



Doctorate program
Milan
EXPERIMENTAL
MEDICINE



Università degli Studi di Milano

**PhD Course in
Experimental Medicine**

CYCLE XXXVI

PhD Thesis

**UNCOVERING IMMUNE EVASION MECHANISMS
FOR THE PROGNOSIS AND THERAPY
OF ENDOMETRIAL AND OVARIAN CANCERS**

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Academic Year 2022-2023

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Abstract

Gynecologic malignancies are a heterogeneous group of solid tumors which include endometrial cancer (EC) and ovarian cancer (OC). EC is the most common gynecological neoplasm and the only gynecologic cancer with increasing incidence and mortality. It is generally diagnosed at an early stage and is well managed with surgery, radiation, and/or chemotherapy. However, EC patients with advanced/recurrent disease have poor outcomes and respond poorly to current management treatments. On the other hand, OC is a devastating disease since the majority of patients, when first diagnosed, are at advanced clinical stages (III/IV). Furthermore, relapse occurs in more than 70% of patients and the 5-year survival rate is lower than 40%. Thus, it is important the development of novel therapeutic approaches. In this context, remarkable progresses have been made in immunotherapy. In fact, several preclinical and clinical studies are ongoing with immunotherapeutic strategies combined or not with classic treatments. To date, the efficacy is not satisfactory, thus a detailed understanding of the biology and interactions between cancer and the immune system is essential for the recognition and development of potential new immunotherapeutic strategies and targets as well as the optimization of existing immunotherapies. Therefore, the aim of this thesis was to characterize the molecular profile, frequency, phenotype and function of immune cells in EC and OC patients.

In particular, considering that the presence of activated tissue-resident gamma delta ($\gamma\delta$) T cells, recognized for their favorable response in various human malignancies, has been associated with a beneficial response to immunotherapies, and that their role in EC progression has not been elucidated yet, we performed a characterization of $\gamma\delta$ T cells in EC. Flow cytometry analysis reveals that V δ 1 is the primary subset of $\gamma\delta$ T cells infiltrating EC lesions in both early and advanced stages. Upon *in vitro* stimulation, these cells maintain their functional activity by producing cytokines, such as interferon (IFN)- γ and tumor necrosis factor (TNF)- α . However, within the tumor, they exhibit increased expression of PD-1, compared to their blood-matched circulating counterparts. Single-cell RNA-sequencing (scRNA-seq) analysis confirmed the upregulation of PD-1 expression in $\gamma\delta$ T cells, exhibiting a progressive increase from peritumor to tumor specimens, in contrast to healthy tissue. Crucially, tumor-infiltrating V δ 1 T cells expressing PD-1 retain their immune competence, which can be enhanced by PD-1 blockade. Indeed, their cytotoxic response against autologous tumor cell targets significantly increases in the presence of the specific blocking anti-PD-1 monoclonal antibody compared to controls. Moreover, scRNA-seq analysis associated the presence and the specific activation of V δ 1 T cells with the response to anti-

PD-1 therapy. These data not only emphasize the specific anti-tumor response of V δ 1 T cells in EC but also indicate the feasibility of targeting $\gamma\delta$ T cells to enhance current immunotherapeutic strategies.

As few studies have been conducted on the early stages (stage I) of OC due to the difficulty in diagnosing this disease, we performed a deep characterization through scRNA-seq of stage I high-grade serous ovarian cancer (HGSOC) tumor microenvironment (TME), the most common subtype of OC, to assess the impact of the TME in OC progression and to identify potential immunotherapeutic targets. Within the HGSOC lesions we detected high enrichment of CD4 regulatory T cells (Tregs) that displayed a spectrum of states linked to their naïve, effector, proliferating and destabilized transcriptomic profiles. The presence of *FOXP3*⁺ Tregs associates with a permissive, immune-suppressive TME, characterized by the prevalence of CD4 Th2 cells, exhaustion of cytotoxic CD8 T cells, and pro-tumoral states of natural killer (NK) cells and tumor-associated macrophages (TAMs). Cell-to-cell communication analysis predicted multiple molecular mechanisms underlying Treg inhibition of T and myeloid immune responses, along with the establishment of reciprocal interactions between Tregs and tumor cells, promoting tumor progression. Trajectory analysis revealed two differentiation paths for Tregs, both leading to immunosuppressive Treg profiles. While the *FOXP3*^{high} profile converges with proliferating Tregs, the second path of cytotoxic *FOXP3*⁺ Tregs converged with *FOXP3*⁻*KLRB1*⁺ ex-Tregs, distinguished for their anti-tumoral *CXCL13*⁺*IFNG*⁺ transcriptomic profile. These findings highlight the pivotal role of Tregs in the early establishment of the immunosuppressive TME conducive to HGSOC, also highlighting the potential of manipulating Treg cell fate and their de-stabilization as a promising target for therapeutic interventions.

Disclosure for research integrity

The research proposed in this manuscript was conducted following the European Code of Conduct for Research Integrity, which includes the values of reliability, rigor, honesty, respect and transparency.

Abbreviations

$\alpha\beta$	Alpha beta
ACK	Ammonium-Chloride-Potassium
ACT	Adoptive cell therapy
APC	Antigen-presenting cell
DC	Dendritic cell
DMSO	Dimethyl Sulfoxide
EC	Endometrial cancer
EMA	European Medicines Agency
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FIGO	International Federation of Gynecology and Obstetrics
$\gamma\delta$	Gamma delta
HBSS	Hanks' Balanced Salt Solution
HC	Healthy control
HEPES	Hydroxyethylpiperazine-ethanesulfonic Acid
HGSOC	High grade serous ovarian cancer
HLA	Human leukocyte antigen
IC	Immune checkpoint
ICIs	Immune checkpoint inhibitors
IFN	Interferon
IL	Interleukin
IHC	Immunohistochemistry
KLRG1	Killer cell lectin-like receptor subfamily G member 1
LAG-3	Lymphocyte activation gene-3
mAb	Monoclonal antibody
MDSC	Myeloid derived suppressor cell
MHC	Major histocompatibility complex
MMR	Mismatch repair
MMRd	Mismatch repair-deficient
MMRp	Mismatch repair-proficient
MSI	Microsatellite instability
MSS	Microsatellite stability
NGS	Next Generation Sequencing

NK	Natural killer
OC	Ovarian cancer
OS	Overall survival
PARP	Poly ADP ribose polymerase
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein-1
PFA	Paraformaldehyde
PFS	Progression-free survival
PMA	Phorbol 12-myristate 13-acetate
<i>POLE</i>	Polymerase- ϵ
RPMI	Roswell Park Memorial Institute
RT	Room temperature
scRNA-seq	Single-cell RNA sequencing
SEER	Surveillance, Epidemiology, and End Results
STIC	Serous tubal intraepithelial carcinoma
TAM	Tumor associated macrophage
TCGA	The Cancer Genome Atlas
TCR	T cell receptor
TF	Transcription factor
TGF-β	Transforming growth factor- β
Th	T helper
TIGIT	T cell immunoglobulin and ITIM domain protein
TILs	Tumor-infiltrating lymphocytes
TKI	Tyrosine kinases inhibitor
TLS	Tertiary lymphoid structure
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TNM	Tumor-Node-Metastasis
Treg	Regulatory T cell
T_{RM}	Tissue-resident memory
UMAP	Uniform manifold approximation and projection
VEGF-A	Vascular endothelial growth factor-A
WHO	World Health Organization

Introduction

Gynecological cancers

Gynecologic malignancies are a heterogeneous group of solid tumors that originate from cervical, ovarian, uterine, vaginal, and vulvar tissues. Among them, EC, also referred to as uterine cancer, is the most common one. According to the World Health Organization (WHO) it is the sixth most common female tumor worldwide with 417,000 new cases reported in 2020 (1). In Italy it is the fourth diagnosed cancer in women, accounting for 5% of cases, following breast, colorectal, and lung cancers (**Figure 1A**). It is noteworthy that EC is the only gynecologic cancer with an increasing incidence and mortality rate (2,3). On the other hand, OC is less common than EC, with 313,959 new cases globally. However, it is one of the leading causes of death in women both worldwide and in Italy (**Figure 1B**).

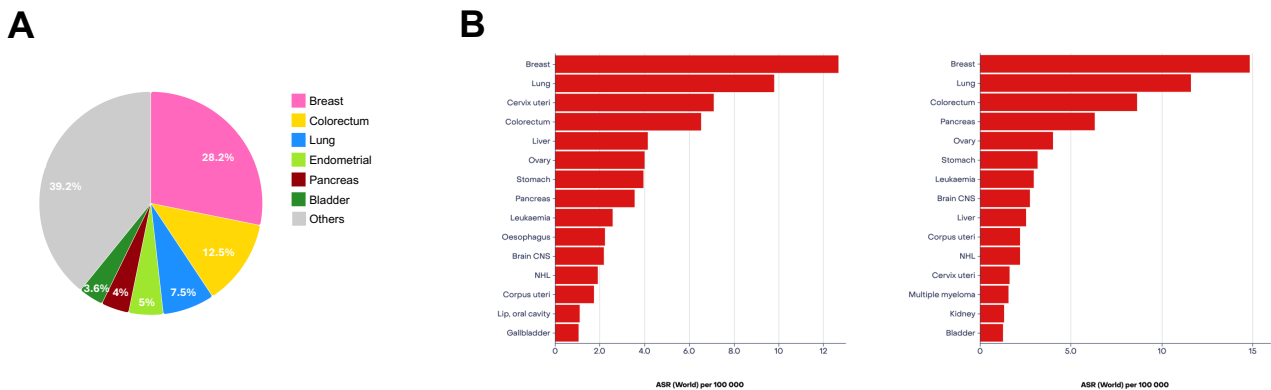


Figure 1 | Cancer statistics of incidence and mortality of tumors in women.

(A) Pie chart representing the distribution of the top six diagnosed cancers in female in Italy. The area of the pie chart corresponds to the proportion of the total number of cases. (B) Bars showing the mortality of different tumors in women worldwide (left panel) and in Italy (right panel). The data refer to the GLOBOCAN 2022 database (<https://gco.iarc.fr/today/en>).

Endometrial cancer

EC is a type of cancer that affects the inner lining of the uterus. Different factors can increase the risk of developing EC, including age (≥ 55 years), certain ethnicities, obesity (high body mass index), endogenous and exogenous estrogen exposure, early age at menarche, late-onset menopause, the use of tamoxifen, metabolic syndrome (e.g., hypertension, diabetes), family history, and genetic predisposition (e.g., Lynch syndrome, *PTEN* mutations) (4). In accordance, it is considered a postmenopausal disease with most cases occurring between the sixth and seventh decades of life and the median age at diagnosis of 61 years old (5,6), even if 15.4% of women with low-grade EC are under the age of 50 years (7). Therefore,

the higher incidence of EC may be linked to the aging of the population, together with the high prevalence of lifestyle risk factors, especially metabolic syndromes (8).

EC classifications

The WHO histopathological classification, which is based on microscopic features, is currently used for the diagnosis of EC. This includes various histological types, such as endometrioid (the most common histological type), serous, mucinous, clear cell, neuroendocrine, and other rare types, like mixed-cell adenocarcinoma, and undifferentiated and dedifferentiated carcinoma (9).

