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Polymerase ϵ (POLE) ultra-mutated tumors induce robust tumor-specific CD4+ T cell responses in endometrial cancer patients

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Abstract

Objective—Around 7–10% of endometrial carcinomas are characterized by Polymerase- ϵ (POLE) exonuclease-domain-mutations, an ultra-mutated-phenotype and a favorable prognosis. It is currently unknown whether POLE ultra-mutated-tumors are more immunogenic when compared to the other groups of endometrial cancers.

Methods—We used autologous-dendritic-cells (DC) pulsed with whole-tumor-extracts to assess the level of CD8+ and CD4+ T-cell-activation induced by POLE-ultramutated (+) and POLE wild-type (–) endometrial cancer cells *in vitro*. T-lymphocyte-proliferations were evaluated using CFSE and/or [³H]thymidine-incorporation-assays while the ability to specifically kill autologous-tumor-cells by cytotoxic-T-lymphocyte (CTL) was tested in standard 4-hr-⁵¹Cr-cytotoxicity-assays. In order to correlate cytotoxic activity and proliferation by CD4+ and CD8+ T-lymphocytes, respectively, with a particular lymphoid subset, two-color-flow-cytometric analysis of intracellular-cytokine-expression (IFN- γ vs IL-4) at the single cell level, was also performed.

Results—DC-pulsed with tumor extracts were able to induce CTL-responses against autologous-tumor-cells in both POLE (+) and POLE (–) cancer patients (P=0.305). These CD8+ T-cell-populations were cytotoxic against tumor-cells but they did not lyse PHA-stimulated-autologous-lymphocytes or autologous-EBV-transformed-lymphoblastoid-control-cell-lines. In contrast, only POLE (+) tumor-lysate-pulsed-DC were able to induce significant proliferation and high IFN- γ expression (i.e., Th1-cytokine-bias) in autologous *in vitro* DC-stimulated CD4+ T-cells as well as naïve CD4+ and CD8+ T-cells from patients-peripheral-blood (P < 0.05).

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Conclusions—POLE ultra-mutated-tumors are significantly more immunogenic when compared to POLE (–) tumors, in particular to the helper arm of the immune system. These data lend support to the hypothesis that the better prognosis of patients with POLE (+) tumors may at least in part be linked to their enhanced immunogenicity.

Keywords

polymerase ϵ ; endometrial carcinoma; uterine serous papillary cancer; whole exome sequencing

Introduction

Endometrial cancers have historically been classified as Type I or Type II [1]. Type I endometrial cancers account for 65–70% of cases and are associated with grade 1–2 endometrioid histology, younger age of onset, retention of estrogen receptor (ER) and progesterone receptor (PR) status and a history of unopposed estrogen. They have more recently been associated with genetic alterations including deletions in k-Ras, PTEN, or mismatch repair mechanisms [1]. In contrast, Type II endometrial cancers are typically serous (USC), clear cell or grade 3 endometrioid histology and associated with loss of ER/PR, black race, absence of unopposed estrogen, presentation at later stage, reduced E-cadherin expression, aneuploidy, mutations in p53 and HER2/Neu overexpression [2]. Type II endometrial cancers such as USC are typically more aggressive than type I cancers and have a poorer prognosis even when diagnosed at early stages [3].

Recently, using an integrated genomic, epigenomic, transcriptomic and proteomic approach, The Cancer Genome Atlas (TCGA) Research Network provided compelling evidence that endometrial cancers result from heterogeneous somatic mutations and classified endometrial cancers into four categories: 1) polymerase epsilon (POLE)-ultramutated, 2) microsatellite instability hypermutated, 3) copy-number low and 4) copy-number high, serous-like [4]. In this landmark study, patients harboring ultra-mutated tumors secondary to mutations in exonuclease domain of POLE were found to have a more favorable progression-free survival as compared with the other three groups of endometrial cancer [4].

POLE and polymerase δ (POLD) constitute the two nuclear DNA polymerases present in eukaryotic cells endowed with intrinsic proofreading activity [5–9]. These polymerases are responsible for the bulk of chromosomal DNA synthesis during cell division, and multiple studies in yeast and mammalian cells have shown that polymerase proofreading as well as post-replication mismatch repair are the primary guardians of DNA replication fidelity [5–9]. Loss of function in one or both of these genes has been demonstrated to dramatically increase the number of spontaneous mutations and to induce a high incidence of spontaneous neoplasms in mice and humans [5–9].

