization (39).

Applying these criteria, we found that 5.9% (7,880/134,473) of the total number of missense mutations in TCGA endometrial cancers were predicted to be potentially antigenic. Of these, 73% (5,767) occurred in POLE-mutant tumors, reflected in a significantly higher number of antigenic mutations per cancer compared with both MSI and MSS subtypes (median 365.5 vs. 16 vs. 2 respectively, \( P < 0.0001 \) all comparisons, Mann–Whitney test; Fig. 4A), though this is likely to underestimate the number of antigenic mutations in MSI tumors as frameshift mutations were not included in our analysis. A substantial majority of antigenic mutations were in tumors with greater than median CD8A expression in both the whole series (78.4%), and the POLE-mutant subgroup (83.9%), though the strength of correlation between the two variables was modest, possibly as a result of immune escape mechanisms (Fig. 4B).

**Discussion**

By complementary analysis of two independent series totaling nearly 400 patients, and including over 60 POLE proofreading-mutant tumors, we have shown that POLE-mutant endometrial cancers are characterized by a striking CD8+ lymphocytic infiltrate, a gene signature of T-cell infiltration, and marked upregulation of cytotoxic T-cell effector markers. Furthermore, we show that, as a consequence of their remarkable mutation burden, POLE proofreading-mutant cancers are predicted to display substantially more antigenic peptides than other tumors, providing a possible explanation for our findings. Although our data demonstrate correlation rather than causation, the strong association between cytotoxic lymphocyte infiltration and favorable outcome in multiple cancers (19–22, 41) leads us to speculate that an enhanced T-cell antitumor response may contribute to the excellent prognosis of POLE-mutant endometrial cancers.

During the last few years, a combination of next-generation sequencing technology, improved *in silico* peptide–MHC-binding prediction (37), and the clinical application of immune checkpoint inhibitors (43–45) have helped facilitate remarkable insights into the mechanisms of tumor immunoeediting and immune escape. The intriguing observation that clinical benefit from CTLA4, PD1, and PD-L1 inhibition is greater in melanoma and cigarette smoking-associated lung cancer than most other
malignancies can now be interpreted in light of the understanding that in these highly mutated tumors, adaptive immune resistance is a key enabler of disease progression (17), and its inhibition can restore the ability of T cells to respond to antigenic peptides presented by these cancers (43). In keeping with this, in melanoma response to checkpoint inhibitors has very recently been shown to correlate both with the number of predicted antigenic tumor mutations (40), and with the degree of cytotoxic T-lymphocyte tumor infiltration before treatment (46). In light of these data, the association between the number of predicted antigenic peptides and T-cell response in POLE-mutant endometrial cancers in our study is noteworthy, as is the marked increase in T-cell exhaustion markers, as these have recently been shown to identify tumor neoantigen–specific CD8+ cells in patients with cancer (47, 48). Despite upregulation of these immunosuppressors in POLE-mutant endometrial cancers, we also found substantial increases in cytotoxic differentiation markers and effectors suggesting that the degree of adaptive immune resistance in these cancers may be insufficient to fully suppress CD8+ T-cell cytotoxicity (49). Collectively, our data suggest a complex interaction between the antigenic landscape of POLE-mutant endometrial cancers and the immune response. In this regard, molecular analysis of recurrences from the few patients with POLE-mutant endometrial cancers who do experience relapse may provide insights into mechanisms of immune escape. Finally, these patients may be good candidates for immune checkpoint inhibitor therapy, as might those patients with POLE proofreading-mutant tumors of other histologies, for which outcomes are more uncertain.

Interestingly, while as anticipated we observed moderately increased T-cell infiltration in MSI tumors (25), this was not associated with the marked increase in cytotoxic effector markers seen in POLE mutants. Although some studies have reported improved prognosis of MSI endometrial cancers, this is inconsistent (50), in contrast with the clear association of MSI with favorable outcome in early colorectal cancer (26). Comparison of the immune response between MSI tumors of both types may provide insights into this discordance.

Our study has limitations. Because of differing sample preservation (FFPE vs. fresh frozen), we were unable to validate either the IHC or RNAseq analysis between series, although the results from both analyses were highly concordant. Furthermore, the retrospective nature of our study meant that we were unable to investigate the repertoire and antigen response of T cells in patients with POLE-mutant cancers. This and other functional analyses will require prospective investigation.

In summary, we have demonstrated that ultramutated POLE proofreading-mutant endometrial cancers are characterized by a robust intratumoral T-cell response, which correlates with, and may be caused by an enrichment of antigenic neoepitopes. Our study provides a plausible mechanism for the excellent prognosis of these cancers, and further evidence of the link between somatic mutation and immunoediting in cancers.

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

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