The main staging classifications for EC are the International Federation of Gynecology and Obstetrics (FIGO), which has been revised in 2023 (10), and the tumor-node-metastasis (TNM)-based Union for International Cancer Control (UICC) criteria (11). Considering different factors including the histological type, the tumor grade, the myometrial invasion, the lymphovascular space and the cervical stroma invasion, the adnexal and the uterine serosal involvement, and the lymph node status, EC is classified into four stages, starting from stage I where the tumor is confined to the uterus to stage IV with an invasive and metastatic tumor which has spread to distant organs (10).

Historically, EC has been categorized into two groups based on grade and histological features, as proposed by Bokhman in 1983 (12). Type I, which is the most common, includes low-grade (grade 1 and 2) endometrioid adenocarcinomas, associated with excess estrogen exposure, metabolic syndrome, and atypical endometrial hyperplasia. This type is usually confined to the uterus at the time of diagnosis, so it has a good prognosis. On the other hand, type II cancers are high-grade (grade 3), with different histological types (e.g., endometrioid, serous, carcinosarcoma, clear cell). They often present at an advanced stage, and they have a poor prognosis, due to their aggressiveness and high rate of metastasis and recurrence (13).

The advent of new technologies, especially Next Generation Sequencing (NGS), enabled a better understanding of EC. In 2013, The Cancer Genome Atlas (TCGA) conducted an integrated genomic and proteomic analysis of 373 EC and identified four molecular groups based on the mutation burden and the copy number alterations: polymerase ϵ (*POLE*) ultramutated, microsatellite instability-high/hypermethylated (MSI), copy number low and copy number high (14). Remarkably, these groups exhibit distinct features and prognoses (**Figure 2**), leading to new stratifications and treatment decisions in the clinic (15). In particular, the approach ProMisE (proactive molecular risk classifier for EC) has

been developed for the identification of mismatch repair (MMR) proteins and p53 and sequences of the *POLE* exonuclease domain using immunohistochemistry (IHC) (16). Based on this, four subgroups, similar to the ones described by TCGA, have been identified for clinical practice: DNA *POLE*, mismatch repair-deficient (MMRd), p53abn (abnormal; which shows aberrant p53 immunohistochemical staining) and p53wt (wild type).

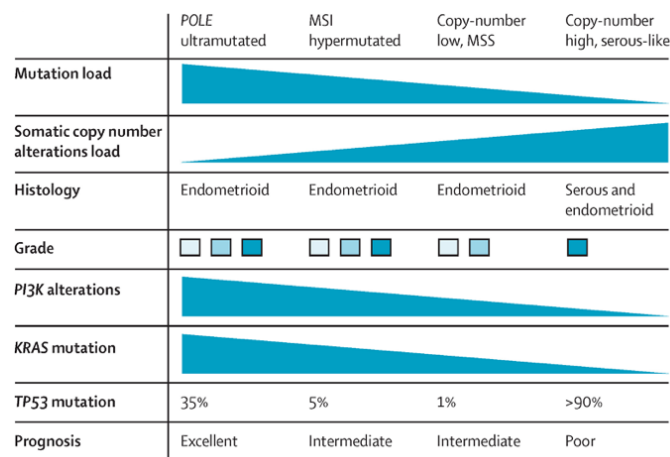


Figure 2 | Molecular subgroups of EC.

Features of the four EC molecular subgroups (*POLE* ultramutated, MSI hypermutated, copy-number low and copy-number high), including the mutation load, the somatic copy number alterations load, the histological type, the grade, PI3K, KRAS and TP53 mutations and the prognosis. For the grade light blue represents grade 1, medium blue represents grade 2 and dark blue represents grade 3. Figure adapted from (5).

Therapeutic approaches

The majority of EC patients are diagnosed at an early stage (stage I), as this tumor is frequently symptomatic already at early stages (5). The standard of care is surgery, which involves total hysterectomy along with bilateral salpingo-oophorectomy (BSO) (17). The pathological evaluation of the surgical staging is important to determine the prognosis and to identify those patients who might benefit from adjuvant radiotherapy and/or chemotherapy (18,19). Indeed, several studies reported that while patients with low-risk diseases respond well to the surgery and didn't show survival benefits from adjuvant radiotherapy (20,21), this is recommended for high-risk patients (22). If, on one hand, radiotherapy shows beneficial effects, on the other hand, the use of chemotherapy is controversial (19). In general, its use is supported for advanced stages of disease, especially in combination with radiotherapy (22). Importantly, the recently introduced molecular classification is now integrated into the risk stratification of EC patients (23) and in the future could help in deciding the type of adjuvant treatment.

Notably, EC patients diagnosed at an early stage (localized tumor) have a high survival rate of 95.6% at five years, while patients with advanced stages have a survival rate of 68.4% and 16.8% if the tumor has spread from the uterus to nearby (regional) or distant sites respectively (based on Surveillance, Epidemiology, and End Results (SEER) data from 2004 to 2019; <https://seer.cancer.gov/statistics-network/explorer/>). This is probably due to the low response rates to the standard of care, based on chemotherapy (whose front-line regimen is platinum-based, with recommendations for carboplatin/paclitaxel) or hormone therapy (suggested for low-grade endometrioid tumors that express estrogen and progesterone receptors), of metastatic and recurrent patients and the lack of second-line treatments (24), thus needing the development of novel therapeutic approaches.

Over the years, numerous targeted therapies have been tested in clinical trials to treat EC by targeting altered molecular signaling pathways, but none have been approved yet. Endocrine treatment is often considered due to the expression of estrogen and progesterone receptors in EC (15), in fact, it is the alternative strategy to standard chemotherapy, thus many studies have been conducted. However, a meta-analysis showed that only a modest response is achieved with this type of therapy (25). Considering that angiogenesis supports tumor growth, antiangiogenic agents, such as bevacizumab targeting vascular endothelial growth factor A (VEGF-A), and lenvatinib, an oral tyrosine kinases inhibitor (TKI), have been tested (26), showing variable responses and thus needing further investigations (27). The most frequently altered pathway in EC is the PI3K/AKT/mTOR pathway, which is involved in proliferation, cellular growth, apoptosis, and angiogenesis, giving rationale for the use of mTOR inhibitors, which target this pathway. Nevertheless, several clinical trials obtained low response rates (28). Novel targeted therapies are demonstrating promising results in EC and are still under investigation. Among them, trastuzumab, a humanized monoclonal antibody against HER2/Neu, obtained improved outcomes in combination with chemotherapy in uterine serous cancers (29). WEE1 inhibitors, targeting *TP53* which encodes for p53, may seem a promising strategy for serous EC, as they are mainly characterized by *TP53* mutations (26). In accordance, the WEE1 inhibitor adavosertib led to effective responses in approximately 30% of patients and is being investigated in a larger phase II study (30,31). Poly ADP ribose polymerase (PARP) inhibitors also showed promising results since EC is often characterized by mutations in *PTEN* and *TP53* genes (32). Furthermore, PARP inhibitors seem to have an immunomodulatory role, thus acting on the immune response within the tumor (33). So, their use, either alone or in combination with other targeted therapies, is being investigated. Despite several studies testing different

therapeutic approaches, there is still no effective treatment for advanced and recurrent EC patients, thus it is important the development of novel therapies.

EC immune tumor microenvironment (TME)

The immunologic characterization of the normal endometrium is dated to experiments performed 20 years ago, suggesting that confirmations are needed with up-to-date technologies (34). However, these data provide evidence that a regulatory immune environment is present in the healthy endometrium, as this is crucial for pregnancy and the defense against pathogens, moreover, it is characterized by shifts due to hormonal exposure (35). Lymphocytes make up the majority of the immune cells. In particular, for what concerns the adaptive immunity, T cells (CD3⁺ cells) are the main population among all immune cells (CD45⁺ cells) (36). Contrarily to the peripheral blood (PB), in the endometrium, the main subpopulation is the one of the cytotoxic CD8 T cells with fewer helper CD4 T cells (37). In addition to the conventional alpha beta ($\alpha\beta$) T cells, the unconventional $\gamma\delta$ and NKT cells (defined as CD3⁺CD56⁺ T cells) are also present in the endometrium, albeit at lower frequencies (37). Remarkably, the innate NK cells constitute one of the major populations in the uterus, with CD56^{bright} cells being the predominant NK population (38). Interestingly, these cells are distinct from both circulating conventional NK cells and NK cells from other peripheral organs (39). Regarding the phagocytic counterpart, granulocytes and myeloid cells (monocytes/macrophages) constitute a minority of the immune cells, whereas B cells are rare (37). The distribution of the immune populations in the normal endometrium is shown in **Figure 3**.

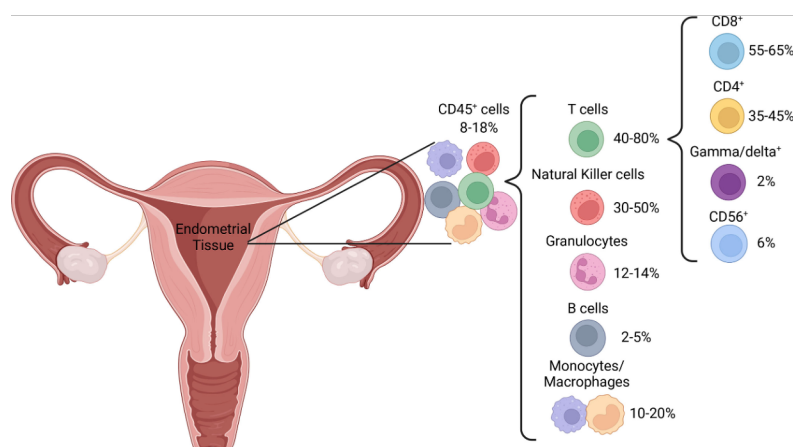


Figure 3 | Distribution of immune populations in normal endometrium.

Distribution represented as frequency of the different immune populations (conventional and unconventional T cells, including CD8, CD4, $\gamma\delta$ and NKT (CD56⁺), NK cells, granulocytes, B cells and monocytes/macrophages). Figure from (34).

The importance of the immune system in the context of EC has been demonstrated by its impact on the prognosis of EC patients. In fact, recently, a meta-analysis revealed that tumor-infiltrating lymphocytes (TILs) and TAMs have a prognostic significance in EC (40). This finding is consistent with other studies that have also reported that the presence of tumor-infiltrating CD3⁺ cells, particularly CD8⁺ cells, identified using immunohistochemistry (IHC), is a favorable prognostic factor in EC patients, leading to better overall survival (OS) for those patients with a high number of cytotoxic CD8 T cells (41,42). Importantly, CD8⁺ cells indicate improved OS regardless of the disease stage of EC patients (41). Conversely, the presence of TAMs is linked to a worse outcome (40). These findings have been confirmed most recently in early-stage EC patients using multiplexed analysis, where more markers were combined to assess the phenotype of the immune cells (43,44). Interestingly, T cells expressing the immune checkpoint (IC) programmed cell death protein 1 (PD-1) are associated with longer relapse-free survival. Moreover, the infiltration of intraepithelial CD103⁺ cells, which are predominantly CD8 T cells and express PD-1, is also correlated with improved survival (45), highlighting the important role of these cells in the control of tumor progression. On the other hand, the role of Tregs seems to be controversial. While some studies report that they predict a worse OS and thus could promote tumor growth (46,47), others associate the presence of Tregs with better OS (44). Remarkably, the prognostic significance of the immune environment in EC has been confirmed by recent studies that take advantage of the huge cohort of EC patients published by TCGA (48–50). So, considering its impact on the prognosis of EC patients, even if with controversial results, the immune system is important for EC progression, however functional studies are needed to clarify the role of the various immune populations infiltrating EC TME.

