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ORIGINAL ARTICLE

Immunosuppressive Tumor Microenvironment Status and Histological Grading of Endometrial Carcinoma

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Abstract

The recent successes of new cancer immunotherapy approaches have led to investigate their relevance in the context of the Endometrial Carcinoma (EC). These therapies, that take the tumor-induced immunosuppressive microenvironment into account, target the tumor immune escape, in particular the inhibitory receptors involved in the regulation of the effector T cells' activity (immune checkpoints). The aim of this study was to identify, in ECs, differences in intergrades immune status that could contribute to the differences in tumor aggressiveness, and could also be used as theranostic tools. The immune status of tumors was assessed by quantitative real-time PCR. We analyzed the expression of specific genes associated to specific leukocytes subpopulations and the expression of reporting genes associated with the tumor escape/resistance. This study highlights significant differences in the EC intergrades immune status especially the tumor-infiltrating cell types and their activation status as well as in the molecular factors produced by the environment. The immune microenvironment of grade 1 ECs hints at a robust tumoricidal milieu while that of higher grades is more evocative of a tolerogenic milieu. This genes-based immunological monitoring of tumors that easily highlights significant intergrade differences relating to the density, composition and functional state of the leukocyte infiltrate, could give solid arguments for choosing the best therapeutic options, especially those targeting immune checkpoints. Moreover it could enable an easy adaptation of individual treatment approaches for each patient.

Keywords Endometrial cancers · Tumor-infiltrating leukocytes · Checkpoints · Immunotherapies

Introduction

Endometrial cancer (EC) is the most frequent gynecologic cancer worldwide, and the fifth most common cancer in women with 320,000 new cases diagnosed in 2018 and a growing incidence in developped countries [1]. EC, which predominantly affects perimenopausal women, is frequently symptomatic at an early stage and thus often diagnosed at stage I, with a 5-year survival rate reaching 71%. However, the prognosis associated with recurring or metastatic disease is poor (5-year survival rate between 9% and 42%) [2] and therapeutic strategies are limited at those stages [3]. Moreover, EC caused 76,000 deaths wordlwide in 2012 [1]. There is therefore a real need to improve our understanding and management of this disease.

Currently, the therapeutic management of the patient depends on the endometrial biopsy which specifies the histological type and the histo-prognostic grade as well as the magnetic resonance imaging which allow the tumor staging. Within the last decade, improvement in technologies such as genomic, transcriptomic and histological analyses, allowed the establishment of new classifications of endometrial carcinomas. The latest classification proposed by The Cancer Genomic Atlas (TCGA) [4], has been made routinely applicable through the international consortium TransPORTEC. It

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consists of 4 groups according to various genetic and epigenetic features, and listed from good to poor prognosis. The Group 1, associated with the best prognosis, included endometrioid endometrial carcinoma (EEC) with somatic inactivating mutations in POLE exonuclease and very high mutation rates (POLE-ultra-mutated). Group 2 included EEC with microsatellite instability (MSI), frequently with MLH-1 promoter hypermethylation and high mutation rates (MSI-hyper-mutated). Group 3 tumors included EEC with low copy number alterations. Group 2 and group 3 are both showing similar progression-free survival rates. Group 4 (Serous-like or copy-number high) showed low mutation rate, but frequent TP53 mutations. This group has the worse prognosis, and included predominantly serous and mixed histology tumors with some high grade EEC [5].

Better understanding of tumor genetic aberrations is essential to define groups of patients with similar outlooks to standardize management and allows comparisons of therapeutic strategies. However, the tumor microenvironment (TME) has been also recognized as critical in the tumor development and progression as well as in the response to therapies, especially immune-checkpoint therapies [6, 7]. Indeed, cancer immunotherapies targeting the immune-checkpoint receptors, especially Cytotoxic T Lymphocyte-Associated protein 4 (CTLA-4) or Programmed cell-Death protein 1 (PD-1), and Programmed cell-Death 1 Ligand 1 (PD-L1) are currently revolutionizing cancer treatment with unprecedented improvements in overall survival in many aggressive diseases [8–12]. However, despite often impressive results, these anti-checkpoint therapies are not beneficial for every single patient, underlying the importance of predictive factors for treatment response and of patient stratification [13]. Whilst CTLA-4 and PD-1 checkpoint inhibitors are the crux of current clinical focus in immunotherapy, other checkpoints with potentially greater potency are emerging and promise to broaden the therapeutic "toolkit" and improve patient benefit [14–18].

The use of checkpoint inhibitors has a strong rationale in EC [19, 20]. Especially for the POLE-ultra-mutated and MSI-hyper-mutated types, which are characterized by a high mutation rate and consequently an active TME expressing high number of neoantigens and an elevated amount of tumor infiltrating lymphocytes. Additionally, among gynecological cancers, ECs show the highest expression of PD-1 and PD-L1 [21], and high mutation load correlates with increased PD1 expression [22]. Indeed, early clinical trials are already showing promising results [23, 24]. However, as for others cancer types, a significant proportion of patients do not respond to PD-1 blockade, suggesting others possible dominant immunosuppressive pathways.

