Somatic POLE exonuclease domain mutations in endometrial cancer : Insights into the biology of POLE-mutant tumors

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Chapter 4

POLE proofreading mutations elicit an antitumor immune response in endometrial cancer

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ABSTRACT

Objective

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.

Methods

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 RNAseq 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 to 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 neopeptides. Our study provides a plausible mechanism for the excellent prognosis of these cancers.

INTRODUCTION

Endometrial cancer is the commonest gynecological malignancy in the Western world, and affects approximately 150,000 women each year in Europe and the US combined.[1] Endometrial cancers have traditionally been classified into endometrioid and non-endometrioid 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 7-12% 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 mutation among the highest in human cancer.[2,8,9] *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 (MSS).[2,4-6,10] 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 PD-1/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 (TILs), 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 pathological 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]

MATERIALS AND METHODS

Patients and tumors

Tumors were selected from the PORTEC-1 and PORTEC-2 studies (n=57[28,29]) and endometrial cancer series from the Leiden University Medical Center (LUMC, Leiden, the Netherlands; n=67) and the University Medical Center 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, microsatellite-stable (MSS); *POLE*-wild-type, MSI; and *POLE* (proofreading)-mutant (Supplementary Table 1). 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 RNAseq data and were informative for this analysis.[2] Of these, 197 were EECs, four mixed EEC/non-endometrioid endometrial cancers (NEECs), and 44 serous (NEEC) cancers. Ethical approval for tumor molecular analysis was granted at the LUMC, UMCG, and by Oxfordshire Research Ethics Committee B (Approval No. 05\Q1605\66).

POLE mutation and microsatellite instability status

DNA was extracted from formalin-fixed paraffin-embedded (FFPE) blocks and sequencing of *POLE* hot spot 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 immunohistochemistry (IHC).[6] Determination of MSI in the TCGA series was by a seven-marker panel, with MSI-High defined as alteration at three or more 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-high 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]

Histological assessment

Tumors were evaluated for the presence/absence of TILs and for Crohn-like reaction by a gynecological pathologist (T.B.) 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; 200µm x 200µm) 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 goat anti-mouse 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[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 RNA sequencing 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[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/genesets.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 (IC_{50}<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 IC_{50}<500 nmol/L, the strongest binder was used for analysis. We defined antigenic mutations as neoepitopes predicted to bind MHC molecules (IC_{50}<500 nmol/L) for which the corresponding wild-type peptide was not predicted to bind MHC (IC_{50}>500 nmol/L).

Statistical analyses

We used the nonparametric Mann–Whitney U 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 Figure 1A-B). 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/MSS subtypes of low and high grade (Supplementary Table 1), 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 Figure 1C). Crohn-like reaction was also significantly more common in *POLE*-mutant than other tumors (*P*<0.001 both comparisons; Supplementary Figure 1D).

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 intrastromal compartments in the CT and the IM were higher in MSI than MSS endometrial cancers ($P<0.0001$ for all comparisons, Mann–Whitney U test), in *POLE*-mutant tumors, the density of $CD8^+$ infiltrate was frequently striking (Figure 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 HPF, $P=0.001$; 26.0 vs. 13.5 intrastromal $CD8^+$ cells, $P=0.002$; Figure 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 MSI (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 Figure 2A-B) and suggested that the infiltrate contained lymphocytes capable of cytotoxic activity (Supplementary Figure 3A-B). Co-immunofluorescence confirmed co-expression of TIA-1 in the $CD8^+$ lymphocytes comprising the *POLE*-mutant tumor infiltrate (Figure 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 cell 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 Figure 4A), while a tendency to increased numbers of $FOXP3^+$ cells in both MSI and *POLE*-mutant tumors (Supplementary Figure 4B) 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 (Figure 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[10]). Forty-three of the MSS tumors were NEECs, while all MSI and *POLE*-mutant endometrial cancers cases were EECs (Supplementary Table 1).

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 ($\rho=0.65$, $P<0.0001$), 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,