Immunotherapies

Immune checkpoint inhibitors for EC treatment

It is noteworthy that the presence of the immune system in EC plays a significant role in tumor development and progression. Furthermore, the tumor microenvironment has the ability to modulate the immune response, which opens new possibilities for EC treatment (51). In particular, immunotherapies that utilize immune checkpoint inhibitors (ICIs) are currently tested in several clinical trials, as a consequence of the upregulation of immune checkpoints and their ligands in EC TME.

Among them, PD-1 and its ligand PD-L1 are highly expressed in EC, with levels of expression that are the highest among gynecological malignancies (52). PD-1 is expressed

by many tumor-infiltrating immune cells and the interaction with its ligands, PD-L1 and PD-L2, inhibits cell activation and differentiation (53). PD-L1 expression on both tumor cells and immune cells has been associated with advanced stages of disease in EC (54), however its impact on the prognosis is controversial. In fact, while some studies reported that PD-L1 expression on tumor cells is associated with a favorable prognosis in high-risk patients (55,56) and longer progression-free survival (PFS) (57), contrarily others showed that its expression on both tumor and immune cells is related to worse OS (56,58). This highlights the importance of evaluating PD-L1 separately on tumor and immune cells to predict EC patients' outcome. Moreover, a high expression of PD-L1 has been observed specifically in POLE-ultramutated and MSI molecular subgroups (57,59). These groups of patients are characterized by a high number of mutations which lead to neoantigen expression and the infiltration and activation of the immune system (57). So, due to their high immunogenicity, these tumors were considered an ideal target for ICIs. In accordance, following the results from different clinical trials which supported the sensitivity of MSI/MMRd to ICIs (60,61), in 2017 the Food and Drug Administration (FDA) approved the use of pembrolizumab, a monoclonal antibody (mAb) targeting PD-1, for the treatment of unresectable or metastatic MSI or MMRd solid tumors, including EC. In addition to pembrolizumab, other antibodies targeting either PD-1 or PD-L1 are currently under evaluation in several clinical trials (62). Although the efficacy and the safety profile of these ICIs obtained important results, there was still the possibility to improve the response rates (52). Consequently, ICIs started to be investigated in combination with other therapies. Based on different clinical trials (63,64), in July 2021, the FDA fully approved the use of pembrolizumab in combination with lenvatinib, a multikinase inhibitor that targets vascular endothelial growth factor receptors 1–3, for advanced EC patients (65). Other trials provided updated results, and these led to the approval by the FDA, in February and March 2023, for dostarlimab, another antibody targeting PD-1, and pembrolizumab as monotherapies for MMRd EC after chemotherapy. Importantly, recent clinical trials demonstrated that the beneficial effect of the addition of ICIs to chemotherapy is not limited to MMRd EC patients, but extends to mismatch repair-proficient (MMRp) EC patients too, which represent the majority of EC patients (66,67). Therefore, this suggests that ICIs could be added to chemotherapy regardless of MMR status of the patients (68).

ICIs targeting CTLA-4 are also investigated in combination with antibodies anti-PD-1 in different clinical trials (NCT03015129, NCT02982486, NCT03015129, NCT03277482, NCT03517488). Nevertheless, up to now only one of them reported their data with only a

modest response in MMRp EC patients, indicating that a further characterization of EC is essential for the identification of those patients that could benefit from specific treatments (69).

In accordance, novel potential immunotherapeutic targets have been identified for EC. CD39 is an enzyme that leads to the release of an immunosuppressive form of adenosine in the tumor microenvironment (70). Its expression could be used to identify tumor-specific T cells (71) and is associated with T cells exhaustion (72). So, it is now investigated as a new immunotherapeutic target in cancer (73). Accordingly, in EC, tumor-specific tissue-resident memory (T_{RM}) T cells characterized by the co-expression of CD39 and CD103 showed an activation profile and cytolytic potential (74). Moreover, the expression of CD39, together with PD-1, CXCL13 and CD103, identifies tumor-reactive CD8 and CD4 TILs and correlates with improved survival in EC (75). In recent years, T cell immunoglobulin and ITIM domain protein (TIGIT) has emerged as a novel IC by suppressing the functions of both NK and T cells (76) and it has the potential to be targeted in EC too (77). In fact, TIGIT has been found to be expressed on tumor resident NK cells ($CD103^+$) with an exhausted phenotype (78). Its co-expression with PD-1 has been observed on T_{RM} T cells with high proliferation rates and reduced cytotoxic activity and the blockade of both PD-1 and TIGIT enhanced T_{RM} cytotoxicity (79). Finally, lymphocyte activation gene-3 (LAG-3), an IC mainly expressed in activated T and NK cells (80), was found in MMRd EC patients (81) and its expression has been associated with a high infiltration of CD8 T cells (82). The blockade of LAG-3 in EC is currently under investigation in a clinical trial that includes patients with MMRd EC (NCT0353802).

In addition to novel targets, it is important to identify biomarkers for the response to immunotherapy. Interestingly, a phase II trial testing the combination of nivolumab (mAb anti-PD-1) with cabozantinib, a multitargeted TKI, in advanced, recurrent, or metastatic EC observed a higher proportion of highly activated tissue-resident $\gamma\delta$ T cells in patients who benefited from the therapy (83). However, the role of $\gamma\delta$ T cells in EC has not been defined yet (84). Thus, elucidating the role of this population could aid in identifying those patients that could benefit from immunotherapy.

$\gamma\delta$ T cells

$\gamma\delta$ T cells are considered a bridge between adaptive and innate immunity with a T cell receptor (TCR) composed by a TCR γ -chain in combination with a TCR δ -chain (85). They originate in the thymus from a common precursor with $\alpha\beta$ T cells (86). During their

development, the rearrangement of the TCR occurs through V (variable), D (diversity; only for the δ -chain), and J (joining) gene segments recombination, also known as V(D)J recombination. Although the number of rearrangements is limited, the introduction of random nucleotides (N additions), along with the incorporation of distinct D segments, increases the potential diversity of the $\gamma\delta$ TCR (87). $\gamma\delta$ T cells development has been extensively studied in mice, and it takes place in different waves that define the tissue-tropism of tissue-resident $\gamma\delta$ T cells (88,89). However, $\gamma\delta$ T cells are not conserved between mice and humans and also their ontogeny is different, with the one of human $\gamma\delta$ T cells being less characterized, even if similarities regarding the development waves were suggested (90). Fortunately, the advent of new technologies allowed to gain insight into this process by analyzing human fetal and postnatal $\gamma\delta$ thymocytes ex-vivo by high-throughput sequencing (91). In particular, a distinct fetal thymic $\gamma\delta$ TCR repertoire has been identified which evidenced that in the fetus effector $\gamma\delta$ thymocytes are committed to three different effector fates with a wave-like pattern, while the pediatric thymus generates naïve $\gamma\delta$ T cells that differentiate into two main subtypes (92). In fact, in humans, $\gamma\delta$ T cells are divided in different subsets based on the TCR δ -chain. V δ 2 T cells, which most frequently pair with the TCR γ -chain TRGV9 and are thus defined V γ 9⁺V δ 2⁺ T cells, are the first $\gamma\delta$ T cells to be generated in the embryonic development, they rapidly expand after birth in response to pathogens and represent the predominant subset in the PB (86,93,94). Their activation is linked to the recognition through their TCR of phosphoantigens, small non-peptidic phosphorylated molecules that are intracellular metabolites of the mevalonate pathway (95). On the other hand, V δ 1 T cells can pair with different γ -chains and are considered more adaptive-like with a private repertoire (96–98). They are more enriched in peripheral tissues, such as gut and liver, however the ligands recognized by their TCR have not been identified yet (99). In addition, V δ 3 T cells constitute a minor subset enriched in the liver and in patients with some chronic viral infections and leukemias and they recognize glycolipid antigens (100,101).

One of the most important features of $\gamma\delta$ T cells is that, despite the presence of the TCR, their activation is independent of major histocompatibility complex (MHC) antigen presentation, they are thus characterized by the direct recognition of antigens and the induction of rapid innate-like responses (85). In accordance, $\gamma\delta$ T cells constitute a first line of defense as they exert cytotoxic activity and release cytokines, including mainly IFN- γ and TNF- α , once they encounter an infected or a tumor cell (102). Moreover, in addition to the TCR, $\gamma\delta$ T cells effector functions could be promoted by natural killer receptors too. In

accordance, the receptor natural killer group 2 member D (NKG2D) mediates the recognition of tumor cells through the binding to its ligands MHC class I-like molecules A and B (MICA and MICB) and UL16-binding proteins (ULBPs) (103). As we have published, the natural cytotoxicity receptor NKp46 identifies a subset of V δ 1 T cells with enhanced cytotoxicity in colorectal cancer (104). While NKp30 and NKp44 can be induced upon stimulation *in vitro* increasing the antitumor activity of $\gamma\delta$ T cells (105). Finally, our laboratory demonstrated that the expression of NKG2A identifies V δ 2 T cells endowed with high antitumor potential (106). The main features of $\gamma\delta$ T cell subsets, compared with conventional $\alpha\beta$ T cells, are shown in **Table 1**.

Feature	$\gamma\delta$ T cells			$\alpha\beta$ T cells	
Subpopulations	V δ 1	V δ 2	V δ 3	CD4	CD8
Development	Thymus, common precursor				
Frequency	25-40% peripheral tissues	1-10% PBMCs	5% peripheral tissues	25-60% PBMCs	5-30% PBMCs
MHC restriction	No MHC restriction			MHC class II	MHC class I
TCR ligands	Unknown	Unprocessed PhAg	Unknown	Processed peptides	
Response	Innate/Adaptive			Adaptive	

Table 1 | Major differences between human unconventional $\gamma\delta$ T cell subsets and conventional $\alpha\beta$ T cells.

Table adapted from (116).

Due to their features $\gamma\delta$ T cells are critical in the context of tumors, in fact in humans they mainly have an antitumor role, but in specific contexts they could exert pro-tumor functions too (107). Remarkably, in 2015, the transcriptomic analysis of 18000 tumor samples, including 39 different human cancers, revealed that they were the most favorable prognostic parameter (108). Following this, several studies confirmed the prognostic role of $\gamma\delta$ T cells, including our laboratory which showed that CD69⁺ V δ 1 T cells impact the overall survival of colorectal liver metastasis patients (109), thus increasing the interest for their application in cancer immunotherapy (110). In fact, due to their MHC-independent activation, their response is relevant in the context of MHC class I-deficient tumors, which often occurs as a mechanism of immune evasion (111). Historically, several clinical trials investigated the

activation and expansion of V δ 2 T cells using aminophosphonates, e.g. zoledronate and pamidronate, to increase phosphoantigen levels, but limited efficacy has been obtained so far (112–114). Recent advances in this field are allowing to develop novel therapeutic approaches. Among them, engineered chimeric antigen receptor (CAR) $\gamma\delta$ T cells or TCR $\gamma\delta$ -transduced T cells, are shaping the new generation of cancer immunotherapies (110). Nevertheless, the impact of the TME should be taken into account. Accordingly, recent studies highlighted the importance of targeting ICs expressed on $\gamma\delta$ T cells in order to restore their activation (115–117).