The aim of this study was to better define the TME of ECs not only to characterize each subtype and/or grade but also to identify reliable biomarkers of response and, thus better orient therapeutic choices, particularly those targeting immune checkpoints. Therefore, we sought to address, in the TME of EC patients, the expression status of PD-1, CTLA4 and T cell Immunoglobulin and Mucin-domain containing-3 (TIM3), regarded as an emerging target in the cancer immunotherapy landscape. In addition, expression of potential checkpoint ligands was addressed at the same time. Moreover, various immune cell populations and expression of cytokines/chemokines were measured to understand, in each grade, the immunological background of the endometrial cancer tumor immune microenvironment.

Materiels and Methods

Patients and Samples

We retrospectively included the EC patients treated in the department of Gynecology and Obstetrics of the Archet 2 Hospital in Nice from January 2014 to January 2018. The biopsies of non-tumoral endometrium were obtained from hysterectomy patients suffering from benign pathologies, such as adenomyosis or leiomyoma (healthy control group) or during hysteroscopic examinations of peri- or postmenopausal patients with a persistent endometrial hyperplasia, or one/several hyperplastic polyps, simple without atypia (hyperplasia group).

Histological Analysis of Surgical Biopsies

After their collection in the department of Gynecology, the surgical biopsies were processed by the Pathology Central Laboratory of the Pasteur University Hospital (Nice, France), formaldehyde-fixed, paraffin-embedded, microtome-cut and Hematoxylin/Eosin-stained, following the standard protocol used in the lab. The histological examination confirmed the diagnosis based on the WHO classification [25].

Tumor Transcriptomics Analysis

Total RNAs Extraction

The automated RNA extractions were performed using the Maxwell 16® instrument (Promega, Lyon, France), following the manufacturer's instructions, on fixed and paraffin-embedded tissues (five 20 μm-sections per specimen), with the purification Maxwell 16® LEV RNA FFPE kit.

cDNA Synthesis and Real Time PCR (RT-PCR)

cDNAs were generated from total RNA (2.5 μg) via reverse transcription, using the Superscript IV enzyme (Invitrogen,

USA) and following the manufacturer's instructions. The use of SuperScript IV showing a high resistance to inhibitors of cDNA synthesis, has proved to be essential to provide reliable and consistent cDNA synthesis in our conditions of formaldehyde-fixed paraffin-embedded samples.

The RT-PCR reactions were performed in a StepOne Plus Real-Time PCR System instrument (Life Technologies, USA). For each investigated gene (Supplementary Table 1), 3 μl of cDNA were mixed with the specific sense (F) and antisense (R) oligonucleotides (Eurogentec, France) in the SYBR Premix Ex Taq II, Tli RNase H Plus buffer (Takara, Japan), according to the manufacturer's instructions. The target gene expression values were normalized to βactin (ΔCt) and used as 1/ΔCt in graphic representations in order to visually achieve proportional values to the amount of target nucleic acid. The comparative Ct (or $2^{-\Delta\Delta Ct}$) method was used to determine the relative levels of gene expression in the samples and expressed as the fold-increase relative to the healthy control.

Statistical Analysis

The statistical comparisons were performed using the non-parametric Mann-Whitney test (U test) with the Bonferroni correction, the p values were considered statistically significant when $p < 0.05/n$ (n = number of comparisons performed; here $n = 6$, thus the difference was significant when $p < 0.83\%$).

Results

Clinicopathological Features of the Cohort

Forty-eight patients were included in this study, 28 specimens of ECs and 20 specimens of non-tumoral endometrium (11 healthy controls and 9 endometrial hyperplasia) (Table 1).

Table 1 Clinical features of cohort

Tissues	Number	Average age
Healthy endometrium	11	51.8 ± 7.8 years
Endometrial hyperplasia	9	55.5 ± 6.7 years
Endometrial cancer	28	
- Grade 1		69,8 ± 9,7 years
Endometrioid type	12	
- Grade 2		64,8 ± 10 years
Endometrioid type	9	
- Grade 3		73,6 ± 8 years
Endometrioid type	2	
Serous type	4	
Mixed Mullerian type	1	

The patients were ranging from age 48 to age 88. The EC specimens were staged from grades 1 to 3, following the FIGO classification [26].

Histological Analysis of the Cohort

The panel b of Fig. 1 presents a representative image of the hyperplasia group's specimens without atypia, showing a simple architecture with endometrial glands of various sizes, irregularly dispersed compared to the healthy control (panel a).