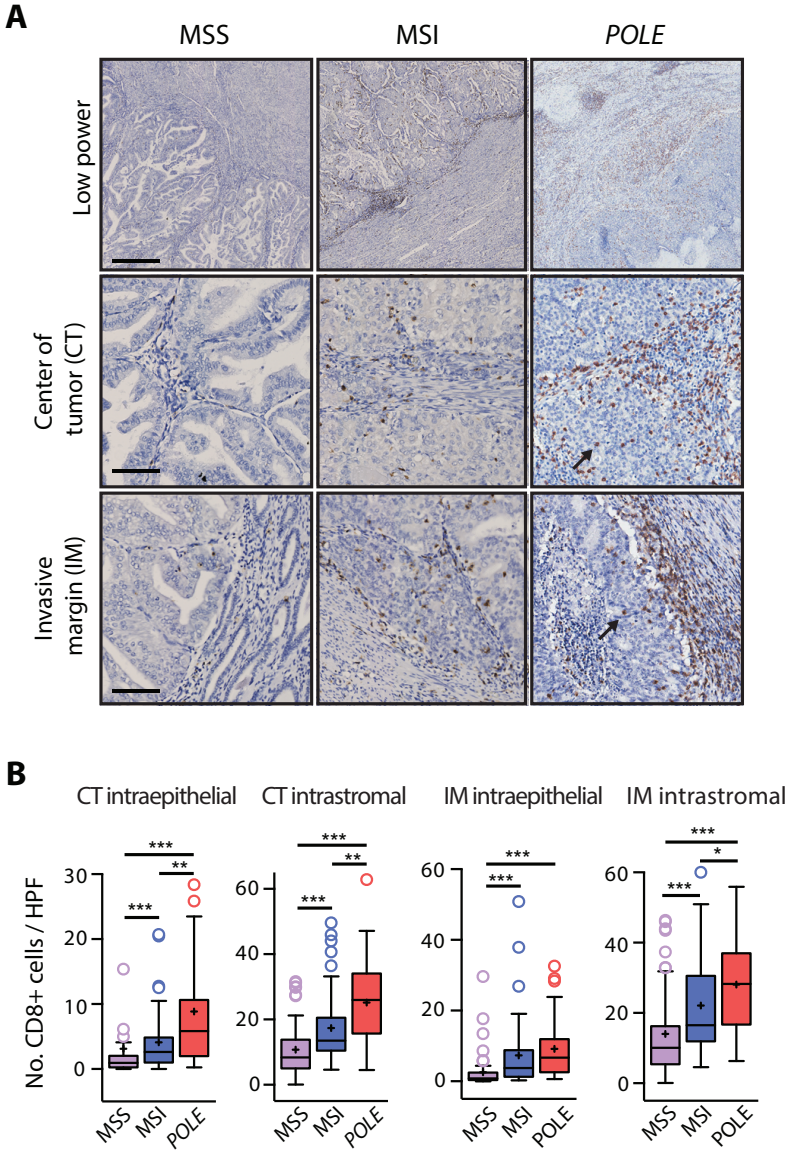

Figure 1. Increased CD8$^+$ lymphocyte infiltration in *POLE*-mutant endometrial cancers.
(A) Results of CD8 IHC by endometrial cancer molecular subtype shown at low magnification (top) and high power views of the center of the tumor (CT) and invasive margin (IM). Arrows highlight intraepithelial CD8$^+$ cells in *POLE*-mutant tumor. Scale bars, 500 µm (upper panel in A) and 100 µm (middle and bottom panels in A). (B) Quantification of CD8$^+$ cell number in intraepithelial and intrastromal compartments in the CT and IM by endometrial cancer molecular subtype. Boxes represent the interquartile range (IQR), with the upper whisker indicating the 75th percentile plus 1.5xIQR, and the lower whisker the 25th percentile minus 1.5xIQR. The median and mean values are indicated by a horizontal line and cross, respectively. Statistical comparison between groups was made by the unadjusted two-sided Mann–Whitney U test; *, **, and *** indicate $P<0.05$, $P<0.01$, and $P<0.001$, respectively.

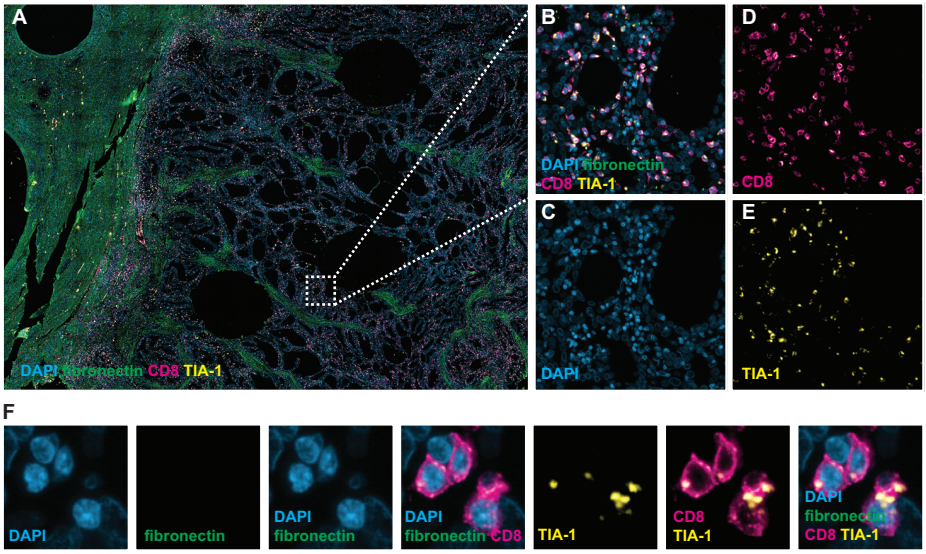

Figure 2. $CD8^+$ TILs in *POLE*-mutant tumors show cytolytic potential.
(A) Low-magnification image of *POLE*-mutant tumor following co-immunofluorescence (co-IF) staining for DAPI (nuclei), fibronectin (extracellular matrix), CD8, and the cytolytic marker TIA-1. (B-F) Co-IF images of the tumor center following staining for all markers (B), DAPI (C), CD8 (D), and TIA-1 (E) confirming co-expression of TIA-1 by tumor-infiltrating $CD8^+$ lymphocytes, also shown at high magnification (F).

Mann–Whitney U 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; Figure 3B), concordant with our previous results. Given the biological 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 immunological 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) 4.12 FDR 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 (Figure 3C).[18]

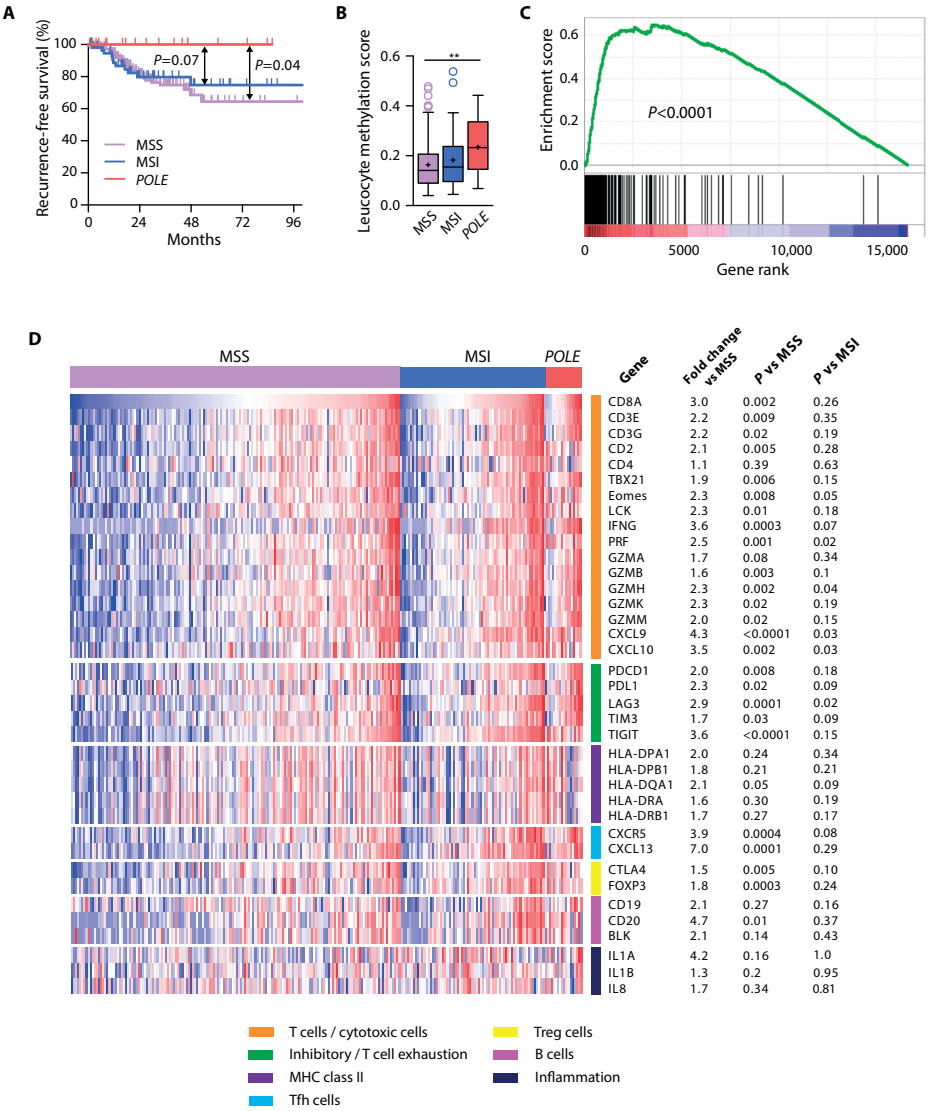

Figure 3. Clinical outcome and T cell response according to tumor molecular subtype in TCGA endometrial cancers.