After the initial TCGA report, multiple studies from our group as well as others have shown that despite commonly being of high histological grade, POLE ultra-mutation is correlated with a favorable prognosis in both Type I and Type II endometrial cancer patients [4,10–14]. It is currently not understood why patients with a POLE ultra-mutated phenotype may have improved outcomes. However, it is possible that the large number of somatic mutations present in these tumors, similarly to what has recently been demonstrated in melanoma and

lung cancer patients [15], may render these cancers highly immunogenic for the host due to the large number of mutated epitopes.

In this study we used autologous dendritic cells (i.e., DC, the most powerful professional antigen presenting cells in the human body) pulsed with whole tumor extracts to assess the level of CD8+ and CD4+ T-cell activation induced by POLE mutated (+) vs POLE wild type (–) endometrial cancers *in vitro*. We report the first evidence that POLE ultra-mutated endometrial tumors may be significantly more immunogenic when compared to POLE wild type endometrial cancers and result in increased immunomodulation through CD4+ activation. These data support the hypothesis that the better prognosis of patients with POLE (+) tumors may, at least in part, be linked to an enhanced immunogenicity of these cancers.

Patients and Methods

Tumor tissue and peripheral blood lymphocytes (PBL) were obtained from a total of 10 patients (5 harboring tumors with POLE mutations and 5 harboring tumors with wild type POLE). Specimens were obtained at the time of surgery through the Gynecologic Oncology Section of the Obstetrics and Gynecology Department and the Pathology Department at the University of Arkansas for Medical Sciences (UAMS), Little Rock, Arkansas, and at Yale University School of Medicine under approval of the Institutional Review Board. Patient characteristics and molecular features of tumors included in the study are found in Table 1. With the exception of UTE4 and UTE9, two endometrial endometrioid carcinomas found to harbor POLE mutations by standard Sanger sequencing, (Table 1), the remaining tumor samples had previously undergone whole exome sequencing [11]. For UTE4 and UTE9, primer sets that cover the exonuclease domain regions (where mutations were previously identified in endometrial cancer) of POLE were used to amplify exonuclease domain genomic regions prior to Sanger sequencing, as previously described [12].

Tumor cell lines

Fresh endometrial tumor cells were obtained from surgical specimens. Single cell suspensions were obtained by processing solid tumor samples under sterile conditions at room temperature as previously described [16]. Briefly, viable tumor tissue was mechanically minced in RPMI 1640 to portions no larger than 1–3 mm³ and washed twice with RPMI 1640. The portions of minced tumor were then placed into 250 ml trypsinizing flasks containing 30 ml of enzyme solution [0.14% collagenase Type I (Sigma, St. Louis, MO) and 0.01% DNase (Sigma, 2000 KU/mg)] in RPMI 1640, and incubated on a magnetic stirring apparatus overnight at 4°C. Enzymatically dissociated tumor was then filtered through 150 µm nylon mesh to generate a single cell suspension. The resultant cell suspension was then washed twice in RPMI 1640 plus 10% autologous plasma. Fresh tumor cell lines were maintained initially in RPMI 1640, supplemented with 10% FBS at 37°C, 5% CO₂. All experiments were performed with fresh or cryopreserved tumor cultures which had at least 90% viability and contained >99% tumor cells.

Preparation of Tumor Lysate

Five to 10×10^6 autologous tumor cells harvested by scraping (or equivalent amount of protein tumor material obtained from fresh frozen tumor specimens) were washed twice with phosphate-buffered saline (PBS, pH 7.4). Cells were lysed by 3 to 4 freeze (in liquid nitrogen) and thaw (room temperature) cycles. Lysis was monitored by light microscopy. Larger particles were removed by centrifugation (10 min, 600 rpm.), supernatants were passed through a 0.2-mm filter, and stored at -80°C until use.