Panels c, d and e of Fig. 1, representative of the grades 1, 2 and 3 ECs, show the progressive replacement of the tumor glandular architecture by solid areas (grade 1: less than 5% of solid areas; grade 2: between 6 and 50%; grade 3: over 50%).

Comparative Analysis of the Leukocyte Infiltrate in Control, Hyperplastic and Tumoral Endometrium

The quantitative assessment of the leukocyte subpopulations infiltrating the tissues was performed using a RT-PCR approach [27]. Cell marker genes that we used were identified as having sufficient cell type specificity that their expression levels can be used to measure immune cell subpopulations in the tumor microenvironment [28]. Thus, by measuring the expression of CD4, CD8, CD19, FoxP3, NCAM1, CD1a, CD68 and CD206, we were able to assess the relative leukocyte infiltrate composition for CD4+ T cells, CD8+ T cells, B cells (CD19), regulatory T cells (FoxP3), NK cells (NCAM1), dendritic cells (CD1a) and macrophages (CD68, CD206), respectively.

As shown in Fig. 2, the respective leukocyte infiltrates in endometrial hyperplasia and in the three endometrial tumor groups, each presented significant differences when compared to the healthy control tissues. However, all the leukocyte subpopulations were not affected. Compared to the control group, the $CD4^+$ T cell content was significantly increased as well in the hyperplasia tissues than in cancer tissues whereas the NK cell population was drastically reduced in all groups compared to the healthy control group (Table 2). In contrast, compared to the control group, the CD8+ T cell content was significantly modified, respectively slightly or strongly increased in the hyperplasia or tumor grade 1 groups, and strongly diminished or unchanged in the grade 2 and 3 tumor groups, respectively.

The B cell population was similar to the control group as well in the hyperplasia endometrium than in tumor endometrium (data not shown).

The overall level of macrophages, reported by CD68 expression, was slightly increased in the ECs when compared to control tissues, with rates significantly higher in the grade 3 than in the grades 1 and 2, whereas the M2 macrophages rates (as reported by CD206 expression) were similar in all tumor groups.

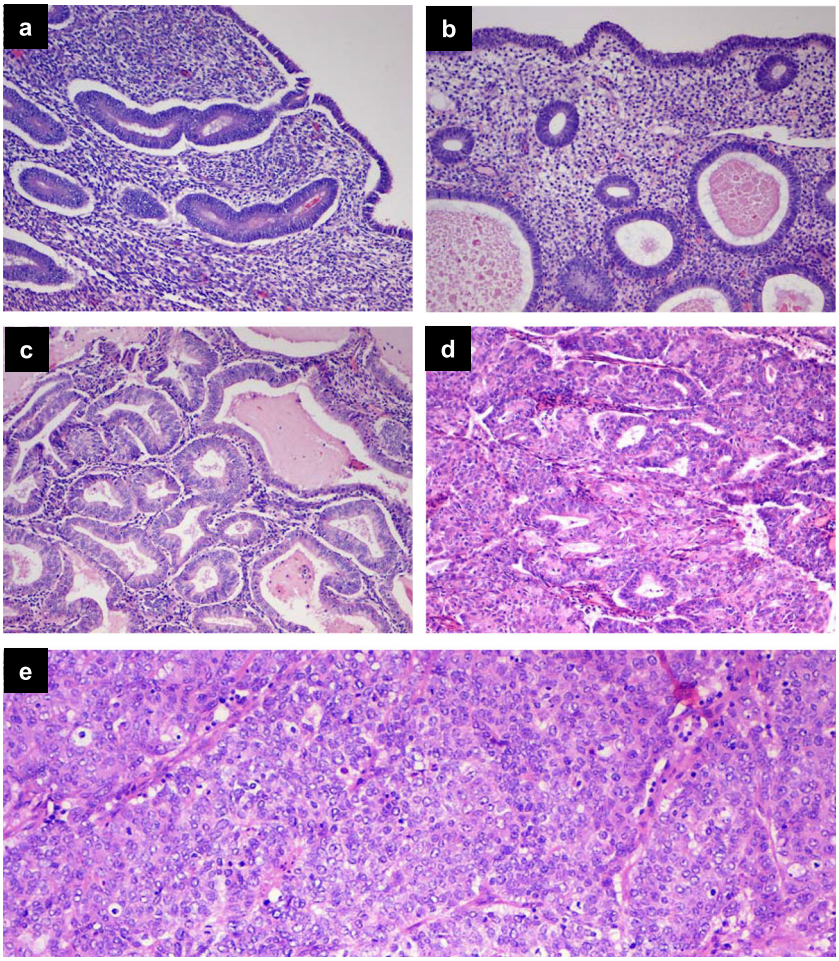

Fig. 1 Representative images of H&E staining of endometrium tissue sections. a Control healthy endometrium. **b** Simple hyperplasia without atypia. **c** Grade 1 EC. **d** Grade 2 EC. **e** Grade 3 EC. Magnification ×100

The regulatory T cell (Treg) population, reported by the expression of the FoxP3 gene, was significantly increased in the tumor groups of all grades compared to the control group. In the hyperplasia group, the Treg levels were similar to the control group.