(A) Kaplan–Meier curves demonstrating recurrence-free survival of *POLE* wild-type, MSS (N=147), MSI (N=63), and *POLE* proofreading domain-mutant (*POLE*, N=18) endometrial cancers in the TCGA series (note that survival data were not available for all cases). (B) Comparison between subgroups was made by the two-sided log-rank test. Leukocyte methylation scores according to endometrial cancer molecular subtype. Unadjusted comparison between groups was made by the two-sided Mann–Whitney U 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 $\log_2$ transformed, zero centered and assigned unit variance. For each gene, the mean fold change in expression in *POLE* mutants relative to MSS endome-

trial cancers was calculated and expression compared between *POLE* mutants, MSS, and MSI endometrial cancers by the unadjusted, two-sided Mann–Whitney U test; ***P*<0.01.

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 IFNγ (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 IFNγ-induced cytokines CXCL9 (4.3-fold, *P*<0.0001) and CXCL10 (3.5-fold, *P*=0.002; Figure 3D and Supplementary Figure 5). 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 CXCR5 (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; CXCL9/10 both *P*=0.03; Figure 3D and Supplementary Figure 5). 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 tumors (20.0%, *P*=0.24) and was not reflected in increased $CD8^+$ cell numbers. Similarly, although we found a tendency to overrepresentation 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 near the F pocket essential for peptide display, and is predicted to be deleterious by both Mutation Assessor and SIFT (Supplementary Table 2). However, the effect of each variant on antigen presentation is, at present, uncertain.

We proceeded to examine whether the cytotoxic T cell response in *POLE*-mutant endometrial cancers may be attenuated by upregulation of immunosuppressive mediators - a phenomenon termed adaptive immune resistance.[17] We found that the T cell exhaustion markers LAG3, TIM-3, and TIGIT, and the T cell inhibitors PD-1 and CTLA-4, were strongly correlated with CD8A expression across all endometrial cancers of all molecular subtypes (ρ=0.65 to 0.87; *P*<0.0001 for all cases), though the correlation with PD-L1 was more modest (ρ=0.34, *P*<0.0001; Supplementary Figure 6A). Interestingly, although expression of these markers in MSI compared with MSS endometrial cancers was either unchanged/minimally altered (TIM-3, CTLA4, PD-L1) or moderately increased (LAG3 1.9-fold, *P*<0.002; TIGIT 2.2-fold, *P*<0.0001), in *POLE* mutants all were significantly and substantially upregulated (e.g., LAG3 2.9-fold, *P*<0.0001 vs. MSS, *P*<0.02 vs. MSI; TIGIT 3.6-fold, *P*<0.0001 vs. MSS, *P*<0.15 vs. MSI; Figure 3D and Supplementary Figure 6B), consistent with prolonged antigen stimulation. However, as noted above, the overall increase in expression of cytotoxic effector markers suggested 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 neoepitopes as a consequence of ultramutation. 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 (IC_{50}<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% (5767) 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, $P<0.0001$ for all comparisons, Mann–Whitney U test; Figure 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 (Figure 4B).

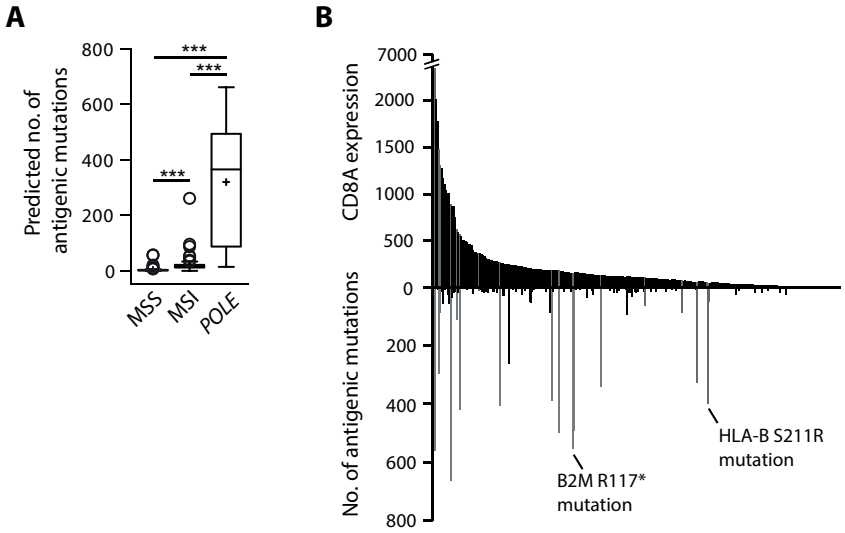

Figure 4. Antigenic mutation burden and tumor CD8A expression according to *POLE* mutation.
(A) The number of mutations predicted to generate antigenic neopeptides in individual TCGA tumors was calculated using exome and RNAseq data (see Materials and methods). Box and whisker plots signify IQR ± 1.5xIQR, mean and median as previously. Comparison between groups was made by the unadjusted two-sided Mann–Whitney U test. (B) Relationship between number of antigenic missense tumor mutations and tumor CD8A expression. *POLE*-mutant samples are highlighted in gray, together with possible mechanisms of immune escape in two cases; ***$P<0.0001$.

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 immunoediting and immune escape. The intriguing observation that clinical benefit from CTLA-4, PD-1, 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 neopeptides. 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.