Isolation of Peripheral Blood Lymphocytes (PBL) and Generation of Dendritic Cells

PBL were separated from heparinized venous blood by Ficoll-Hypaque (Sigma) density gradient centrifugation and either cryopreserved in RPMI 1640 (Gibco-BRL) plus 10% DMSO and 30% autologous plasma, or immediately used for DC generation. Briefly, PBMC obtained from 20–35 ml of peripheral blood were placed in 6-well culture plates (Costar, Cambridge, MA) in AIM-V (Invitrogen) at $0.5\text{--}1 \times 10^7/3$ ml per well. After 2 h at 37°C , non-adherent cells were removed, and the adherent cells were cultured at 37°C in a humidified 5% $\text{CO}_2/95\%$ air incubator, in AIM-V medium supplemented with recombinant human GM-CSF [(800 U/ml), Immunex, Seattle, WA] and IL-4 [(1000 U/ml) Genzyme Cambridge, MA. Every 2 days, 1 ml of spent medium was replaced by 1.5 ml of fresh medium containing 1600 U/ml GM-CSF and 1000 U/ml IL-4, to yield final concentrations of 800 U/ml and 500 U/ml, respectively. After 5 days' culture, DC maturation was induced by addition of $\text{TNF}\alpha$, IL- 1β (both from R & D Systems, Minneapolis, MN) and PGE_2 (Sigma) for 48 hrs as previously described (16–18). The phenotype of mature DC was determined by flow cytometric analysis of HLA-DR, CD86, CD80, and CD83 expression. Viability was determined by trypan blue exclusion. All preparations were highly enriched in mature DC with a large and veiled appearance, expressing HLA-DR(+++) CD86(+++) CD80(++) CD40(++). Most DC (i.e., 85%) also expressed CD83, a maturation marker. This phenotype was stable after it was pulsed with tumor lysate as described below.

Dendritic Cells Pulsing

Following culture, DC were washed twice in AIM-V and added to 50 ml polypropylene tubes (Falcon, Oxnard, CA). The cationic lipid DOTAP (Boehringer Mannheim, Indianapolis, IN) was used to deliver the total cell extract to DC. 500 μL of total cell extract derived from 5 to 10×10^6 tumor cells in AIM-V and DOTAP (125 mg in 500 μL of AIM-V) were mixed in 12×75 mm polystyrene tubes at room temperature for 20 min. The complex was added to the DC in a total volume of 2–5 ml of AIM-V and incubated at 37°C in an incubator with occasional agitation for 3 h. The cells were washed twice with PBS and resuspended in AIM-V as described below.

In Vitro Generation of Tumor-specific CTLs

Fresh or cryopreserved responder PBL were washed and resuspended in AIM-V at $10\text{--}20 \times 10^6$ cells/well in 6-well culture plates (Costar, Cambridge, MA) with tumor lysate-pulsed autologous DC (ratios from 20:1 to 30:1 responders PBL:DC). The cultures were supplemented with recombinant human IL-2 (10U/ml Aldesleukin, Chiron Therapeutics, Emeryville, CA) and incubated at 37°C . Human rIL-2 (10 U/ml) was added to the cultures

thereafter every 4 days. At day 21, CD8⁺ cells were separated from the bulk cultures by positive selection with CD8-Dynabeads (DynaL Inc., Lake Success, NY) before being assayed for CTL activity. As negative control targets, autologous lymphoblasts were prepared by 3-day stimulation with PHA (GIBCO-BRL, 1 mg/ml) in RPMI-1640 plus IL-2 (25 U/ml), while EBV-transformed autologous lymphoblastoid B-cell lines (LCL) were established by coculture of PBMCs with EBV-containing supernatant from the B95.8 cell line in the presence of 1 mg/ml cyclosporin A (Sandoz, Camberley, UK) and were maintained in AIM-V supplemented with 10% human AB serum (Gemini Bioproducts).