Finally, considering the high DC content (reported by CD1a expression), the grade 1 EC group was different from the control group unlike the hyperplasia and grades 2 & 3 EC groups (Table 2).

Immune Checkpoints Expression in Control, Hyperplastic and Tumoral Endometrial Tissues

We focused on the study of the expression of CTLA4, PD-1, PD-L1, PD-L2, TIM-3 and galectin-9, using a RT-PCR strategy.

As shown in Fig. 3, CTLA4, PD-1 and TIM-3 expression was significantly increased in the three tumor groups when compared to the control group with significant intergrade difference only in the case of PD-1. Moreover, the expression of both PD-1 ligands, PD-L1 and PD-L2, was modified in endometrial tumors compared to control endometrium. Indeed, PD-L1 expression significantly increased in all tumor groups, with the highest level in grade 2, whereas only the grades 2 and 3 EC groups exhibited a significant rise of PD-L2 expression (Table 2).

In contrast, the expression of CTLA4, PD-1, PD-L1, PD-L2 and TIM-3 was unchanged in the hyperplasia group.

Concerning TIM-3's ligand, galectin-9 was significantly increased when compared to the control group in the hyperplasia group, as well as in the tumor groups of grades 1 and 2. In contrast, galectin-9 was unchanged in the grades 3 (Table 2).

Comparative Study of the Cytokine Microenvironment Profiles in Control, Hyperplasia, and Tumoral Endometrial Tissues

Several different signals are able to regulate the expression of immune checkpoint ligand/receptor pairs. We focused on the cytokines IL1β, IL6, IL10, IL12, IFNγ, the chemokines CCL2, CCL5, CCL17, CCL21, CX3CL1 and the growth factors M-CSF, TGFβ, TNFα, VEGF-A and -C.

As shown in Fig. 4, IL1 β and IL6 expression was significantly increased in the hyperplasia and endometrial tumors of any grades compared to the control group whereas those of

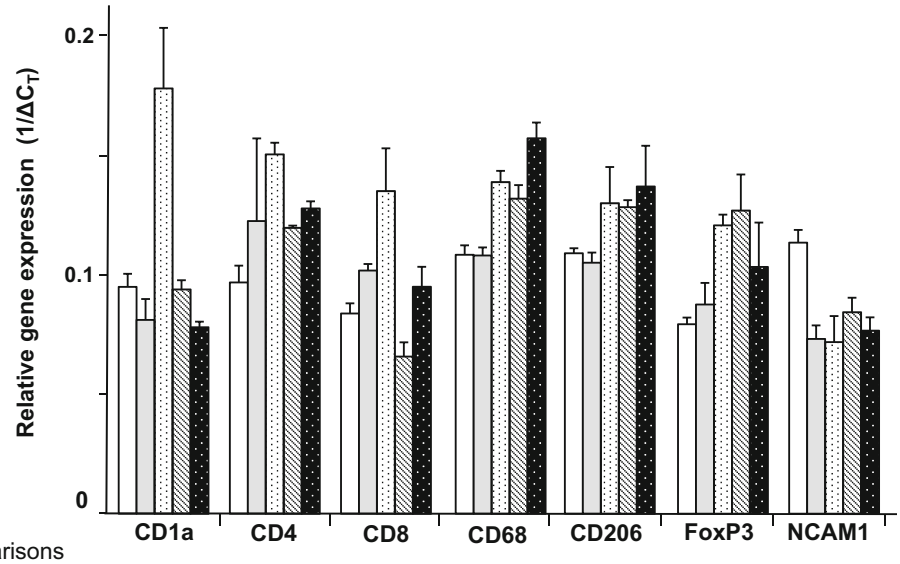

Fig. 2 Immune cells profile of endometrium tissue sections. RT-PCR analysis of the content in dendritic cells (CD1a), CD4+ T cells, CD8+ T cells, monocytes/ macrophages (CD68/CD206), Treg (FoxP3) and NK cells (NCAM1), in the control healthy endometrium (white column), endometrial hyperplasia (gray columns), grade 1 ECs (dotted white columns), grade 2 ECs (hatched colomns) and grades 3 ECs (dotted black columns). Samples were normalized to β actin. HE: healthy endometrium; Hyperplasia: endometrial hyperplasia; Gr: grade; EC: endometrial cancer. * $p < 0.0083$

Comparisons	CD1a	CD4	CD8	CD68	CD206	FoxP3	NCAM1
HE/Hyperplasia	ns	*	*	ns	ns	ns	*
HE/Gr1 EC	*	*	*	*	*	*	*
HE/Gr2 EC	ns	*	*	*	*	*	*
HE/Gr3 EC	ns	*	ns	*	*	*	*
Gr1 EC/ Gr2 EC	*	ns	*	ns	ns	ns	ns
Gr1 EC/ Gr3 EC	*	ns	*	*	ns	ns	ns

IL12 gradually increase from both control and hyperplasia tissues to the grade 1 EC and then decrease from the grade 2 to the grade 3 (Table 2). For IL10, its expression compared to the control group is increased only in the tumor groups, regardless the grade. In contrast, only the grade 1 EC group showed a significant rise of IFNγ expression.