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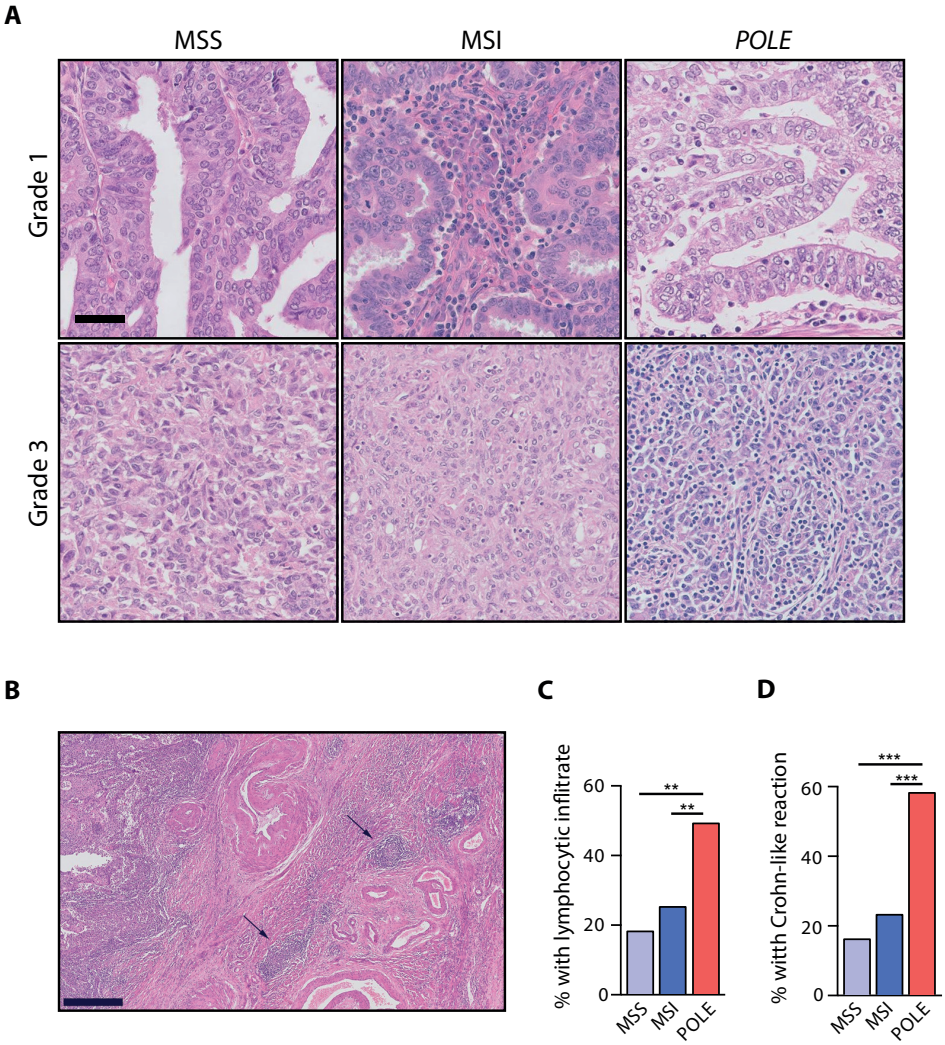
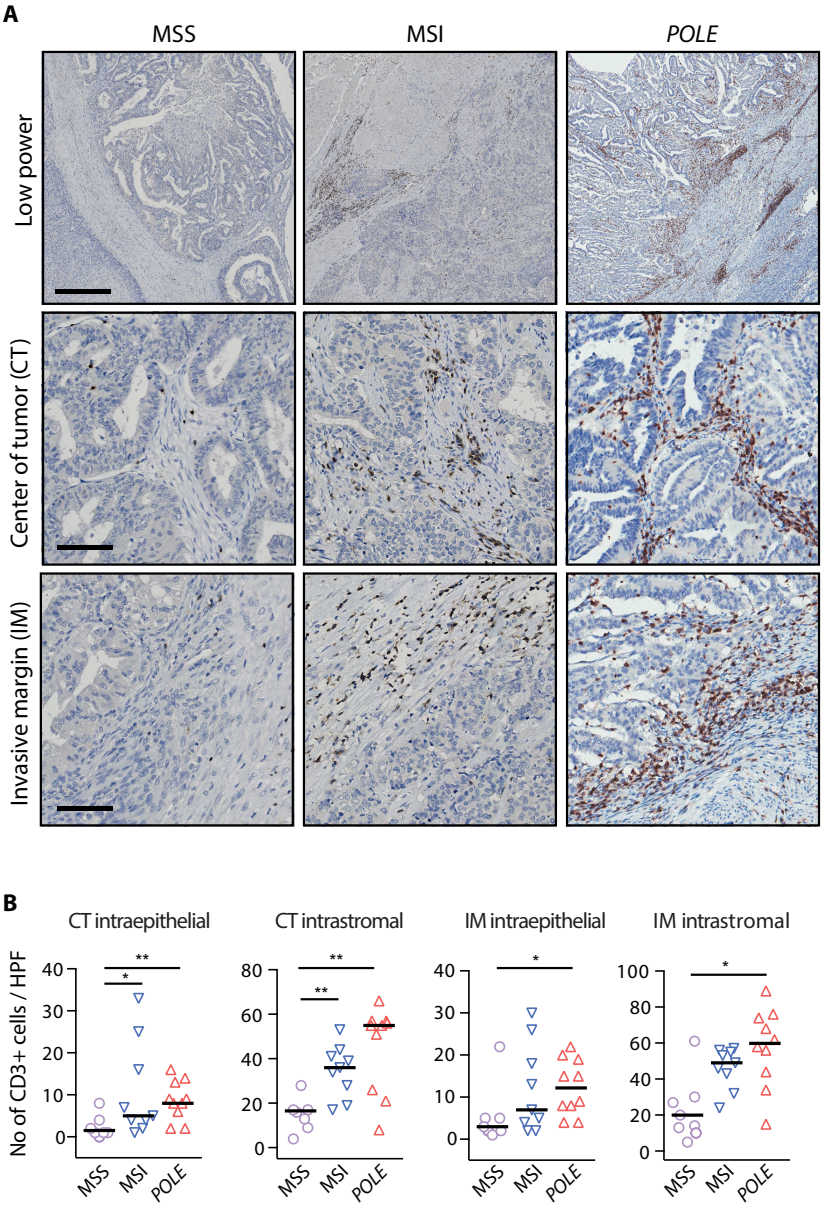
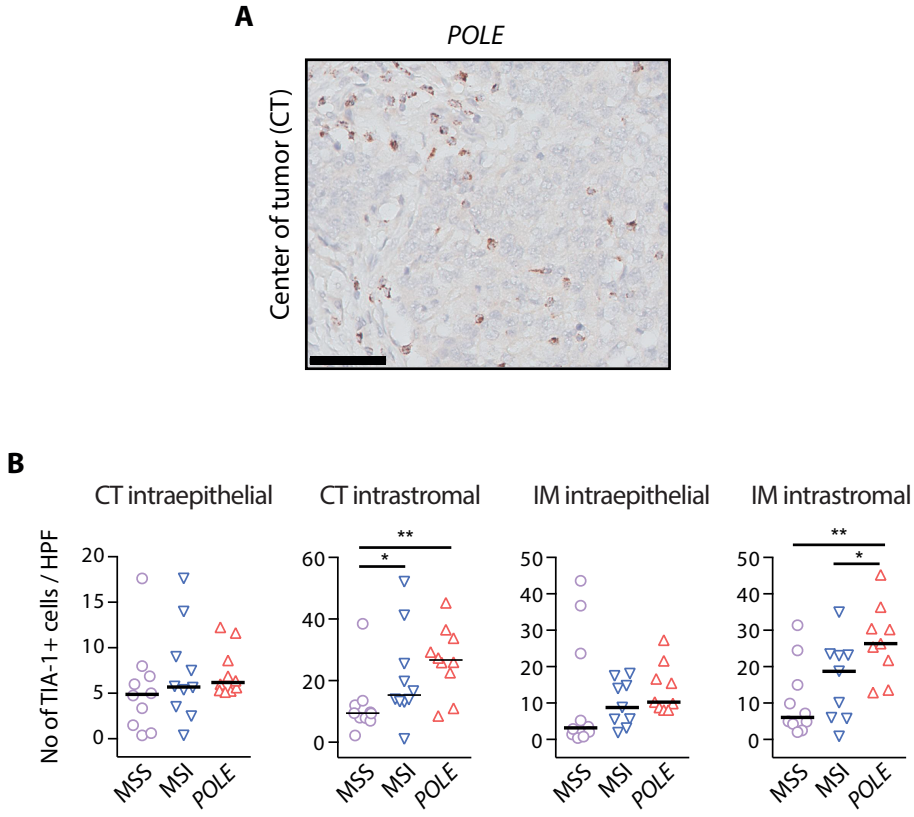