Ovarian cancer

OC is not a single disease entity, but it is a heterogeneous disease composed of different histological subtypes that constitute distinct pathologies with varying risk factors, origins, molecular compositions, clinical features, and therapeutic approaches (shown in **Table 2**) (118). Epithelial cancers represent the majority of OC (90%) and include serous, endometrioid, clear cell, and mucinous carcinomas, with HGSOC being the most common one (70%) (119). Histologically and clinically HGSOC is different compared with low-grade serous ovarian carcinoma and thus are considered distinct pathologies (120). Accordingly, OC is broadly divided into two groups: type I are mostly early-stage tumors characterized by a low-grade, slow growing, genetic stability, as they lack *TP53* mutations and are associated with mutations in other genes, such as *KRAS*, *BRAF*, *PTEN* and *β -catenin*, and include low-grade serous and endometrioid carcinoma, clear cell and mucinous carcinoma; on the other hand, type II present at advanced stages and are characterized by a high-grade, aggressivity, genetic instability harboring typically *TP53* mutations, and include HGSOC and high-grade endometrioid carcinoma (121). The differences among the histologic subtypes reflect on the origin of these tumors too, in fact even if the pathogenesis of OC is not clear and is highly debated, it has been proposed that serous tumors generate from the implantation of epithelium from the fallopian tube, while endometrioid and clear cell tumors have been associated with endometriosis (122). In particular, for HGSOC, the serous tubal intraepithelial carcinoma (STIC) has been proposed as a precursor, as it is a noninvasive tumor lesion that originate preferentially in the distal fallopian tube epithelium and could progress in the fallopian tube or after implanting on the ovary or the peritoneum and give origin to HGSOC (123).

The risk factors for the development of OC include genetic mutations, age, hormonal therapy, infertility and nulliparity (118). Following *TP53*, which is the predominant genetic

alteration in epithelial ovarian cancer (124), particularly in HGSOC (125), germline *BRCA1* and *BRCA2* mutations are the most significant ones for OC and HGSOC is the most common subtype associated with them (126,127). Also Lynch syndrome can increase the risk to develop OC (128). However, even if some mutations are recurrent in OC, some tumors, especially HGSOC, are highly heterogeneous (129).

Histologic subtype	Clinical findings	Genetic characteristics	Treatment options
High-grade serous carcinoma and high-grade endometrioid carcinoma	• Can present with peritoneal carcinomatosis, ascites and/or pelvic mass • Typically advanced stage at presentation	• Deficiencies in homologous recombination (50% of tumors) • Associated with <i>BRCA</i> and <i>TP53</i> mutations	• Platinum-based chemotherapy and PARP inhibitors • Tumors are initially sensitive to platinum-based chemotherapy, but most patients with advanced-stage cancer will recur
Low-grade serous carcinoma	• Presents in younger patients • Can be early or late stage at presentation	• Associated with <i>KRAS</i> and <i>BRAF</i> mutations • Tumors have genomic stability	• MEK inhibitors (currently being tested in clinical trials) and hormonal therapies
Low-grade endometrioid carcinoma	• Can be associated with endometriosis	• Associated with <i>PTEN</i>, <i>ARID1A</i> and <i>PIK3CA</i> mutations • Can have microsatellite instability	• Possible hormonal therapies (not yet established)
Clear-cell carcinoma	• Can present with parenchymal metastases • Can be associated with hypercoagulability and hypercalcemia	• Associated with <i>ARID1A</i> and <i>PIK3CA</i> mutations	• Immunotherapy agents • Can be resistant to platinum-based chemotherapy
Mucinous carcinoma	• Presents in younger patients and is typically early stage at presentation	• Associated with <i>KRAS</i> mutations	• Tends to be insensitive to chemotherapy but is still treated initially with cytotoxic chemotherapy

Table 2 | Characteristics of OC by histologic subtypes, including clinical features, molecular characteristics and therapeutic options.

Table adapted from (118).

The median age at diagnosis for OC patients is 63 years old and most women have a symptomatic presentation of the disease with ascites and gastrointestinal dysfunction. However, considering that the symptoms are general and nonspecific, they might be initially missed or attributed to other diseases. This, together with the lack of screening methods, leads to a late diagnosis that frequently occurs when the cancer is already at a late disease stage (stage III-IV) (130). As a consequence, the early diagnosis is the most important prognostic factor (131), as early-stage patients have a five-year survival rate of 92.4%, while for advanced-stage patients it is only 31.5% (based on SEER data from 2013 to 2019; <https://seer.cancer.gov/statistics-network/explorer/>).

Treatment options

The primary treatment option for OC is cytoreductive/debulking surgery, which is essential for the staging, made through the FIGO staging system, and the resection of the tumor (132,133). The indication of platinum-based adjuvant chemotherapy depends on the stage, the grade and the histologic subtype. Accordingly, while many patients with low-stage and grade disease do not receive adjuvant chemotherapy after the surgery, those with higher stages and grades and specific histologic subtypes, such as HGSOc and clear cell carcinoma, are recommended to receive it (134). Moreover, trials have demonstrated that neoadjuvant chemotherapy followed by surgery and postoperative chemotherapy obtained similar results to first-line surgery followed by chemotherapy (135–137). This is an alternative treatment for those patients who cannot undergo surgery or if the tumor is too extensive to allow complete resection.

Studies have also investigated the efficacy of the combination of different treatments as maintenance therapy to optimize adjuvant chemotherapy, including platinum-based chemotherapy and anti-angiogenic agents (138). These led to the approval in 2011 by the European Medicines Agency (EMA) for the use of bevacizumab in combination with chemotherapy for advanced-stage OC patients (139–141). However, one of the main challenges is that the majority of advanced OC patients experience recurrence. The use of PARP inhibitors is a recent therapeutic option for OC (142). At the beginning, PARP inhibitors, including olaparib and rucaparib, have been approved by the FDA for the treatment of *BRCA*-mutated OC patients, alone or in combination with other therapies (143–146). But in 2017 the FDA approval for the use of PARP inhibitors extended to recurrent OC independently from *BRCA* status (147). So, they are now used as both first line and maintenance therapy, however, one of the limitations, as for platinum-based chemotherapy,

is the development of resistance to these therapies, thus needing their combination with other therapeutic options or the development of novel innovative therapies (148,149). In this context, immunotherapy is a widely investigated strategy, which obtained promising results for several tumors, but in OC up to now has obtained low response rates, needing further investigations (150,151).

Immunobiology of HGSOC

HGSOC, the most common OC subtype, is known for its aggressiveness, high recurrence rate, high capacity to metastasize, and resistance to conventional therapies. In fact, it is often diagnosed at advanced stages when it has already spread to the peritoneum (152). In order to improve the treatment and the clinical outcome, HGSOC has been categorized into subgroups. Tothill et al. identified four molecular subtypes with distinct survival, clinical and tumor characteristics. Importantly while a reactive stromal signature predicts a poor prognosis, the group defined by an immune signature, called immunoreactive, and characterized by a high proportion of infiltrating lymphocytes correlates with a better prognosis (153,154). Accordingly, HGSOC has been associated with mutations in the tumor suppressor gene *TP53*, which have been correlated with an inflammatory TME (155). This suggests that the immune system plays an important role in HGSOC progression and thus elucidating the tumor immune microenvironment could help in identifying novel therapeutic targets. Indeed, up to now, HGSOC patients are initially treated in the same way, despite these new classifications, but many patients could relapse and become resistant to therapies.

Several studies reported that the infiltration of cytotoxic T lymphocytes, especially in the tumor islets, correlates with improved OS (156,157). At the transcriptional level, these cells displayed a tissue-residency profile, characterized by the expression of the integrin CD103, and the expression of cytotoxic and exhaustion molecules, including CD39 and PD-1 (158,159). Supporting their favorable role, they have been associated with an increased survival (160,161). Remarkably, the immune system impacts on tumor progression, in fact it has been described that OC separates into three TILs subtypes: one with little or no immune infiltration, one with stromal infiltration and the last one with combined epithelial and stromal infiltration; interestingly the highest levels of epithelial immune infiltration inversely correlate with the malignant clone diversity, neoantigen depletion and subclonal loss of heterozygosity at human leukocyte antigen (HLA) loci (162). Among the CD8 T cells, the cells with showed superior antitumoral activities are those with a T_{RM} phenotype, as they

showed clonal enrichment and effector activity, supporting their important role, once effector T_{RM} become exhausted they can be replenished by stem-like T_{RM} (163). The tumor antigens recognized by TILs are still under investigations, however, although HGSOC has a low mutation burden, both neoantigen specific CD8 and CD4 T cells have been identified (164,165). Importantly, the clones with high affinity TCRs localize preferentially in the tumor (164). In addition, CD4 and CD8 populations showed specificities for shared tumor-associated antigens, these cells are present at high frequencies and participate in the tumor control (166). These data highlight the important anti-tumor role exerted by TILs, nevertheless, the prognosis of OC remains poor and the mechanisms that explain not only the resistance to conventional therapies, but also the low response of OC to ICIs, are still to be determined (167).

Other factors and the interaction of TILs with other components of the TME are essential for understanding OC progression and the response to therapies. In accordance, the cellular interactions of effector T cells and fibroblasts, which are major components of the TME, affect the chemoresistance (168). On the other hand, several studies reported the impact of neoadjuvant chemotherapy on the immune infiltrate. In particular, chemotherapy has been shown to induce multiple immune markers (169), but only if the tumor is already characterized by a high lymphocytic infiltration (170). In addition, this reflects an increase of cytotoxic immunogenic activity and of T cells and TCR clonality after neoadjuvant chemotherapy (171). Notably, it has been suggested that chemotherapy could induce or unmask pre-existing common antigens, as an increased number of shared TCRs was observed between patients after chemotherapy (171). Moreover, chemotherapy induces the reversal of the immune profile from a pro-tumor phenotype towards an anti-tumor in late recurrence patients (172). In addition to chemotherapy, the cellular interactions dictate the state of TILs in OC TME. Various intraepithelial myeloid antigen-presenting cells (APCs) have proved to provide CD28 co-stimulation signals to exhausted CD8 T cells, with consequent activation due to improved effector functions, memory, survival and proliferation (166). This interaction allowed also the activation of TILs upon PD-1 blockade, elucidating mechanisms that can improve ICIs approaches. Tumor growth factor- β (TGF- β) showed an important role in both tumor and fibroblast cells to promote T-cell exclusion and immunosuppression, so contributing to an overall immunosuppressive TME in the T-cell-excluded tumors and being a promising target to overcome immune escape mechanisms (173). The presence of tertiary lymphoid structures (TLSs) in HGSOC tumors has been associated with cytotoxic tumor-specific T cell activation and proliferation (174). Moreover,

the frequency and the level of maturation of TLSs have an impact on the phenotypic and functional properties of intratumoral T cells, in particular it has been reported that in HGSOc the limited number of intratumoral TLSs characterized by relatively elevated tumor mutational burden and increased density of effector CD8 T cells together with the prevalence of TLSs which support an ICI-resistant TIM-3⁺PD-1⁺ phenotype could explain the limited sensitivity to ICIs (175). This study confirms, as already published, that also B cells exert important functions for TILs (176). Thus, the appropriate activation of TILs mediated by the TME is essential for sustaining the anticancer immune responses and understanding the complexity of the TME could help in it (177).