When compared to the control group, the expression of CCL17 was significantly increased, in similar proportion, in hyperplasia and all tumor groups whereas only the grade 1 groups exhibited a significant rise of CCL2 expression. Besides, the CX3CL1 expression was increased in the hyperplasia, as well as in the tumor groups of all grades, when compared to the control group (Table 2).

Finally, the expression levels of the CCL5 and CCL21 chemokines, as well as those of the growth factors M-CSF, TGFβ, TNFα, VEGF-A and –C, in the 4 groups (hyperplasia and endometrial tumors of any grades) were similar to the control group 's levels (data not shown).

Discussion

Tapping into the resources of the immune system to counter tumor development is a challenge about to be won in the antitumor fight, as highlighted by the recent successes of anti-checkpoint therapies. The tumors' immune status contributes to the response to these treatments, however it is not yet routinely investigated in clinical practice.

In order to better guide the future therapeutic choices, we were therefore interested in defining the EC immune status in each grade. Because the immune status of a tumor is difficult to define, the criteria to describe it should be multiple, numerous and overlapping to ensure accurate evaluation and, thereby constitute a relevant tool for guiding the therapeutic strategy. The RT-PCR approach enables the easy analysis of multiple genes/ markers that, for technical, economic and/or practical reasons, could not be routinely completely investigated using other procedures. In the current study, the tumor immune status was supported by near 30 genes associated with the leukocyte subpopulations and/or with the tumor escape/resistance.

In cellular terms, given the overall increase of the leukocyte infiltrate, an immune response was triggered in all EC grades. However, the leukocyte populations present in the grade 1 ECs hinted at tumoricidal capabilities while the populations seen in the grades 2 and 3 were more evocative of tolerogenic functions.

The number of CD4+ T cells was similarly increased in all EC grades compared to controls whereas the number of CD8+ T cells was only increased in the grade 1 ECs and decreased or unchanged in the others grades. However, even though the CD4+ T cells count was similar in all EC grades, the analysis

Table 2 Relative levels of gene expression in the samples expressed as the fold-change relative to the healthy control ($2^{-\Delta\Delta Ct}$). (+) up-regulation, (−) down-regulation, nc: no change

Genes	Endometrial hyperplasia	Grade 1 EC	Grade 2 EC	Grade 3 EC
CCL2	nc	4.42	2,53	nc
CCL17	6.94	+36.37	+17.80	8.58
CD1a	nc	+24.48	nc	nc
CD4	+5.31	+8.88	+3.98	5.3
CD8b	+3.3	+24.6	−10.18	nc
CD68	nc	+3.31	+3.08	+5.87
CD206	nc	+2.87	+2.33	+4.24
CTLA4	nc	+10.11	+16.78	15.73
CX3CL1	nc	+7.98	+6.56	+5.81
FoxP3	nc	+24.68	+36.91	+25
Galectin-9	2,57	+4.65	+3,88	nc
IFNγ	nc	+43.8	nc	nc
IL1β	+7.6	+9,45	+27.95	+101.91
IL6	+17.45	+17.37	+12.94	+29.46
IL10	nc	+45.65	+45.35	41.76
IL12	+8.15	+83.8	+30	nc
NCAM1	−22.46	−17.01	−10.08	−12.26
PD-1	nc	+15.33	+79.67	59.99
PD-L1	nc	+43.70	+284	50.16
PD-L2	nc	nc	+22.15	49.12
TIM-3	nc	+3,69	+2,61	+3,99

of the cytokines profile predicted different phenotypes depending on the grade. Indeed, only the grade 1 ECs showed a significant increase of IFNγ expression when compared to control, suggesting the presence of Th1 CD4+ T cells in these tumors. Coherently, the cytotoxic Th1 cells, known to be able to stimulate the cytotoxic activity of CD8+ T cells, were only increased in the grade 1 ECs. Moreover, in line with a TME contributing to a Th1 profile of at least a part of the CD4+ T