Supplementary Figure 1. Tumor-infiltrating lymphocytes and Crohn-like reaction in *POLE*-mutant endometrioid endometrial cancers.

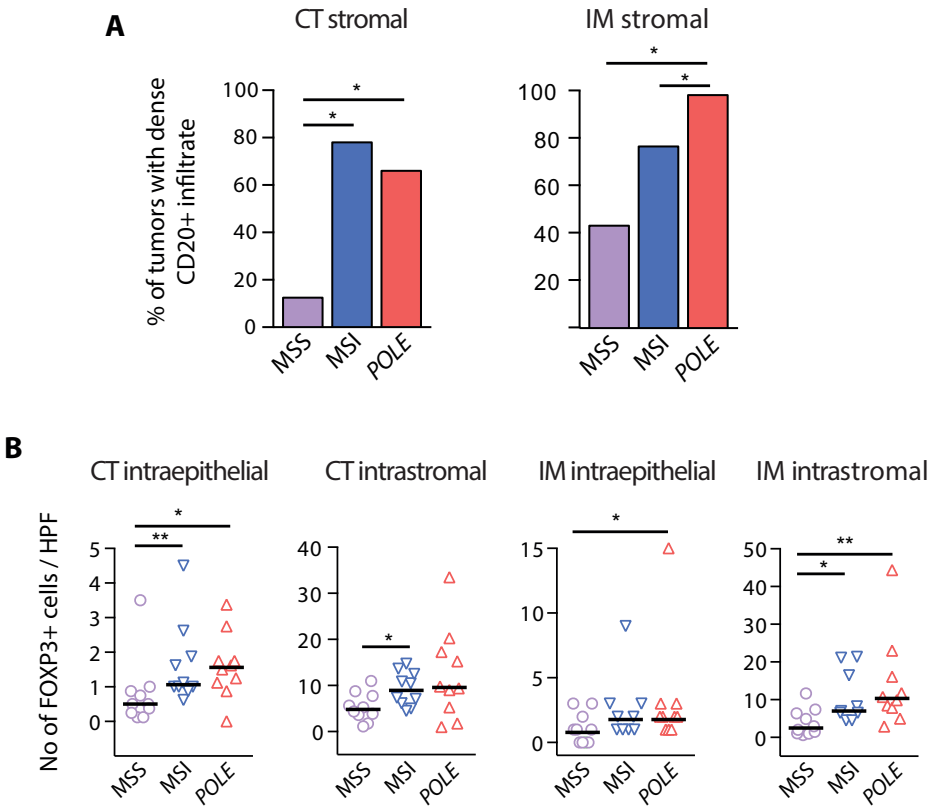
(A) Representative H&E-stained sections demonstrating tumor-infiltrating lymphocytes (TILs) in *POLE*-wild-type, microsatellite-stable (MSS), *POLE*-wild-type, microsatellite-unstable (MSI) and *POLE* proofreading domain-mutant (*POLE*) ECs in both low- and high-grade tumors. Scale bar corresponds to 50µm. (B) Representative H&E stained section demonstrating Crohn-like reaction (indicated by arrows) in a *POLE*-mutant tumor. Scale bar corresponds to 500µm. (C) Frequency of lymphocytic infiltrate by EC molecular subtype. (D) Frequency of Crohn-like reaction by EC molecular subtype. Comparison between groups in C and D was made by two-sided Fisher's exact test; ** and *** indicate $P<0.01$, and $P<0.001$, respectively.



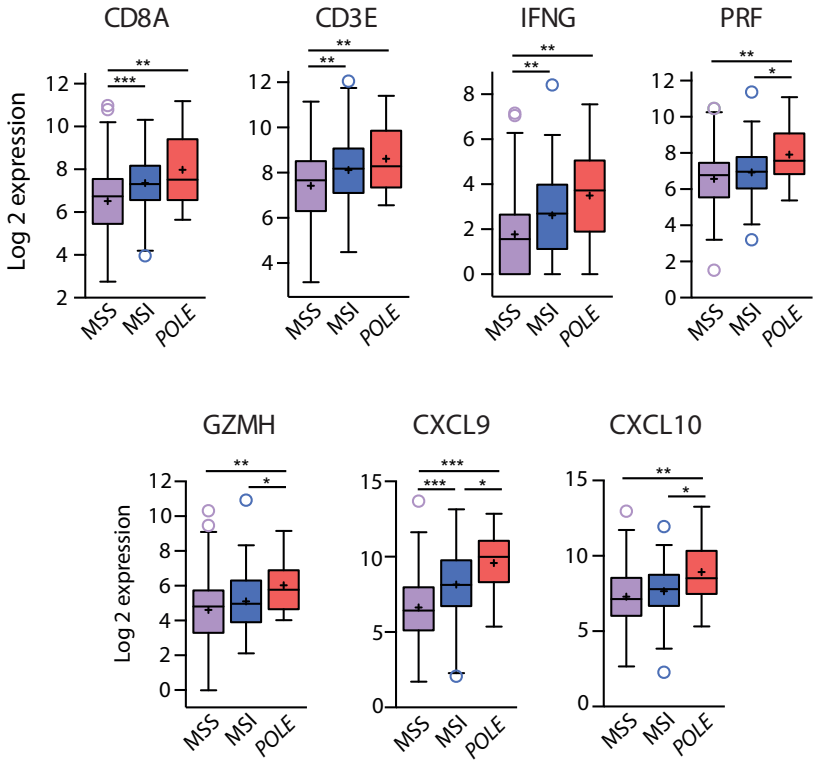
Supplementary Figure 2. $CD3^+$ tumor infiltrate according to EC molecular subtype.
(A) Representative images of $CD3^+$ staining in microsatellite-stable and -unstable *POLE*-wild-type cancers (MSS & MSI) compared to a *POLE* proofreading domain-mutant (*POLE*) tumor at low magnification (upper panels) and high magnification in the center of the tumor (CT) and invasive margin (IM) (middle and lower panels). Scale bar in upper panel indicates 500µm, bar in middle and lower panels indicates 100µm. (B) Quantification of $CD3^+$ cell numbers per high power (200 x 200µm) field in intraepithelial and intrastromal compartments within the center of the tumor (CT) and invasive margin (IM) according to EC molecular subtype. Horizontal bars indicate the median. Comparison between groups was made by unadjusted Mann-Whitney test; * and ** indicate $P<0.05$, and $P<0.01$, respectively.