Among TILs, not only conventional T cells are associated with antitumor properties, but also other immune populations, such as NK cells and unconventional $\gamma\delta$ T cells, could be involved in the control of OC (**Figure 4**) (178). In fact, $\gamma\delta$ T cells are enriched in OC tissues, however, controversial results have been reported. While, on one hand their presence has been associated with both overall survival and reduced residual tumor burden after primary surgery and the expression of immune checkpoints as PD-1 and CD39 makes them an ideal target for immunotherapies (179,180), on the other hand they have been shown to promote tumor progression and immune evasion by secreting the inflammatory cytokine interleukin (IL)-17 and enhancing immunosuppressive mechanisms (181). The role of NK cells is debated to. Indeed, alterations of NK cell phenotype and functions have been reported in OC. The acquired expression of CD9 results in reduced anti-tumor cytokine production and cytotoxicity (182). Supporting their regulatory potential, it has been shown that NK cells suppress TILs expansion and alter TILs cytokine production (183). Nevertheless, studies investigating the use of NK cells in immunotherapeutic approaches revealed that they can inhibit systemic metastasis of ovarian cancer cells and improve the survival of mice and that expanded NK cells from OC patients are cytotoxic against the autologous tumor in a patient-derived xenograft murine model (184,185). The antitumor potential of NK cells in OC has been recently confirmed by identifying tissue-resident NK cells expressing CD103, CD49a and NKG2A highly responsive towards ovarian cancer cells (186).

One of the main issues of OC is the presence of a highly suppressive immune microenvironment characterized by TAMs, myeloid derived suppressor cells (MDSCs) and Tregs that exert pro-tumor properties (187–189). TAMs are the most abundant immune cells in advanced stage OC and foster tumor growth, invasion, angiogenesis, metastasis, and

drug resistance (190). Tregs have been associated with a poor outcome, they suppress tumor-specific T cell immunity and contribute to tumor growth (191,192).

Remarkably, based on gene expression profiling analyses of immune cell and stromal activation, four subtypes have been identified for HGSOC named differentiated, immunoreactive, mesenchymal and proliferative (125). These groups correlate with OS (193), however this classification has not yet been integrated into the routine clinical practice.

The majority of the studies regards advanced stages OC, as its clinical detection occurs in late stages, and there is few knowledge regarding early stages of the disease. Therefore, elucidating the pathogenesis and the impact of the immune microenvironment in early cancer development and relapse is fundamental for identifying biomarkers for early detection and for the exploration of novel cancer prevention strategies.

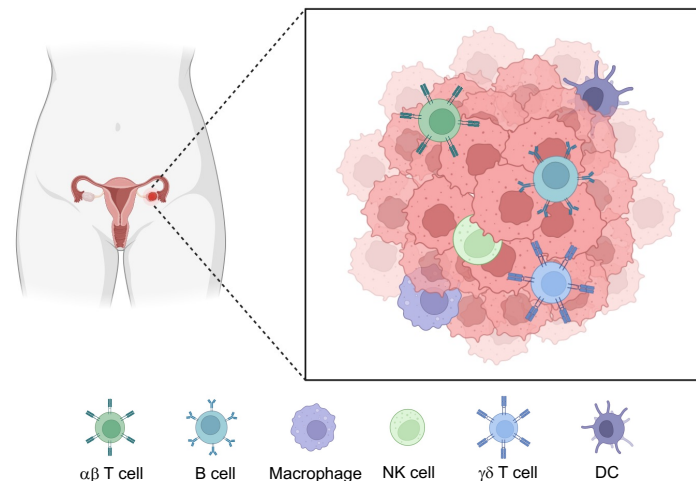


Figure 4 | OC immune microenvironment.

Composition of OC immune microenvironment composed by both adaptive and innate immunity, comprising conventional $\alpha\beta$ T cells (cytotoxic CD8 and helper CD4 T cells), unconventional $\gamma\delta$ T cells, B cells, NK cells, macrophages and antigen presenting cells including DCs. Figure created with Biorender.

Immunotherapies for OC

The response to ICIs has been disappointing in OC, so novel immunotherapeutic approaches need to be investigated. Considering the complexity of the OC immune microenvironment with the interplay of various players, combination treatments are promising. For example, the blockade of CTLA-4 could synergize with PD-1 blockade to reinvigorate tumor-specific CD8 TILs (166). Accordingly, the results of a randomized phase II trial showed that the combination of nivolumab and ipilimumab resulted in a response rate superior to nivolumab alone in patients with recurrent OC, although the gain in progression-free survival was limited (194). In addition to CTLA-4 other ICs could be targeted in

combination with PD-1, in fact a synergistic effect has been observed combining PD-1 with LAG-3 blockers (195). Acting on the myeloid compartment, which has an essential role in regulating CD8 response, is an alternative strategy. Targeting CD40 could have powerful effects inducing an immunostimulatory environment and thus promoting the infiltration and the activation of cytotoxic CD8 cells and APCs (196,197). Moreover, blocking the triggering receptor expressed on myeloid cells 2 (TREM2) restored the response to ICIs monotherapy in mice (198) and now its use is currently investigated in a phase I clinical trial (NCT04691375).

Preliminary promising results derive from adoptive cell therapy (ACT) using autologous TILs expanded *in vitro*. In fact, TILs harvested from metastases led to the regression of the target lesions but did not suppress the appearance of new lesions (199). The possible involvement of PD-1 gave the rationale to combine ICIs with ACT (199), in accordance, the addition of ipilimumab improved the T cell expansion and increased the CD8 T cell tumor reactivity (200). An important challenge for TILs ACT relates to intratumoral heterogeneity, as multiple distinct tumor immune microenvironments can co-exist within a single patient (201). In this context, tumor debulking offers the opportunity to harvest TILs from multiple tumor sites, moreover, ascites fluid samples also contain tumor-specific T cells (202).

For cold or immune-excluded tumors the restoration of T cell inflammation is important for immunotherapies. Low-dose radiotherapy has been reported to enable the responsiveness to combinatorial immunotherapy, as it mobilizes both adaptive and innate immunity (203). Whether also chemotherapy induces T cell inflammation is under investigation (204). While, preclinical data showed that PARP inhibitors synergize with ICIs in OC (205) and the combination of PARP inhibitors with ICIs is currently tested in a phase III clinical trial (NCT03522246) (206).

Aim of the thesis

Gynecologic malignancies are a heterogeneous group of solid tumors which include EC and OC. EC is the most common gynecological neoplasm and the only gynecologic cancer with increasing incidence and mortality. It is generally diagnosed at an early stage and is well managed with surgery, radiation, and/or chemotherapy. However, EC patients with advanced/recurrent disease have poor outcomes and respond poorly to current management treatments. On the other hand, OC is a devastating disease since the majority of patients, when first diagnosed, are at advanced clinical stages (III/IV). Furthermore, relapse occurs in more than 70% of patients and the 5-year survival rate is lower than 40%. Thus, it is important the development of novel therapeutic approaches. In this context, remarkable progresses have been made in immunotherapy. In fact, several preclinical and clinical studies are ongoing with immunotherapeutic strategies combined or not with classic treatments. To date, the efficacy is not satisfactory, thus a detailed understanding of the biology and interactions between cancer and the immune system is essential for the recognition and development of potential new immunotherapeutic strategies and targets as well as the optimization of existing immunotherapies. Therefore, the aim of this PhD project was to characterize the molecular profile, frequency, phenotype and function of immune cells in EC and OC patients.

In particular, considering that the presence of activated tissue-resident $\gamma\delta$ T cells has been associated with a beneficial response to immunotherapies and that their role in EC progression has not been elucidated yet, we performed a characterization of $\gamma\delta$ T cells in EC. A phenotypical, transcriptional and functional characterization of $\gamma\delta$ T cells through flow cytometry and single-cell RNA sequencing elucidated their role in EC and their association with the prognosis and the response to immunotherapies.

Moreover, as few studies have been conducted on the early stages (stage I) of OC due to the difficulty in diagnosing this disease, we performed a deep characterization through scRNA-seq of stage I HGSOC TME to assess the impact of the TME in OC progression and to identify potential immunotherapeutic targets.

Materials and Methods

Patients' enrollment and specimen processing

Ethical statement

Patients' recruitment and samples' collection have been performed in accordance with the Declaration of Helsinki. The collection of human samples for research purposes was ethically approved by the Institutional Review Board (IRB) of Humanitas Research Hospital (HRH) for PB from buffy coats of healthy controls (HCs) (approval of 30/01/2007) and PB and tissue samples from EC and OC patients undergoing hysterectomy and ovariectomy (approval 225/20). All participants signed a consent form which clarifies that the donation of specimens for this research project did not affect in any way the diagnosis, the therapies and the prognosis of the disease.

Clinical features

In collaboration with the Gynecology Unit of HRH, between 2020 and 2024, we enrolled EC and OC patients undergoing hysterectomy and ovariectomy. From each patient we collected the PB and the tumor tissue at the surgery. In total, we analyzed 28 EC patients and 2 OC patients. For both EC and OC, the patients are represented by elderly patients (mean age of 66 and 54 years old for EC and OC, respectively) and they were not treated before the surgery.

The vast majority of EC patients have an early-stage of the disease (stage I in 69% of cases) and an endometrioid histological subtype (68% of cases), while they equally distributed for the grading (57% G1-G2 versus 42% G3). 6 patients presented MSI, assessed by the Unit of Pathological Anatomy of HRH through the evaluation of the status of the mismatch repair proteins MutS homolog 2 (MSH2), MSH6, MutL protein homolog 1 (MLH1) and PMS1 Homolog 2 (PMS2). The loss of positivity for at least one of these markers indicates instability of microsatellites. The features of the patients are reported in **Table 3**.

Feature	Variable	Patients (n=28)
Age – mean (range)		66 (51-88)
FIGO stage – n° (%)	Stage I	22 (69)
	Stage II	0 (0)
	Stage III	6 (21)
	Stage IV	0 (0)
Grading – n° (%)	G1-G2	16 (57)
	G3	12 (43)
Histological subtype – n° (%)	Endometrioid	19 (68)
	Others	9 (32)
Microsatellite instability – n° (%)	MSS	12 (43)
	MSI	6 (21)
	NA	10 (36)

Table 3 | Clinical characteristics of EC patients.

Clinical characteristics of the 28 EC patients analyzed including age, FIGO stage, grading, histological subtype and microsatellite instability.

As early stages of OC are extremely difficult to diagnose, we enrolled 2 stage I HGSOC patients (**Table 4**). According to FIGO staging one patient (P1) was classified as stage IC (tumor involves one or both ovaries with any of the following: capsule rupture, tumor on the surface, positive washings/ascites) and the other one (P2) as stage IA (tumor limited to one ovary, capsule intact, no tumor on the surface, negative washings/ascites).