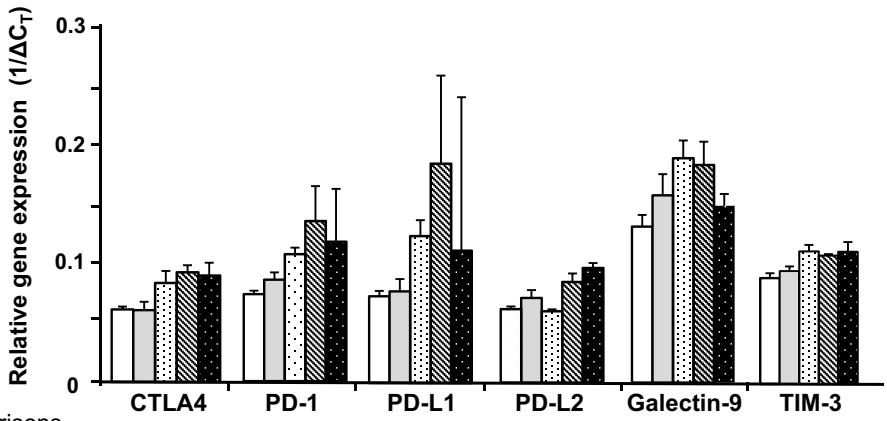

Fig. 3 Immune checkpoints profile of endometrium tissue sections. RT-PCR analysis of immune checkpoints gene expression in the control endometrium (white column), endometrial hyperplasia (gray columns), grade 1 ECs (dotted white columns), grade 2 ECs (hatched colomns) and grade 3 ECs (dotted black columns). Samples were normalized to β actin. HE: healthy endometrium; Hyperplasia: endometrial hyperplasia; Gr: grade; EC: endometrial cancer. * $p < 0.0083$

Comparisons						
HE/Hyperplasia	ns	ns	ns	ns	*	ns
HE/Gr1 EC	*	*	*	ns	*	*
HE/Gr2 EC	*	*	*	*	*	*
HE/Gr3 EC	*	*	*	*	ns	*
Gr1 EC/ Gr2 EC	ns	*	*	*	ns	ns
Gr1 EC/ Gr3 EC	ns	*	ns	*	*	ns

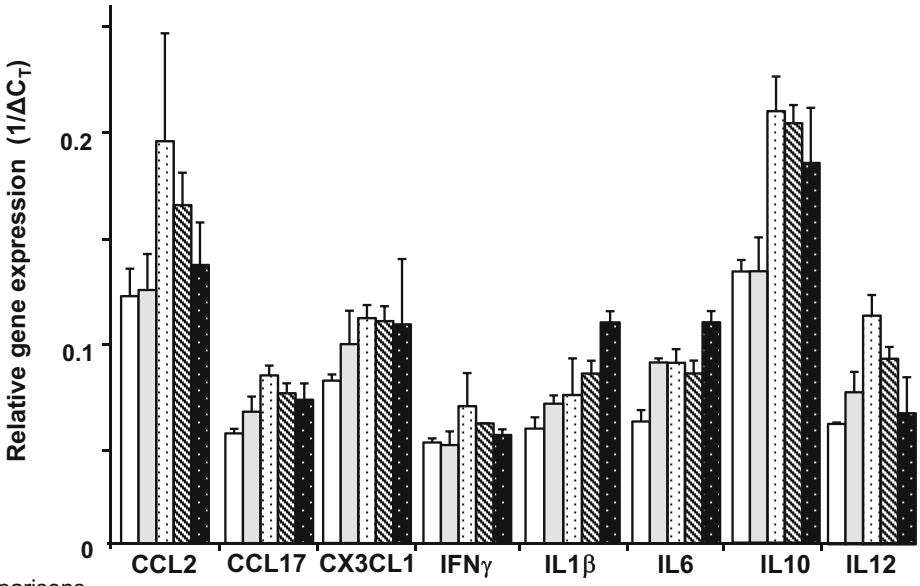

Fig. 4 Cytokine profile of endometrium tissue sections. RT-PCR analysis of cytokine gene expression in the control endometrium (white columns), endometrial hyperplasia (gray columns), grade 1 ECs (dotted white columns), grade 2 ECs (hatched colomns) and grade 3 ECs (dotted black columns). Samples were normalized to βactin. HE: healthy endometrium; Hyperplasia: endometrial hyperplasia; Gr: grade; EC: endometrial cancer. * $p < 0.0083$

Comparisons

HE/Hyperplasia	ns	*	ns	ns	ns	*	ns	*
HE/Gr1 EC	*	*	*	*	*	*	*	*
HE/Gr2 EC	*	*	*	ns	*	*	*	*
HE/Gr3 EC	ns	*	*	ns	*	*	*	ns
Gr1 EC/ Gr2 EC	*	ns	ns	*	ns	ns	ns	*
Gr1 EC/ Gr3 EC	*	ns	ns	*	*	ns	ns	*

cell population, the grade 1 ECs exhibited both high levels of IL12 and strong increase of myeloid DCs (CD1a-reported), which constitute a major source of IL12 when mature [29]. Coherently, the grade 1 ECs exhibited high contents of CCL2, which has been described as an essentiel actor of the DC maturation process via its binding to CCR2 [30].