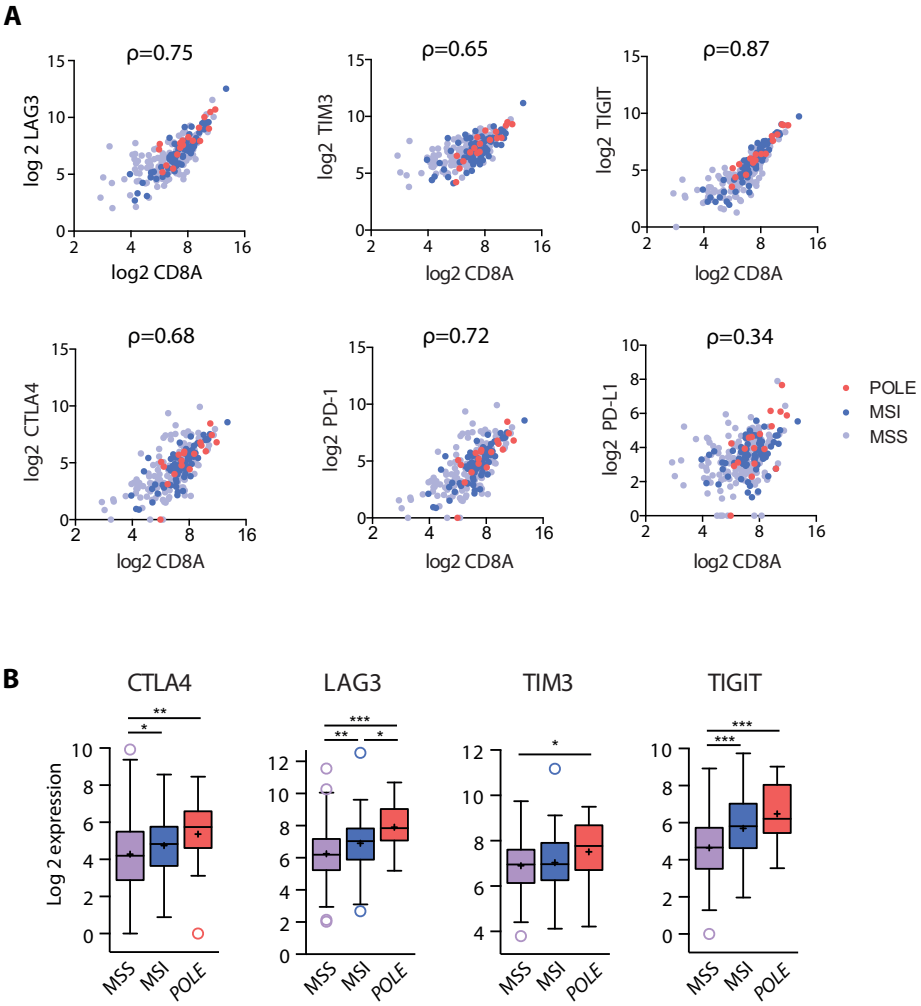
Supplementary Figure 3. Number of TIA-1$^+$ cytolytic T cells according to molecular subtype.
(A) Representative high magnification image of TIA-1$^+$ staining in a *POLE* proofreading domain-mutant (*POLE*) tumor. Scale bar indicates 50µm. (B) Quantification of TIA-1$^+$ cell numbers per high power (200x200µm) field in intraepithelial and intrastromal compartments within the center of the tumor (CT) and invasive margin (IM) for MSS, MSI and *POLE*-mutant tumors. Horizontal bars indicate the median. Comparison between groups was made by unadjusted Mann-Whitney test; * and ** indicate $P<0.05$, and $P<0.01$, respectively.



Supplementary Figure 4. CD20$^+$ and FOXP3$^+$ tumor infiltrate according to EC molecular subtype. (A) Percentages of tumors displaying dense intrastromal CD20$^+$ infiltrate in the center of the tumor (CT) and invasive margin (IM). (B) Quantification of number of FOXP3$^+$ cells in tumor regions by EC molecular subtype. Horizontal bars indicate the median. Statistical comparisons in A and B were made by unadjusted two-sided Mann-Whitney test; * and ** indicate $P<0.05$ and $P<0.01$, respectively.



Supplementary Figure 5. Expression of T cell markers and cytotoxic effectors by EC molecular subtype. Expression of genes encoding T cell subunits CD8α (CD8A), CD3ε (CD3E), cytotoxic effector molecules interferon γ (IFN), perforin (PRF) and granzyme H (GZMH), and IFNγ-induced cytokines CXCL9 and CXCL10 was compared between molecular subtypes using RSEM normalized TCGA RNAseq data. Box and whiskers signify IQR ± 1.5xIQR, mean and median as previously. Statistical comparison between groups was made by unadjusted two-sided Mann-Whitney test; *, ** and *** indicate $P<0.05$, $P<0.01$ and $P<0.001$, respectively.



Supplementary Figure 6. T cell exhaustion markers according to tumor CD8A expression and EC molecular subtype.

(A) Correlation between CD8A expression and T cell exhaustion markers ($P<0.0001$, all cases). (B) T cell exhaustion makers according to EC molecular subtype. Box and whiskers signify IQR ± 1.5xIQR, mean and median as previously. Statistical comparison between groups was made by unadjusted Mann-Whitney test; *, **, and *** correspond to $P<0.05$, $P<0.01$, and $P<0.001$, respectively.