T-cell Proliferation Assay

CD4⁺ T cells, derived from day 21 CD8⁺ depleted T cell populations were re-stimulated once with tumor lysate-pulsed DC at a 20:1 ratio, and 2–3 weeks later further purified by positive selection with CD4-Dynabeads (DynaL Inc. Lake Success, NY) to obtain a population more than 99% pure. Lymphoproliferative responses were tested using autologous DC and/or LCL pulsed with tumor lysate from POLE mutated vs POLE wild type tumors. Briefly tumor lysate-pulsed or control PHA-activated peripheral blood lymphocytes lysate-pulsed or unpulsed autologous DC or LCL were seeded in a 96 well/plates (2×10^4 cell/well). CD4⁺ T cells (2×10^4 cell/well) were tested for specific proliferation after 72 h. Cultures were pulsed with 1 mCi/well of [³H] thymidine for the last 16 h, and incorporated radioactivity was measured as described [17]. All assays were carried out in triplicate wells. In additional experiments tumor-lysate stimulated CD4⁺ T cells or unstimulated PBL from POLE mutated vs POLE wild type patients were stained with carboxyfluorescein succinimidyl ester (CFSE) (CellTrace CFSE Cell Proliferation Kit, Invitrogen, Carlsbad, CA) at a working concentration of 10 micromolar as previously described [19] in the absence of IL2. The CFSE labeled cells were plated and cultured in the presence of tumor lysate-pulsed or PHA-activated peripheral blood lymphocytes lysate-pulsed autologous DC or LCL for 5 to 6 days. Cells were collected and labeled with CD8 and CD4 (Becton Dickinson) and analyzed on a flow cytometer (FacsCalibur, Becton Dickinson) using CellQuest. The percentage and numbers of CD8⁺ and CD4⁺ T cells in the control PHA-activated peripheral blood lymphocytes lysate-pulsed autologous DC vs tumor lysate-pulsed DC treated wells were calculated after analysis by flow cytometry to determine CD8⁺ and CD4⁺ T cell proliferation induced in this co-culture system.

Cytotoxic Activity

A standard 4-hour chromium (⁵¹Cr) release assay was performed to measure the cytotoxic reactivity of DC-tumor lysate stimulated CD8⁺ T lymphocytes. PHA-activated peripheral blood lymphocytes and/or EBV-transformed LCL were used as autologous control targets. The percentage cytotoxicity was calculated by the following formula: % cytotoxicity = $100 \times [(ES)/(T-S)]$, where E is the experimental release, S is the spontaneous release by effector cells, T is the maximum release by target cells lysed with 1% SDS.

Flow Cytometric Analysis of Intracellular Cytokines

Flow cytometric analysis of intracellular cytokine expression in CD4⁺ and CD8⁺ T cells were tested at about 4–6 weeks after priming, after resting for 14 days after the last antigen stimulation prior to activation with autologous DC pulsed with PHA-activated peripheral

blood lymphocyte-lysate or tumor-lysate from POLE mutated vs POLE wild type tumors. OKT3-stimulated T cells were used as positive controls in all the experiments. Briefly, T cells ($5 \times 10^5/\text{ml}$) were incubated at 37°C for 12 h in AIM-V plus 5% autologous plasma with PHA-activated peripheral blood lymphocytes or tumor lysate pulsed DC (30:1 T cell to DC ratio) and $10\text{ }\mu\text{g/ml}$ Brefeldin A. The cells were harvested, washed and fixed with 2% paraformaldehyde in PBS for 20 min at room temperature, after which they were washed and stored overnight in PBS at 4°C . For intracellular staining, the cells were washed and permeabilized by incubation in PBS plus 1% BSA and 0.5% saponin (S-7900, Sigma) for 10 min at room temperature. Activated and control cells were stained with FITC-anti-IFN- γ , and PE-anti-IL-4, and isotype-matched controls (FITC-anti-IgG2a and PE-anti-IgG1) from Becton-Dickinson. After staining, cells were washed twice with PBS plus 0.5% BSA. Analysis was conducted on a flow cytometer (FacsCalibur, Becton Dickinson) using CellQuest.

Statistics

Student t-test was used to evaluate differences in cytotoxic activity, cytokine secretion and proliferation by CD8+ T cells and CD8+ and CD4+ DC-stimulated T cells, respectively. Statistical analysis was performed using SPSS version 18. A P-value of < 0.05 was considered as the level of statistical significance.

Results

Tumor-Specific CD8+ Cytotoxic T cell Responses against POLE mutated vs POLE wild type cancers

Cytotoxicity assays were conducted after a minimum of 4 weeks after initiation of T lymphocyte cultures as described in the methods. The results presented in Figure 1 represent the mean of 2 to 5 separate cytotoxicity assays for each patient. CD8+ T cell cytotoxicity against autologous tumor-cell targets was demonstrated in all patients where viable autologous tumor target cells were available (i.e., three harboring POLE mutated tumors vs three harboring POLE wild type tumors, Table 1). Autologous tumor cell killing ranged from 26.2 to 67.6% in POLE mutated patients vs. 18.3 to 40.2% in POLE wild type at 20 effectors per target. Cytotoxicity against autologous PHA-stimulated or autologous EBV-transformed LCL was detectable at low to negligible levels in all patients (Figure 1). The absence of significant cytotoxicity showed that, although these cells were highly cytotoxic against autologous tumor cells, they failed to kill normal cells or autologous cells infected with EBV. Importantly, when the cytotoxic activity of tumor lysate pulsed DC-stimulated CD8+ T cells from POLE mutated vs. POLE wild type cancer patients was compared we found CTLs to be similarly effective in inducing killing of the autologous tumor targets (mean cytotoxicity $\pm$ SEM: $43.4 \pm 7.3\%$ versus $33.1 \pm 5.1\%$ in POLE mutated vs POLE wild type ($P=0.305$) (Figure 1).