If the myeloid DCs may reinforce a wide range of inflammatory and immune reactions required for the control of malignant cells, in contrast, the tumor-associated macrophages mostly support the tumor growth [31]. IL-1 β has emerged as a key component of tumor promoting-inflammation by shaping, besides tumor infiltrating myeloid cell recruitment, different tumor process such angiogenesis and skewing/ suppression of anti-tumor immunity [32]. Consistent with a role in tumor-promoting inflammation in ECs, the highest IL1β levels were associated with the highest macrophage contents in the grade 3 ECs.

In contrast to myeloid cells, the number of Tregs, the immune tolerance's major actors, was similarly increased in all EC grades compared to controls. Consistent with a potential Treg accumulation within the tumors, IL10 and CCL17 were concomitantly increased in the tumor microenvironment [33], not in hyperplasia tissue microenvironment. Nevertheless, the grade 1 ECs showed the higher CD8+ T cells/Tregs ratio, indicator of better prognosis in the most tumor types [34, 35].

As previously described by others, NK cells, the largest representative of the innate system were under-represented in all EC grades, regardless of the grade, when compared to control tissues. Endometrial NK cells (eNK cells) express unusual repertoire of activating receptors and other cell surface markers [36], especially, they lack the expresssion of CD16, but express high level of NCAM1 (CD56), hence its use in this study. It appeared that their cytotoxic activity was low as well as their ability to produce cytokines. Even though the anti-tumor activity of the eNK cells is low, it can be assumed that the decrease in NK cells infiltration into ECs indicates a reduction in immune cells with tumor-destructive properties. Recently, the presence of NK cells in the uterine tumors was reported to correlate with a beneficial outcome [37]. This provide motivation for the use of therapies able to stimulate the activity and/or the recruitment of eNK cells ECs, which remain to be explored.

Taken together, it is clear that the major molecular (IFNγ, IL12) and cellular (Th1 CD4+, cytotoxic CD8+ T cells, myeloid DCs) actors potentially able to delay tumor progression were more weakly represented (or absent) in the grades 2 and 3 ECs than in the grades 1. Coherently, in endometrial hyperplasia, currently regarded as a spectrum of morphologic alterations ranging from benign change induced by an abnormal environment to premalignant disease, the immune cell environment was close to the grade 1 EC.

Consistent with molecular and cellular data, the analysis of the main checkpoint/ligand pairs seems indicate that the immune brakes provided by some pairs are more expressed in the grades 2 and 3 ECs than in the grades 1 or in endometrial hyperplasia.

CTLA4 expression is restricted to T cells and its action allows the inhibition of the cytotoxic and helper T cells and the reinforcement of the Tregs' immunosuppressive activity [38]. The T cell contents as well the CTLA4 levels were increased in all EC grades when compared to control tissues. Nevertheless, if the CTLA4 and Treg contents were similar in all EC grades, the CD8+ T cells seemed significantly higher in the grade 1 than in the grades 2 and 3. These results led us to theorize that an anti-CTLA4 therapy could be benificial for all EC grades, especially for the grade 1, since in addition to the immunosuppressive Treg cells inhibition, expected in all grades, the theoretically functional but exhausted antitumor CD8+ T cells would thus be reactivated.

However, based on previous studies showing that CTLA-4 pathway blockade resulted in IFN-γ production by T cells [39], anti-CTLA-4 treatment could be also relevant in the grades 2 and 3 to drive Th1 immune responses required to tumor rejection, provided that there is no IFN-γ signaling defects [40]. Even if the role of IFN-γ signaling in tumor cells in the setting of anti-CTLA-4 therapy still remains poorly known, recent data however highlight that genomic defects in IFN-γ pathway genes are associated with primary resistance to anti-CTLA-4 therapy. It would also appear that the loss of IFN-γ-signaling in tumor cells represents a common mechanism for tumor resistance to immune checkpoint therapy, especially to CTLA-4 and PD-1 blockade treatments [41]. Consequently, searching/identifiying genomic defects in the genes of the interferon γ pathway seems essential to detecting the patients likely to better respond to these anti-checkpoint immunotherapies, emphazing the importance of combining both transcriptomic and genomic analyzes.

Compared to CTLA4, PD-1 is more widely expressed in leukocytes (T cells, B cells and NK cells) [42], with major functions to inhibit effector T cell activity and enhance the function and development of Tregs.