Supplementary Table 1. Demographic and clinicopathological characteristics of cases by EC molecular subtype.

	MSS	MSI	*POLE*	Total
PORTEC, LUMC & UMCG cases				
Age, median (range), years	62.5 (36-84)	68.8 (35-89)	63.5 (46-82)	65.0 (35-89)
FIGO stage (2009), n				
I/II	48	39	45	132
III/IV	6	10	2	18
Grade, n				
1/2	45	39	33	117
3	9	10	14	33
Type, n				
EEC	54	49	47	150
NEEC	0	0	0	0
Total, n	54	49	47	150
TCGA series*				
Age, median (range), years	64 (34-90)	63 (35-88)	57 (33-87)	63 (33-90)
FIGO stage (2009), n				
I/II	116	57	14	187
III/IV	40	12	4	56
Unknown	1	1	0	2
Grade, n				
1/2	97	45	9	151
3	60	24	9	93
Type, n				
EEC	114	69	18	201
NEEC	43	0	0	43
Total, n	157	69	18	244

*TCGA analysis excludes one NEEC for which MSI status could not be determined. Secondly, all *POLE*-proofreading mutant ECs were MSS with exception of one TCGA tumor (TCGA-AP-A051) which was assigned to the *POLE*-mutant subgroup on basis of characteristic mutation signature.

Abbreviations: MSS, microsatellite-stable *POLE*-wild-type; MSI, microsatellite-unstable *POLE*-wild-type; *POLE*, *POLE* proofreading-mutant; EEC, endometrioid endometrial cancer; NEEC, non-endometrioid endometrial cancer.

Supplementary Table 2. Antigen presentation pathway mutations in TCGA *POLE* proofreading-mutant ECs.

TCGA ID	*POLE* mutation	Exonic mutations, n	CD8A expression*	Antigen presentation pathway mutations	Variant allele frequency	Predicted functional effect	
						Mutation assessor	SIFT
TCGA-B5-A0JY	P286R	8890	High	HLA-B E253K	0.33	Medium	Deleterious (0.00)
				B2M R117*	0.59	N/A	N/A
				PDIA3 R344C	0.36	Medium	Deleterious (0.00)
				PDIA3 E384D	0.30	Neutral	Deleterious (0.00)
TCGA-BS-A0TC	M444K	1203	Low	None			
TCGA-D1-A103	A456P	5965	High	PDIA3 R344C	0.30	Medium	Deleterious (0.00)
TCGA-D1-A16X	P286R	1755	High	None			
TCGA-D1-A16Y	V411L	952	Low	None			
TCGA-A5-A0GP	V411L	1235	Low	None			
TCGA-AP-A051	L424I	6716	High	PDIA3 A69T	0.31	Low	Deleterious (0.04)
TCGA-AP-A056	V411L	7226	High	HLA-C E288K	0.16	Low	Tolerated (0.06)
				CALR S300Y	0.34	Neutral	Deleterious (0.00)
TCGA-AP-A059	S297F	9187	High	None			
TCGA-AP-A0LM	V411L	10489	High	B2M A6V	0.13	Low	Tolerated (1.0)
				CANX K182N	0.20	Medium	Deleterious (0.00)
TCGA-AX-A05Z	P286R	5677	High	None			
TCGA-AX-A0J0	P286R	6913	Low	HLA-B S112R	0.71	High	Deleterious (0.00)
				CALR K62R	0.50	Medium	Deleterious (0.00)
				TAPBP R383Q	0.57	Medium	Tolerated (0.34)
TCGA-B5-A11E	V411L	8210	High	PDIA3 E384*	0.44	N/A	N/A
TCGA-B5-A11N	P286R	1557	High	None			
TCGA-BG-A0VX	L424V	163	High	None			
TCGA-BS-A0UF	P286R	7391	High	CANX D37N	0.33	Medium	Deleterious (0.00)
TCGA-BS-A0UV	P286R	7801	High	None			

Supplementary Table 2. (continued) Antigen presentation pathway mutations in TCGA *POLE* proofreading-mutant ECs.

TCGA ID	*POLE* mutation	Exonic mutations, n	CD8A expression*	Antigen presentation pathway mutations	Variant allele frequency	Predicted functional effect	
						Mutation assessor	SIFT
TCGA-D1-A17Q	P286R	5141	Low	CALR Y128C	0.13	High	Deleterious (0.00)
				CANX Q508H	0.48	Medium	Deleterious (0.00)
				TAPBP R383Q	0.38	Medium	Tolerated (0.34)

*Relative to median CD8A expression in all TCGA ECs.

POLE proofreading mutation, immune response and prognosis in endometrial cancer

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ABSTRACT

Endometrial cancers (ECs) with *POLE* proofreading mutations are typified by ultramutation and excellent prognosis. We investigated whether these were related, and found that *POLE*-mutant ECs display a robust T cell response that corresponds to an enrichment of antigenic tumor neopeptides. Enhanced immunogenicity may explain the favorable outcome of *POLE*-mutant ECs.

POLE PROOFREADING MUTATION, IMMUNE RESPONSE AND PROGNOSIS IN ENDOMETRIAL CANCER

The proofreading exonuclease activity intrinsic to the replicative DNA polymerases epsilon and delta (Pols ε and δ) is essential to maintain fidelity of DNA replication and prevent mutagenesis. While a role for defective polymerase proofreading in human cancer has long been postulated, this has only recently been confirmed, with the demonstration that germline mutations in the exonuclease domains of *POLE* and *POLD1* (which encode the principal subunits of Pols ε and δ respectively) predispose to cancer.[1] Subsequently, we and others have shown that somatic *POLE* proofreading mutations occur in 7-12% endometrial cancers (ECs),[2,3] 1-2% colorectal cancers (CRCs), as well as cancers of the brain, stomach and pancreas (TCGA unpublished, http://www.cbioportal.org, accessed June 2015). In keeping with the essential contribution of polymerase proofreading to replication fidelity, *POLE* proofreading-mutant ECs are ultramutated.[3] However, perhaps less predictably, they also have an excellent prognosis.[3,4] We hypothesized that these two characteristics may be related – more specifically, that tumor neopeptides caused by ultramutation may stimulate an cytolytic immune response, analogous to previous observations in hypermutated mismatch repair-deficient CRCs.[5] In a recent study,[6] we investigated this in two large EC cohorts.