Tumor-Specific proliferations of CD4+ T cells from POLE mutated vs POLE wild type cancer patients

Tumor lysate-pulsed DC stimulated CD4+ T cells (purity $>99\%$) from POLE (+) and POLE (–) cancer patients were tested for specific proliferation against tumor-lysate pulsed

autologous DC or LCL. As controls, PHA-activated peripheral blood lymphocytes lysate pulsed autologous DC or LCL or autologous unpulsed DC or LCL were used. Proliferation assays were conducted after a minimum of two *in vitro* stimulations with tumor lysate-pulsed DC as described in the methods section. As shown in Figure 2, consistent proliferations of autologous CD4+ T cells were detectable only after stimulation with DC pulsed with tumor lysate derived from POLE (+) endometrial cancer patients but not POLE (–) cancer patients. Indeed, only in POLE mutated cancer patients CD4 +T cell proliferations were significantly higher than those induced in CD4+ T cells stimulated by DC controls (Figure 2, $P = 0.03$).

Tumor-Specific proliferations of naïve CD8+ and CD4+ T cells from PBL

In additional experiments we evaluated whether freshly collected (i.e., naïve) PBL from POLE (+) and POLE (–) cancer patients may proliferate when cultured with tumor-lysate pulsed autologous DC in vitro. As controls, PHA-activated peripheral blood lymphocytes lysate pulsed autologous DC or LCL or autologous unpulsed DC or LCL were used. As clearly depicted in Figure 3A and 3B, a significant proliferation of both CD4+ and CD8+ freshly collected T cells from autologous PBL was detected only after stimulation with DC pulsed with tumor lysate derived from POLE (+) endometrial tumors but not POLE (–) cancer patients. Indeed, as shown in Figure 3A and 3B, only in this group of patients were proliferation of CD4+ and CD8+ cells significantly higher than those induced in CD8+ and CD4+ T cells by PHA-activated peripheral blood lymphocytes lysate pulsed autologous DC or LCL alone or unpulsed DC controls (CD4+ $P = 0.04$; CD8+ $P = 0.04$).

Intracellular Cytokine Expression by Tumor-Specific CD4+ T cells

To evaluate whether cytokine expression from tumor-lysate stimulated CD4+ T cells from POLE (+) and POLE (–) cancer patients segregated in discrete IFN- γ /IL-4– and IFN- γ /IL-4+ subsets, we took advantage of flow cytometric techniques for the detection of intracellular cytokine expression at the single cell level. Two color flow cytometric analyses of intracellular IFN- γ and IL-4 expression by CD4+ T were performed after at least 6 weeks of culture. This was completed after overnight stimulation with tumor-lysate pulsed or unpulsed DC in the presence of Brefeldin A, as described in the methods section. As representatively shown in Figure 4, only CD4+ T cells from POLE (+) mutated patients were found to express detectable amounts of intracellular IFN- γ after overnight stimulation with DC-loaded with POLE mutated tumor lysate ($P < 0.005$). Consistently, among the populations of CD4+ T cells in these patients, we found more IFN- γ expressors than IL-4 secretors (Figure 4 and data not shown). Importantly, DC pulsed with lysate from autologous control PBL or fibroblast (data not shown) or unpulsed DC controls stimulated only minimal IFN- γ expression in CD4+ T cells from POLE mutated patients after overnight stimulation (Figure 4).

Discussion

Our group, the TCGA Network and others have recently shown that 7–10% of sporadic endometrial carcinoma may harbor somatic mutations in the proofreading exonuclease domain of DNA polymerase epsilon (POLE), the leading strand replicase [4–13, 20].