	Age	FIGO Stage	Grading	Histological subtype	Note
P1	63	IC	High grade	HGSOC	With previous breast cancer (BRCA mut)
P2	45	IA	High grade	HGSOC	

Table 4 | Clinical features of OC patients.

Clinical features of the 2 OC patients analyzed including age, FIGO stage, grading, histological subtype and other relevant features.

Peripheral blood mononuclear cells (PBMCs) isolation

Blood samples were obtained from EC and OC patients before the surgical resection. PBMCs from HCs and EC and OC patients were isolated by density-gradient centrifugation using Lympholyte®-H Cell Separation Media. According to the protocol for PBMCs isolation, PB was first diluted with Hank's Balanced Salt Solution without calcium and magnesium

(HBSS-/-) in a ratio 1:1 (volume-to-volume). Then, the diluted blood was stratified on 17,5 mL of Lympholyte®-H Media in a Falcon® 50 ml conical tube (**Figure 5**) and then centrifuged for 30 minutes at 400 relative centrifugation force (rcf) at room temperature (RT) without acceleration and break. After the centrifugation, PBMCs were in the interphase ring located at the interface between Lympholyte®-H and plasma (**Figure 5**) and were collected, washed with HBSS-/- and centrifuged for 10 minutes at 400 rcf. When necessary, cells were resuspended in Ammonium-Chloride-Potassium (ACK) lysis buffer for 5 min at 4°C to remove red blood cells. Subsequently, PBMCs were counted and frozen in cryovials in 1 ml of Fetal Bovine Serum (FBS) with 10% of the cryoprotective Dimethyl Sulfoxide (DMSO).

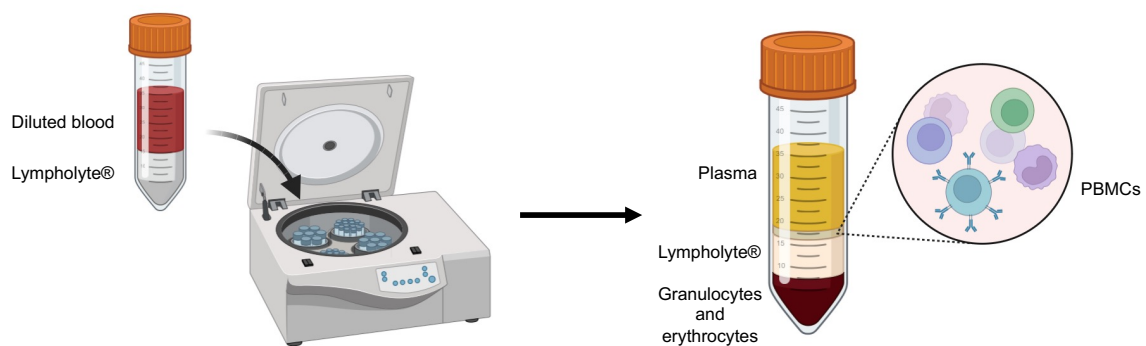


Figure 5 | Schematic representation of density-gradient centrifugation for PBMCs isolation. On the left is shown the blood stratification on Lympholyte® with the subsequent centrifugation, while on the right is shown the separation of the different phases after the centrifugation with the presence of the interphase ring containing PBMCs between the plasma and the Lympholyte®.

Isolation of single cell suspensions from EC and OC tumor tissues

Human tumor tissue samples were obtained from EC and OC patients who underwent hysterectomy and ovariectomy by the Gynecology Unit at HRH. Tissue samples were conserved in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% FBS, 1% ultraglutamine, 2% hydroxyethylpiperazine-ethanesulfonic Acid (HEPES) buffer 1 M, 1% penicillin/streptomycin/amphotericin B (P/S/A), 0.1% geneticin, until use. Samples were weighted and cut into smaller pieces of 2-3 mm to favor the digestion. Tissue dissociation was obtained by mechanical dissection using gentleMACS™ Dissociator and enzymatic digestion with 2 mg/mL of collagenase D and for OC tissues with the addition of 100 µg/ml of hyaluronidase in RPMI-1640 with 1% of FBS for 45 minutes at 37°C with 5% of CO₂ under rotation. 5 ml of enzymatic solution was used for each gram of tissue and up to 2 g were placed in gentleMACS™ C-Tubes. After enzymatic incubation, the obtained cells were filtered using 100 µm and 70 µm cell strainers and washed in HBSS-/- . When

necessary, cells were resuspended in ACK lysis buffer for 5 min at 4°C to remove red blood cells. At the end, the single cell suspensions were counted and preserved with the same procedure described for PBMCs.

Isolation of primary tumor cells from EC tumor tissues

When the EC tumor tissue was big enough (at least 0.5 g), it was divided into two parts, one for the isolation of single cell suspensions as described in the previous paragraph, and the other one for the isolation of primary tumor cells. To obtain them, the tissue was dissociated with the Human Tumor Dissociation Kit according to the datasheet. Briefly, the dissociation was obtained by mechanical dissection using gentleMACS™ Dissociator and enzymatic digestion with the enzymes provided by the kit in RPMI-1640 at 37°C with 5% of CO₂ under rotation. 5 ml of enzymatic solution was used for each gram of tissue and up to 2 g were placed in gentleMACS™ C-Tubes. After enzymatic incubation, the obtained cells were filtered using 70 µm cell strainer and washed in HBSS-/-. After isolation, the cells were counted and put in culture in RPMI-1640 supplemented with 10% FBS, 10% human serum, 1% P/S/A, 1% ultraglutamine, 1% non-essential amino acids (NEAA), 25 mM HEPES and 10 µg/ml gentamicine to expand them. Complete RPMI was filtered using a bottle-top vacuum filter with 0.22 µm pores and then stored at 4°C. Cells were kept in cell culture flasks with filter cups in a humidified atmosphere at 37°C with 5% of CO₂. The medium was changed every 2-3 days and subculture was done when the cells reached confluence. Cells kept in culture were used for co-culture experiments, as described in the Materials and Methods section “Co-culture experiments”.

High-dimensional multiparametric flow cytometry

All flow cytometry experiments were performed in batches on frozen samples to minimize technical variability and were acquired at BD FACSymphony™ A5 flow cytometer.

The anti-human mAbs listed in **Table 5** were used for the analysis of the phenotype and function of $\gamma\delta$ T cells in EC patients. All antibodies were titrated on human PBMCs and used at the concentration giving the highest signal-to-noise ratio over background, as described in (207).

Marker	Fluorochrome	Clone	Company	Surface / Intracellular staining	Experiment
CD3	BUV661	UCHT1	BD	SUR	Phenotype, Functional
V δ 1 TCR	PE-Vio770	REA173	Miltenyi	SUR	Phenotype
V δ 1 TCR	PE	REA173	Miltenyi	SUR	Phenotype, Functional
V δ 2 TCR	BUV395	B6	BD	SUR	Phenotype, Functional
V δ 3 TCR	APC	D3P11.5B	Beckman Coulter	SUR	Phenotype
CD27	APC- eFluor780	323	eBioscience	SUR	Phenotype, Functional
CD45RA	BUV737	HI100	BD	SUR	Phenotype, Functional
CD16	BUV496	348	BD	SUR	Phenotype
CD56	BUV563	NCAM16.2	BD	SUR	Phenotype
CD57	BV605	QA17A04	BioLegend	SUR	Phenotype
CD28	PE-Cy7	CD28.2	BioLegend	SUR	Phenotype
CX3CR1	FITC	2A9-1	BioLegend	SUR	Phenotype
CX3CR1	BUV805	2A9-1	BD	SUR	Phenotype
CD69	PE-CF594	FN50	BD	SUR	Phenotype
CD69	BV605	FN50	BioLegend	SUR	Phenotype
CD103	BUV805	Ber-ACT8	BD	SUR	Phenotype, Functional
CXCR6	BV650	13B 1E5	BD	SUR	Phenotype
CD161	PE/Dazzle 594	HP-3G10	BioLegend	SUR	Phenotype
CD161	BV421	HP-3G10	BioLegend	SUR	Phenotype
CD39	PE/Dazzle 594	A1	BioLegend	SUR	Phenotype
CD39	BUV615	TU66	BD	SUR	Phenotype
ILT-2	PE-Cy5	GHI/75	BD	SUR	Phenotype
KLRG1	BV785	2F1/KLRG1	BioLegend	SUR	Phenotype
TIM-3	BV711	7D3	BD	SUR	Phenotype
TIM-3	BV650	7D3	BD	SUR	Phenotype
TIGIT	BV421	741182	BD	SUR	Phenotype
LAG-3	APC-R700	T47-530	BD	SUR	Phenotype
NKG2A	APC	REA110	Miltenyi	SUR	Phenotype
NKG2A	VioBright-FITC	REA110	Miltenyi	SUR	Phenotype
PD-1	BV711	EH12.1	BD	SUR	Phenotype
IFN- γ	PE-Cy7	B27	BioLegend	IC	Functional
TNF- α	BV786	MAb11	BioLegend	IC	Functional
CD107a	PE-Cy5	H4A3	BD	SUR	Functional

Table 5 | List of mAbs used in the polychromatic flow cytometry panels.

The list indicates all the mAbs used in the study with the corresponding: conjugated-fluorochrome, clone, company name, type of staining (surface (SUR) or intracellular (IC)) and the experiment in which each antibody has been used (phenotype or functional).

Surface staining

Frozen PBMCs were thawed in RPMI-1640 supplemented with 10% of FBS, 1% penicillin/streptomycin (P/S) and 1% ultraglutamine (RPMI-1640 complete) and washed in HBSS-/-. After pelleting cells by centrifugation for 5 minutes at 400 rcf, they were stained for live/dead discrimination with Zombie Aqua™ fixable viability kit in a final volume of 100 µl (for a maximum of 10×10^6 cells) for 15 minutes at RT in the dark. The Aqua mix was freshly prepared diluting 0.5 µl of the Zombie Aqua™ fixable viability kit with 19.5 µl of sterile water and 380 µl of HBSS-/-.

The Zombie Aqua™ is a fluorescent reactive dye that permeates into cells with compromised membranes (i.e. dead or necrotic cells) and reacts with amines located intracellularly in dead cells, resulting in a bright positive fluorescent signal. On the contrary, in viable cells (with intact cell membranes) Zombie Aqua™ dye only reacts with amines on the cell surface, resulting in a dim fluorescent signal. Hence, the use of the Zombie Aqua™ kit allows the identification of dead cells and their consequent exclusion from the analysis, avoiding unspecific binding and false positive events due to dead cell autofluorescence.

At the end of the incubation, cells were washed with 1 ml of FACS Buffer (HBSS/- with 2% of FBS) and centrifuged for 5 minutes at 400 rcf. Then, pelleted cells were incubated for 20 minutes at RT in the dark with the mix containing the anti-human mAbs chosen for the surface staining, prepared in FACS Buffer, in a final volume of 100 µl (for a maximum of 10×10^6 cells). Finally, cells were washed with 1 ml of FACS Buffer and fixed in 1% of paraformaldehyde (PFA) for further acquisition at the flow cytometer.