Given the expression of PD-1 and its 2 ligands in the different grades of ECs, it seems obvious that this system of immune brakes was more powerfull in the grades 2 and 3 compared to grade 1. Therefore, it is reasonable to suppose that anti-PD-1, anti-PD-L1, and anti-PD-L2 therapies could be particularly suited for the treatment of higher grade ECs. However, it should be noted that in addition to potential negative impacts induced by defects in IFN-γ signaling, the anti–PD-1 therapies could be impaired by the strong presence of PD-1–expressing Treg, as recently described in some aggressive subtypes of breast cancer [43]. Provide simultaneous information of the PD-1 expression and the Treg presence in the tumor could lead to the development of relevant strategies coupling T-reg depletion and anti-PD1 therapies. Moreover, in the cases of PD-L1/PD-L2 co-expression, single anti-PD-L1 or anti-PD-L2 immunotherapies might not be sufficient to effectively impact the PD-1 pathway.

A variety of other molecules, including TIM3, regulate T cell activation [44]. To date, four relevant ligands are known to interact with TIM3, among which galectin-9 itself is described as having several binding partners. TIM3/galectin-9 expression and functions are not limited to T-cells, the TIM3/galectin-9 pathways differ between immune and non-immune cells and may be associated with inhibitory as well as stimulatory immune functions [45, 46]. Consequently, it is not yet clear under what circumstances TIM3 and/or galectin-9 exhibit inhibitory or stimulatory effects on antitumor immunity.

In EC tumors, TIM3 expression was significantly higher than in non-tumoral endometrial tissues, regardless of the grade. To date, limited data are available to predict success of TIM3 inhibition in EC, and further investigations must be conducted to address this challenge. However, given the fact that PD-1 blockade may lead to up-regulation of TIM3, blockade of TIM3 following development of adaptative resistance to PD-1 or other checkpoint inhibitors already holds promise in developing combination therapies in which anti-TIM3 is a key component.

Based on our data, it is concevable that the galectin-9 expression in EC could be associated to inhibition rather than promotion of tumor development. In line with this assumption, a recent meta-analysis highlighted the association between galectin-9 expression and a better prognosis for solid tumor patients and, indicated galectin-9 as both prognostic biomarker and emerging therapeutic target against solid tumors [47]. Interestingly, this study notably supports galectin-9-induced anti-tumor immune response. Indeed, consistent with the data highlighted in the grades 1, galectin-9 induces DC maturation in vitro, as revealed by up-regulation of IL12 production, and galectin-9-matured dendritic cells elicite the production of Th1 cytokines (especially IFN-γ) by CD4+ T cells [48–50]. In the grades 2, despite similar galectin-9 levels, the poor DC contents might not be sufficient to obtain significant IL12 increase, itself not sufficient to significantly boost the cytotoxic Th1 cytokine production and consequently the cytotoxic activity of CD8+ T cells. As additional pejorative factor, the CD8 content itself is significatively lower in the grades 2 and 3 than in the grades 1. Nevertheless, other mechanisms, such as the induction of tumor cell apoptosis, described to support the negative impact of galactin-9 in tumor development could take place in ECs, mostly in the grades 1 and 2 and not in the grades 3.

Clearly, significant differences have emerged between tumor tissues and healthy control tissues, as well as between tissues of different tumor grades. Especially, the microenvironment of the grade 3 EC produces several brakes and few boosters of the immune response suggesting an immunosuppressive microenvironment. In constrast, low brakes and more

boosters in the grade 1 EC are evocative of a tumoricidal microenvironment. Based on investigated targets, the grade 2 EC seems in an intermediate status between the grades 1 and 3.

Overall, differences of immune status seems to emerge between the grades, as least between the grades 1 and 3. These differences that may contribute to the differences in tumor aggressiveness could be used as theranostics tools. Moreover, these data underline the importance of having a deep knowledge of tumor to predict the success of therapies or, to understand and anticipate their failure.

The TCGA classification is a huge step forward towards individualized therapies to assess which subsets of EC patients are more likely to benefit from an immunotherapeutic approach. Nevertheless, further investigations should include the identification of which dominant immunosuppressive pathway characterizes each subtype, grade or tumor in order to better identify reliable biomarkers of response. It is also crucial to identify biomarkers of response for the ongoing clinical trials which, following evidences derived from others cancers, are exploring combinations of checkpoints inhibitors or associations of checkpoints inhibitors with others therapies. Our transcriptomic analysis specifying the individual immune status of each tumor, which could be enriched with any other markers of interest, was designed to meet these expectations. In the future, it would be however interesting to pair this data with cell numbers using notably immunohistochemical techniques, which would integrate easily into the anatomopathological approach.

Performed in addition to the tumor grading and combined with the tumor genetic background, this analysis seems essential to ensure the best therapeutic option. Moreover, on a case-by-case basis, additional genes could be easily submitted to transcriptomic analysis to access new clarifications of the tumor microenvironment, specific to each patient.

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Compliance with Ethical Standards

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standarts.

For this study formal consent is not requiered.

Conflict of Interest The authors declare that they have no conflicts of interest.

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