Polymerase proofreading is vital to ensure replication fidelity, and in keeping with this, cancers with POLE exonuclease mutations display striking levels of mutations (i.e., ultramutator phenotype). Importantly, recent reports in both Type I and Type II endometrial cancer patients have provided strong evidence that patients harboring tumors with POLE exonuclease-mutations may experience a significantly better prognosis when compared to the remaining group of endometrial cancer patients [4–13, 20].

The basis of improved outcome in POLE ultramutated cancers is currently poorly understood. However, multiple non-mutually exclusive hypotheses have been used to explain the favorable prognosis of POLE mutated endometrial cancer patients. First, these tumors may be less fit than other cancers, possibly as a result of their extremely high number of mutations, to quickly spread and metastasize in the body. Second, because of their defective DNA repair, they may be more sensitive to standard anti-cancer treatments such as chemotherapy or radiation therapy. Finally, due to their extraordinary number of somatic mutations these cancers may be highly immunogenic for the host due to the large number of mutated epitopes.

Dendritic cells are the most potent antigen presenting cells known in humans and play a crucial role during the priming and reactivation of antigen specific immune responses [21]. This unique function as well as the recent standardization of DC culture conditions *in vitro* has provided the opportunity to evaluate their potential for the immunological treatment of cancer patients. Accordingly, using tumor-lysate pulsed autologous DC-activated lymphocytes in this paper we report the results of an evaluation of the intrinsic immunogenicity of POLE mutated versus POLE wild type endometrial cancers using completely autologous human cancer models. Here, we provide the first evidence that while both POLE + and POLE – endometrial cancers may be able to elicit significant cytotoxic T cell responses *in vitro* against autologous tumors only the POLE ultra-mutated are able to consistently induce strong proliferation in both naïve CD4+ and CD8+ T cells and tumor specific CD4+ T cells. Furthermore, only POLE mutated tumor-lysate pulsed autologous DC were able to induce significant IFN- γ cytokine secretion and a Type I cytokine bias in proliferating CD4+ T lymphocytes from endometrial cancer patients. Taken together, these findings may, at least in part, explain the better prognosis of this subset of endometrial cancer patients [4,10–13,20] and therefore may have important implications for the treatment of human tumors harboring POLE driver mutations.

Generation of potent CTL immune responses, in particular against weak antigens such as tumor-specific antigens (which are normally encountered outside an inflammatory context), requires the presence of CD4 helper T cells and the presence of both helper and CTL determinants on the same APC [22–24]. Indeed, the inability to mount a potent antitumor immune response against tumors has often been attributed to the lack of generation of sufficient tumor-specific T cell help [22–24]. Consistent with this view, in clinical studies, the *in vivo* persistence of adoptively transferred antigen specific CD8+ T cells against cytomegalovirus [25], or the enhanced generation of hepatitis B specific CTLs [26] was dependent upon endogenous CD4 responses. Moreover, the generation of tumor reactive T helper cells has been shown to be particularly important for the immunotherapy of established (i.e., vascularized) tumors and metastatic disease in several murine tumor

models [27–29]. Accordingly, our *in vitro* experimental results suggest that POLE ultra-mutated tumors may trigger strong immunity *in vivo* because of the combined activation of both the helper and the cytotoxic arms of the immune system.

POLE proofreading-mutant endometrial cancers are ultra-mutated, with a base substitution mutation frequency among the highest found in human tumors (i.e., the number of somatic mutations in these tumors exceed by far those found in endometrial and colorectal cancers with micro-satellite instability (MSI)) [4,11]. Importantly, a high load of somatic mutations has been recently associated with a survival benefit from immune checkpoint abrogation in human tumors [15]. These data combined with our findings and the recent discovery that POLE mutated tumors display prominent immune infiltrate *in vivo* [19], suggest but not prove that POLE ultra-mutated tumors, similar to melanoma and lung cancer patients with high mutation burden, may potentially strongly benefit from the use of novel immunotherapeutic approaches based on blocking immune check-points antibodies (i.e., anti-CTLA4-ipilimumab, anti-PD1-nivolumab, Bristol Meyers Squibb, Wallingford, CT). Additional studies will be necessary to validate this hypothesis.

In conclusion, we report the first evidence that POLE ultra-mutated tumors may be significantly more immunogenic when compared to POLE (–) endometrial tumors, in particular to the helper arm of the immune system. These data lend support to the hypothesis that the better prognosis of patients with POLE (+) tumors may at least in part be linked to an enhanced immunogenicity of these malignancies.