Intracellular staining

The intracellular staining has been done with Cytofix/Cytoperm™ and Perm/Wash™ kits according to the manufacturer's instructions. After the surface mix incubation, cells were washed with 1 ml of FACS Buffer and centrifuged for 5 minutes at 400 rcf. Pelleted cells were resuspended in 150 µl of Cytofix/Cytoperm™ and incubated for 30 minutes at 4°C in the dark. Cytofix/Cytoperm™ simultaneously fixes and permeabilizes cells, allowing the following intracellular staining. At the end of the incubation, cells were washed with 1 ml of saponin-containing Perm/Wash™ Buffer 1X and centrifuged for 5 minutes at 400 rcf. Pelleted cells were incubated for 30 minutes at 4°C with the Perm/Wash™ Buffer containing the mix of antibodies chosen for the intracellular staining, in a final volume of 100 µl (for a maximum of 10×10^6 cells). At the end, cells were washed with 1 ml of Perm/Wash™ Buffer,

centrifuged for 5 minutes at 400 rcf and resuspended in 100-150 µl for the further acquisition at the flow cytometer.

Cell sorting

Frozen cells isolated from the tumor tissue of the two stage I HGSOc patients were thawed in RPMI-1640 complete, washed with HBSS-/- and stained as described in the paragraph “Surface staining” using the APC-Vio770-conjugated anti-CD45 mAb (Miltenyi, clone 5B1) in order to FACS sort CD45⁺ and CD45⁻ cells and perform single-cell RNA sequencing (scRNA-seq). Cells were sorted on a BD FACS Melody cell sorter.

Functional assays

Cytokines production assay

Frozen PBMCs and cells isolated from tumor tissue from EC patients were thawed in RPMI-1640 complete and washed in HBSS-/- . Then, cells were plated in a U-bottom 96 multi-wells plate at a concentration of 1x10⁶ cells/ml in RPMI-1640 complete supplemented with 10 ng/ml of IL-15. The day after, for the detection of IFN- γ and TNF- α intracellular accumulation, the cells were stimulated with 0,5 µg/ml of phorbol 12-myristate 13-acetate (PMA) and 0,1 µg/ml of ionomycin, moreover protein transport inhibitor Golgi Plug (1 µg/ml) was added in each well. After 4 hours of incubation in a humidified atmosphere at 37°C with 5% of CO₂, cells were collected and washed with HBSS-/- . The intracellular staining was done using Cytofix/CytopermTM and Perm/WashTM kits as described above in the paragraph “Intracellular staining” and using specific anti-human mAbs for TNF- α and IFN- γ .

Co-culture experiments and CD107a degranulation assay

In order to test the cytotoxic activity of human isolated $\gamma\delta$ T cells from PB and tumor tissue, co-culture experiments with autologous and heterologous primary tumor cells isolated from EC patients (as described in the paragraph “Isolation of primary tumor cells from EC tumor tissues”) were performed.

Frozen PBMCs and cells isolated from tumor tissue were thawed in RPMI-1640 complete and washed in HBSS-/- . Then, cells were plated in a U-bottom 96 multi-wells plate at a concentration of 1x10⁶ cells/ml in RPMI-1640 complete supplemented with 10 ng/ml of IL-15. The day after, cells were pre-incubated with a mAb targeting PD-1 (alone or in combination with a mAb targeting TIGIT) or left untreated for 30 minutes in a humidified

atmosphere at 37°C with 5% of CO₂, and then were put in the presence or not of target primary tumor cells isolated from EC patients.

Thus, PE-Cy5-conjugated anti-CD107a mAb was added in each well to detect the degranulation capacity of $\gamma\delta$ T cells. After 4 hours of incubation in a humidified atmosphere at 37°C with 5% of CO₂, cells were collected, washed with HBSS-/- and stained for cellular membrane markers flow cytometry analysis, as described above in the Materials and Methods section “Surface staining”.

Flow cytometry data analysis

FlowJo

Flow Cytometry Standard (FCS) 3.0 files, obtained from BD FACS Symphony flow cytometer, were analyzed using the FlowJo software (TreeStar Inc), version 10.10.1.

High dimensional flow cytometry data

After their generation, raw data were processed using FlowJo 10.10.1 software. First, FCS files were compensated using single-stained controls. Then, their analysis was performed by manual single gating strategy, in particular this was made in order to remove debris, dead cells, doublets and other cell populations and identify $\gamma\delta$ T subsets. In addition to the single gating, an unbiased computational approach was applied to combine all markers information at the same time and to assess cells similarity in the samples. This process was based on the use of uniform manifold approximation and projection (UMAP) algorithm that allows the identification of novel cell subpopulations or biomarkers, which would be hardly detected by manual analysis.

High dimensional flow cytometry data visualization through UMAP

Data from each sample were concatenated in a single matrix to perform the UMAP algorithm. This algorithm is based on non-linear dimensionality reduction analysis, well-suited for embedding high-dimensional data for visualization in a two-dimensional scatter plot (UMAP map), named UMAP1 and UMAP2. On the UMAP map, cells that are phenotypically similar are located closely together and correspond to different cellular clusters, thus enabling the visualization of different cellular subpopulations.

Single-cell RNA sequencing

scRNA-seq analysis of tissue EC public datasets

Processing scRNA-seq data

The sequencing raw data (FastQ) of two public datasets were downloaded from NCBI SRA database under the accession numbers PRJNA650549 and SRP349751. The FastQ files were processed with Cell Ranger 7.1.0 (10X Genomics), using default parameters. The scRNA-seq reads were aligned to the GRCh38 (version refdata-gex-GRCh38-2020-A, 10X Genomics) human genome reference. Only mapped reads with valid barcodes and unique molecular identifiers (UMIs) were retained to compute a gene expression matrix containing the number of UMI for every cell and gene. Gene counts were then imported in Python (v3.9.7) and processed with Scanpy (v1.9.3) (208). Raw data matrices of all samples (n=18) were merged in one single anndata object, and cells with fewer than 500 and higher than 4000 expressed genes, or lower than 950 and higher than 12000 UMIs, and greater than 10% mitochondrial genes were removed. Cell multiples were computationally identified and removed using Single-Cell Remover of Doublets (209). Data were then log-normalized with a scale factor of 10000. Highly variable genes (HVGs, n=4000) were identified across each sample using the *Seurat_v3* dispersion-based methods on raw counts, and data from each sample were integrated and batch corrected with the single-cell Variational Inference (scVI)-tools algorithm (v1.0.0) (210), setting the *patient_ID* and *dataset_of_origin* as covariates. The clustering analysis was performed using the Leiden algorithm (211), and the clusters were then embedded in two dimensions by UMAP, using the scVI latent space.

Differentially expressed genes analysis

Differentially expressed genes (DEGs) were computed using the *FindAllMarkers()*, to identify the cluster-specific genes, and *FindMarkers()* functions implemented in Seurat (v4.4.0) (212) under R v4.3.2. DEGs were computed on raw counts with the *negbinom* statistical test, and significance was defined based on FDR-adjusted p-value <0.05 and Log2-FoldChangeGene >|0.25| values. DEGs were then visualized by volcano plot with the R *EnhancedVolcano()* function.

Gene ontology analysis

Gene ontology analysis was performed using the R package clusterProfiler (v4.8.3). The pathways of cell type were enriched using DEGs with adjusted p-value <0.05 and Log2-

FoldChangeGene >0.25. Only pathways with qvalue <0.05 have been selected as significant and visualized by ggplot2 package under R v4.3.2.

scRNA-seq analysis of PB EC public dataset

Processing scRNA-seq data

The samples' raw gene counts of the public dataset were downloaded from the Gene Expression Omnibus (GEO) under the accession number GSE212217, and then imported in Python (v3.9.7) and processed with Scanpy (v1.9.3) (208). Raw data matrices of all samples (n=46) were merged in one single anndata object, and cells with fewer than 500 and higher than 4000 expressed genes, or lower than 1100 and higher than 17000 UMIs, and greater than 10% mitochondrial genes or lower than 0.05% ribosomal genes were removed. Cell multiples were computationally identified and removed using Single-Cell Remover of Doublets (209). Data were then log-normalized with a scale factor of 10000. Highly variable genes (HVGs, n=4000) were identified across each sample using the *Seurat_v3* dispersion-based methods on raw counts, and data from each sample were integrated and batch corrected with the scVI-tools algorithm (v1.0.0) (210), setting the *patient_ID* as covariate. The clustering analysis was performed using the Leiden algorithm (211), and the clusters were then embedded in two dimensions by UMAP, using the scVI latent space.

Differentially expressed genes analysis

DEGs were computed using the *Wilcoxon rank-sum test* with the *sc.tl.rank_genes_groups()* function implemented in Scanpy, to identify the cluster-specific genes and to compute specific statistical comparisons. Genes with an FDR-adjusted $P < 0.05$ and expressed by at least 10% of cells in the cluster at a minimum Log2-Fold Change of $|0.25|$ were considered significant. DEGs were then visualized by volcano plot with the R *EnhancedVolcano()* function, showing statistical significance (adjusted P value) versus magnitude of change (Log2-Fold Change).

Gene ontology and functional enrichment analysis

Gene ontology analysis was performed using the R package clusterProfiler (v4.8.3). The pathways of cell type were enriched using DEGs with adjusted p-value <0.05 and Log2-FoldChangeGene >0.25. Only pathways with qvalue <0.05 have been selected as significant and visualized by ggplot2 package under R v4.3.2.

Reactome enrichment analysis was performed using the Reactome web tool (<https://reactome.org/>). The Reactome pathways of cell type were enriched using DEGs with FDR-adjusted $P < 0.05$ and $\text{Log}_2\text{-Fold Change} > |0.25|$, and only enriched terms with $\text{FDR} < 0.05$ were selected as significant and visualized by R package ggplot2 v3.4.0 under R v4.2.1.

Cell cycle and proliferation scores

To evaluate the cell cycle phase, we first have calculated for each cell a score based on the expression of G2/M and S phase markers with the Scanpy implemented *scanpy.tl.score_genes_cell_cycle()* function, and cells expressing neither of them have been associated to the G1 phase (213).

Gene module scores were calculated with specific gene lists with the Scanpy implemented *scanpy.tl.score_genes()* function.

scRNA-seq analysis of OC

Library preparation

Libraries for scRNA-seq were prepared using the Chromium Single Cell 3' Library & Gel Bead Kit v3.1 (10x Genomics) according to the manufacturer's guidelines. Cells (PBMCs, tumor-associated CD45⁺ immune cells, and their CD45⁻ tumor counterpart) were resuspended in a volume equivalent to 8,000 target cells for each sample and were individually loaded onto a Chromium single-cell controller (10X Genomics) to generate single-cell gel beads-in-emulsion (GEMs).

Captured cells were then lysed and the released RNA was barcoded through reverse transcription in individual GEMs. Complementary DNAs were amplified, and the quality was assessed using an Agilent 4200 TapeStation. The scRNA-seq libraries of patient 1 (batch_id = 1) were sequenced using the Illumina NextSeq500 sequencer with a paired-end 150-bp (PE150) reading strategy while the scRNA-seq libraries of patient 2 (batch_id = 2) were sequenced using the Illumina NextSeq2000 sequencer with a paired-end 150-bp (PE150) reading strategy.