Following the observation that *POLE* proofreading mutants had a higher density of tumor-infiltrating lymphocytes (TILs) than other ECs, we confirmed that this represented a $CD8^+$ cytotoxic T cell infiltrate likely to be capable of cytolytic activity, as evidenced by co-staining for the activation marker TIA-1. Consistent with these data, examination of RNAseq data from the independent TCGA EC series confirmed significant enrichment for immune-related pathways and a highly specific 200-gene tumor T cell infiltration signature in *POLE* proofreading-mutant ECs. This analysis also demonstrated that *POLE*-mutant tumors displayed significantly increased expression of CD8A and other T cell cytotoxic differentiation and effector markers known to predict favorable outcome in cancer,[7] including T-bet, Eomes, IFN-γ, perforin, and granzymes B, H, K and M. Using a bioinformatic approach to investigate the possible contribution of antigenic tumor neopeptides to the antitumor immune response, we found that *POLE* proofreading-mutant ECs were predicted to display substantially more antigenic peptides than other ECs, providing a potential explanation for our findings.

Taken together, our data suggest that enhanced immunogenicity contributes to the excellent prognosis of *POLE* proofreading-mutant ECs, and are concordant with a recent study, which showed that dendritic cells pulsed by *POLE*-mutant tumor lysates stimulated greater $CD4^+$ and $CD8^+$ cell proliferation than those pulsed by ECs lacking

POLE mutations.[8] However, this begs the question of why *POLE*-mutant ECs are not eliminated by this enhanced cytotoxic T cell response? We found no evidence of an increased frequency of loss of HLA class I protein expression in *POLE*-mutant ECs, and functional mutations in the antigen presentation machinery also appeared relatively uncommon (2 of 18 cases). In contrast, we found striking increases in the expression of immunosuppressive checkpoint molecules and regulatory T cell markers, including LAG3, TIM-3, TIGIT, PD-1, CTLA-4 and FOXP3, in *POLE*-mutant ECs, suggesting that this may be the principal mechanism of immune evasion in these tumors.

In short, our data suggest that *POLE* proofreading-mutant ECs evoke a striking antitumor immune response, which is likely to contribute at least partly to their excellent prognosis (Figure 1). In addition to validating our results in further independent EC series, it will be important to determine whether an enhanced cytotoxic T cell reaction also occurs in other *POLE* proofreading-mutant cancers. Interestingly, recent data suggest this may be the case in glioblastomas.[9] Given the association between benefit from immune checkpoint inhibitors and tumor mutation burden,[10] our study also suggests that the few patients with advanced or recurrent *POLE* proofreading-mutant cancers may be promising candidates for these agents. Finally, a pressing question is why some *POLE*-mutant tumors do not appear to stimulate as potent an immune reaction as others? Is it simply that these tumors are less mutated? Or do they harbor novel mechanisms of immune escape? Thus, while much remains unknown, further study of *POLE*-mutant cancers may provide insights into antitumor immune response and evasion that are generalizable more broadly, with potential benefits for a wide range of cancer patients.

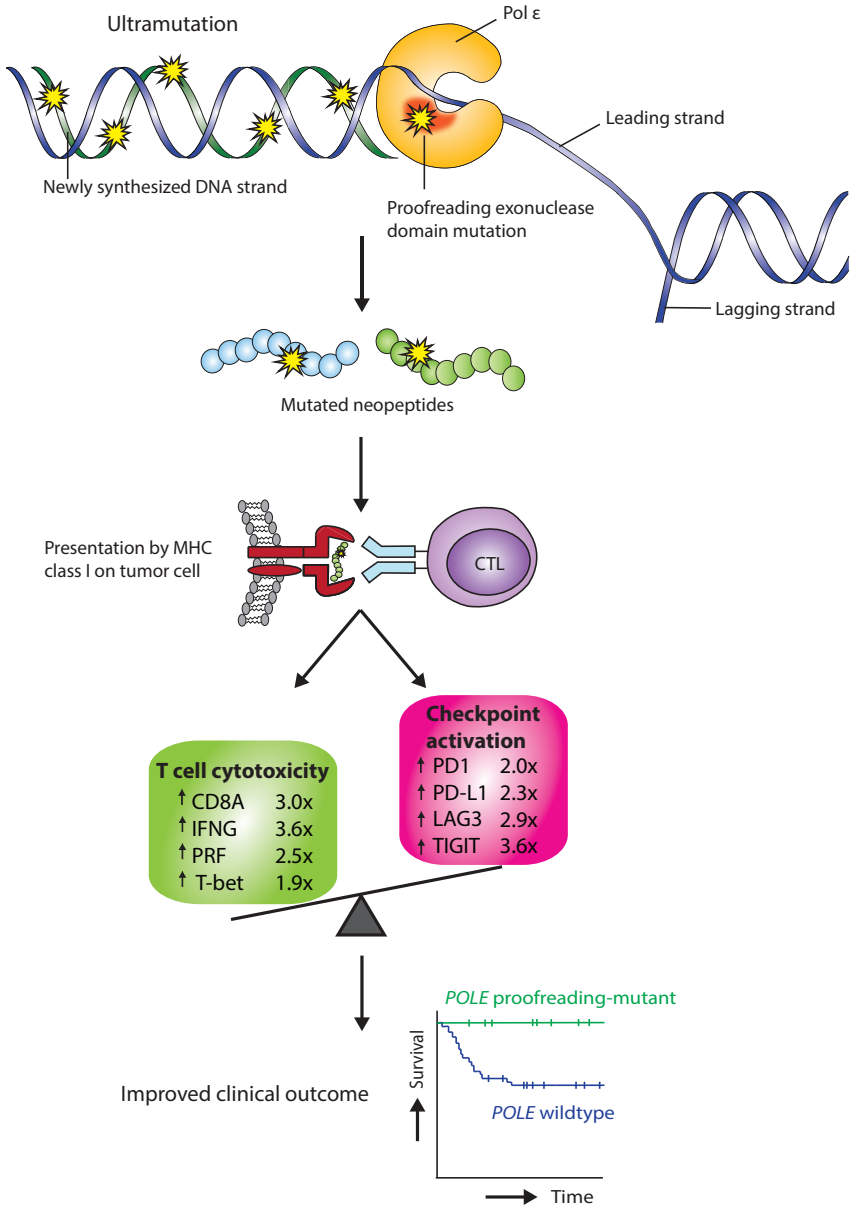

Figure 1. Possible mechanism linking *POLE* proofreading mutation, immune response and favorable endometrial cancer prognosis.

POLE encodes the catalytic and proofreading subunit of DNA polymerase ε (Pol ε), the leading strand replicase in humans. Cancer-associated *POLE* exonuclease domain mutations perturb proofreading activity, resulting in tumor ultramutation. Enhanced presentation of mutated antigenic neopeptides stimulates both a cytolytic T cell response and upregulation of immunosuppressive checkpoints; however, increased effector cytokine expression (not shown) suggests that the T cell response is functional and at least partly contributes to the favorable prognosis of *POLE* proofreading domain-mutant endometrial cancers.

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