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Abbreviations

USC	uterine serous carcinoma
POLE	Polymerase ε
CTL	cytotoxic T lymphocytes

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Research Highlights

- 7–10% of endometrial carcinomas contain Polymerase ϵ (POLE) exonuclease-domain-mutations, have an ultra-mutated phenotype, and a favorable prognosis.
- POLE ultra-mutated tumors are more immunogenic when compared to wild-type POLE (–) endometrial tumors.
- Better prognosis of patients with POLE (+) tumors may be linked, at least in part, to enhanced immunogenicity.

Figure 1.

Tumor specific CD8+ CTL responses induced by tumor lysate-pulsed DCs in POLE mutated vs POLE wild type endometrial cancer patients in vitro, measured in 4 h ^{51}Cr -release assays. Percentage lysis (+ standard deviation) at a 10: 1 and 20:1 effector/target cell ratio are shown against autologous tumor cells and autologous PHA-stimulated normal lymphocytes or LCL for the two groups of endometrial cancer patients. Although in most of the experiments CD8+ T cells from POLE mutated patients were found more cytotoxic against autologous tumor cells when compared to CTLs from POLE wild type endometrial cancer patients, this difference was not significant ($P=0.305$).

Figure 2.

Tumor specific CD4+ T proliferation induced by tumor lysate-pulsed DCs in POLE mutated vs POLE wild type endometrial cancer patients in vitro, measured in CFSE based-assays after 5 days. Data are presented as mean proliferation index (+ standard deviation) of CD4+ T cell-stimulated with tumor lysate-pulsed DCs vs PHA-activated peripheral blood lymphocytes lysate pulsed control DCs for the two groups of endometrial cancer patients. Proliferation assays were conducted after a minimum of two in vitro stimulation with tumor lysate-pulsed DC as described in the methods section.

Figure 3.

Tumor specific peripheral blood CD8+ and CD4+ T proliferations induced by tumor lysate-pulsed DCs in naïve (i.e., fresh PBL) obtained from POLE mutated vs POLE wild type endometrial cancers, measured by CFSE based-assays after 5 days. Data are presented as mean proliferation index (+ standard deviation) of CD8+ and CD4+ T cells stimulated with

tumor lysate-pulsed DCs vs PHA-activated peripheral blood lymphocytes lysate pulsed control DCs for the two groups of endometrial cancer patients.

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Figure 4.

Two-color flow cytometric analysis of intracellular IFN- γ and IL-4 expression by tumor specific CD4⁺ T cells after overnight re-stimulation by tumor lysate-pulsed DC vs control DC pulsed with lysate from normal PBL in the presence of Brefeldin A. CD4⁺ T cells stimulated with OKT3 were used as positive control. A representative experiment using CD4⁺ T cells from a POLE mutated vs POLE wild type endometrial cancer patient is shown. Assays were conducted after a minimum of two in vitro stimulation with tumor lysate-pulsed DC as described in Methods. Upper panel: OKT3-restimulated CD4⁺ T cells. Middle Panel: control PBL-pulsed DC-restimulated CD4⁺ T cells. Lower Panel: Tumor-pulsed DC-restimulated CD4⁺ T cells.

Table 1

Sample ID	Histology	Grade	Stage	Age	Race	somatic mutation #	Somatic missense POLE mutations	PBL	Tumor culture
FF40	USC	G3	IB	57	W	4715	V411L-Y470H-S928I-F1099S-F1672L	yes	yes
FF9	USC	G3	IIIC	56	W	17344	R77C-V411L-R742C-T1052M-V1452A	yes	no
UTE4	ACA END	G3	IVA	65	W	n/a	A957V	yes	yes
ARK6	USC	G3	IB	48	W	13356	H76N-D368Y-A832T-C1642Y-A1967B-G2076V-L2207I	yes	yes
UTE9	ACA END	G1	IA	43	O	n/a	A456G	yes	no
ARK1	USC	G3	IVA	62	B	59	not present	yes	yes
ARK2	USC	G3	IVB	63	B	84	not present	yes	yes
FF16	USC	G3	IIIC	54	W	25	not present	yes	no
FF6	USC	G3	IA	63	B	39	not present	yes	no
ARK15N	USC	G3	IIIC	67	W	109	not present	yes	yes