Processing scRNA-seq data

The scRNA-seq reads were aligned to the GRCh38 (version refdata-gex-GRCh38-2020-A, 10X Genomics) human genome reference, and UMIs were quantified using Cell Ranger 6.1.1 (10X Genomics). Subsequent analyses were performed using the Python package

Scanpy (v1.9.3) under Python v3.9.7, if not stated otherwise. Raw data matrices of all samples were merged and cells with fewer than 400 expressed genes or 400 UMIs, or greater than 10% mitochondrial genes were removed. Sample “Lib_57”, for which many cells with a low number of detected genes were sequenced, was filtered by removing all cells with fewer than 1000 expressed genes. Data were then log-normalized with a scale factor of 10,000. Highly variable genes (HVGs) were identified using the *Seurat* dispersion-based methods. Principal component analysis (PCA) was performed using HVGs via ARPACK implementation of singular value decomposition (SVD). To limit the risk of biases, multiple sample-data integration was performed using the *harmonypy* package (v0.0.5). More in detail, the 6 samples were integrated using the *batch_id* as the key covariate in the formula.

Neighbors were identified using the top 6 components of Harmony-corrected PCA embeddings and clustering was performed using the *Leiden* algorithm with an initial resolution of 2. Clusters were then embedded in two dimensions by UMAP.

Cluster marker identification and cell-type annotation

Differential expression (DE) analysis between clusters was carried out to find markers for each of the identified clusters by using the Wilcoxon rank-sum test (*Scanpy*). Genes with an FDR-adjusted p-value <0.05 and expressed by at least 10% of cells in the cluster at a minimum Log2-FoldChangeGene of [0.25] were considered significant.

A total of 21697 cells were profiled and annotated based on the expression of canonical cell-type markers, among them, 10244, 5528, and 5925 originated from tumor-associated CD45⁺ immune cells, their CD45⁻ tumor counterparts, and matched PBMCs, respectively. Identified clusters of malignant cells, CD4⁺ T cells, cytotoxic lymphocytes, and myeloid cells were independently subjected to the next round of scaling, dimensionality reduction, and unsupervised clustering, as described above.

Pseudotime Trajectory Analysis

Pseudotime analysis was performed separately on malignant epithelial cells and tumor-infiltrating Treg subtypes by Monocle (v2.8.0). For malignant epithelial cells, a trajectory was constructed for each patient by using HVGs as the ordering genes. The proliferative cells of each patient (cT4 and cT6 respectively) were selected as the root nodes of the graph. For tumor-infiltrating Tregs a unique trajectory was constructed for the two patients using HVGs as the ordering genes and selecting the most naïve Treg cells (c12) as the root node. The

BEAM function was used to identify genes with branch-dependent expression. Node 2 was selected as the branch point and a gene was defined as branch-dependent in its estimated q value was lower than 0.01. The expression of significantly branch-dependent genes was visualized on a heatmap, using the *plot_genes_branched_heatmap()* function.

Survival analysis

SurvivalGenie is a web tool to perform survival analysis on single-cell RNA-seq data from tumors and normal samples from The Cancer Genome Atlas (TCGA, <https://tcgadata.nci.nih.gov/tcga/>). Survival analyses were performed separately on clusters of malignant epithelial cells and clusters of tumor-infiltrating Tregs. For epithelial cells, the survival analysis was performed on cT5 and cT0 by uploading a specific gene set for each cluster. The gene sets were obtained by performing DE analysis between all clusters of epithelial cells separately for each patient. A list of top 35 marker genes for each cluster was identified by selecting genes with adjusted p-value <0.01 and Log2 Fold-Change >0.25. For tumor-infiltrating Tregs, the survival analysis was performed on c3 and c9 by uploading one specific gene for each cluster independently. The TCGA has been selected as the cancer program, and ovarian cancer (OV) from the primary tumor as the dataset (n=372). The Cutp method was selected to define high and low groups. The results were visualized by Kaplan-Meier curves with corresponding Forest plots. P value was calculated using the log-rank test. The significant differences between groups are indicated by the p value: *p-value <0.05.

Single-cell gene regulatory network inference

Single-cell gene regulatory network (GNR) inference was performed on tumor-infiltrating Tregs by using *pySCENIC* package (v0.11.2), a Python implementation of the SCENIC pipeline. GNR was inferred using the GRNBoost2 algorithm given a predefined list of transcription factors (TFs) (All_TFs_hg38.txt). Candidate regulons (i.e., a TF and its predicted target genes) from TF-target gene interaction, motif enrichment, and TF-regulon prediction were computed using the *pyscenic cxt* function. The activity of the predicted regulons in the individual cells was computed by using the *AUCell* method. The default values were used for all parameters. The cluster-specific regulons were identified by the *regulon_specificity_scores()* function. The regulon specificity score (RSS) (from 0-1, 1 indicating complete specificity of the regulon for the cell type), was calculated for each cell type separately, and the top 10 regulons were shown for each cluster.

RNA velocity analysis

RNA velocity analysis was performed using the Python package *scVelo* (v0.2.4) to infer the future states of a cell from unspliced and spliced mRNA counts. The matrices of spliced and unspliced counts were quantified using the Python library *velocity* (v0.17) from the aligned bam file generated by Cell Ranger. Counts matrices in loom file format were loaded to *scVelo* and merged into the *anndata* object. Genes detected in less than 20 unspliced and spliced counts were filtered out, and the counts were then normalized. The *filter_genes_dispersion()* function was used to identify the top 4000 HVGs. The *scv.pp.moments()* function was used to compute the first- and second-order moments among nearest neighbors in the PCA space. The velocities and velocity graphs were estimated using the generalized dynamical model (*scv.tl.recover_dynamics()*, *scv.tl.velocity()*, *scv.tl.velocity_graph()*). Velocities were visualized on UMAP embedding.

Functional enrichment analysis

Reactome enrichment analysis was performed using the Reactome webtool (<https://reactome.org/>), while KEGG pathway functional enrichment was performed by the R package *clusterProfiler* (v4.8.3). The pathways of cell type were enriched using DEGs with FDR-adjusted p-value <0.05 and Log2-FoldChangeGene >|0.25|. Only enriched terms with FDR (Reactome) or qvalue (KEGG) <0.05 were selected as significant.

Cell-cell communication

Cell-cell interaction analysis was performed using *nichenetr* (v2.0.1), the R implementation of the NicheNet method. NicheNet analysis was applied to investigate the cell-cell interactions (CCIs) between *FOXP3^{high}* Tregs in c3, designated as the sender population, and the remaining cell populations, regarded as the receiver populations, as well as between *FOXP3⁺* Tregs in c8, identified as the sender population, and the other cell populations acting as receivers, and similarly for ex-Tregs in c9. Only genes expressed in at least 10% of the sender or receiver population were considered. The gene sets of interest for each receiver population were identified as the marker genes with adjusted p-value <0.05 and Log2-FoldChange >0.5. Ligand activities were predicted using the *predict_ligand_activities()* function and ranked according to the Pearson correlation coefficient. The ligand-receptor network was predicted by looking at the prior interaction potential. The top 25 ligand-receptor pairs were visualized with dotplot.

Cell cycle and proliferation scores

Specific cell cycle phase scores, including G1M, G2M, and S score, were computed on CD4 Th cells, proliferative Tregs in c14, blood CD4 T cells, and myeloid cells, by summing the scaled expression values of a list of cell cycle marker genes using the R package *Seurat* (v4.4.0) under R v4.3.2. The total proliferation score was calculated based on proliferative signature (*MIK67*, *TOPA2*, *UBE2C*) and computed on epithelial cells using the *AddModuleScore()* function provided by *Seurat*.

Statistical analysis

The statistical analysis was performed using GraphPad PRISM, version 10.2.1 (La Jolla, California, USA), software. The statistical difference between two groups of data following normal distribution was assessed by the two-way, paired or unpaired t-test. For non-normal distribution paired or unpaired Wilcoxon and Mann-Whitney tests were used, respectively. For more than two groups Kruskal-Wallis test with Dunn's multiple comparison was used. Results are represented as mean $\pm$ Standard Error of the Mean (SEM). For correlation analysis Pearson's or Spearman's coefficient was applied. Statistically significant P values were represented with GraphPad style and summarized with following number of asterisks (*): * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

List of reagents

All reagents used for the present manuscript are listed in the **Table 6**.

Reagent	Source
Lympholyte®-H Cell Separation Media	Cedarlane Laboratories
Hank's Balanced salt solution buffer without Ca^{2+} and Mg^{2+} (HBSS -/-)	Euroclone SpA
Falcon® 50 ml conical tube	Corning
Cryovials	Globe Scientific INC.
Fetal Bovine Serum (FBS)	Sigma Aldrich
Dimethyl Sulfoxide (DMSO)	Sigma Aldrich
Roswell Park Memorial Institute (RPMI)-1640 medium	Euroclone SpA
Ultraglutamine	Lonza
Hydroxyethylpiperazine-ethanesulfonic Acid (HEPES) buffer 1 M	Lonza
Penicillin/streptomycin/amphotericin B (P/S/A)	Lonza
Geneticin	Sigma Aldrich
GentleMACS™ Dissociator	Miltenyi
Collagenase D from Clostridium histolyticum	Sigma Aldrich
Hyaluronidase from bovin testes	Merck
GentleMACS™ C-Tubes	Miltenyi
Falcon® 100 µm Cell Strainer	Corning
Falcon® 70 µm Cell Strainer	Corning
Human Tumor Dissociation Kit	Miltenyi
Human serum	Euroclone SpA
Non-essential amino acids (NEAA) Mixture (100X)	Lonza
Gentamicine	Lonza
Penicillin-Streptomycin (P/S)	Lonza
Zombie Aqua™ fixable viability kit	BioLegend
Paraformaldehyde solution 4% in PBS	Santa Cruz Biotechnology
Cytofix/Cytoperm Fixation/Permeabilization kit	BD Biosciences
Falcon® 96-well Clear Round Bottom TC-treated Cell Culture Microplate	Corning
Recombinant human IL-15	Peprtech
Phorbol 12-myristate 13-acetate (PMA)	Sigma Aldrich
Ionomycin	Sigma Aldrich
GolgiPlug Protein Transport Inhibitor	BD Biosciences
Ultra-LEAF™ Purified anti-human CD279 (PD-1) Antibody (clone EH12.2H7)	BioLegend
Human TIGIT biosimilar antibody - Human IgG1	InvivoGen

Table 6 | List of all reagents used.

The list indicates all reagents used in the study with the corresponding company from which they were purchased.

Results

Exploring PD-1 targeting on $\gamma\delta$ T cells in EC

$\gamma\delta$ T cells infiltrate EC tumor tissue

To assess the frequency and the phenotype of $\gamma\delta$ T cells in EC patients, we designed a multiparameter flow cytometry panel, to stain PBMCs and tumor-infiltrating lymphocytes from EC patients as well as PBMCs from age- and sex-matched HCs. The gating strategy used for the identification of $\gamma\delta$ T cells is shown in **Figure 6**: following the exclusion of double events (using the forward scatter channel (FSC)-A versus FSC-H) and dead cells (cells positive for the viability marker Zombie), lymphocytes were selected based on size (FSC-A) and the intracellular complexity (side scatter channel (SSC)-A). $\gamma\delta$ T cells were then identified among CD3⁺ cell population.

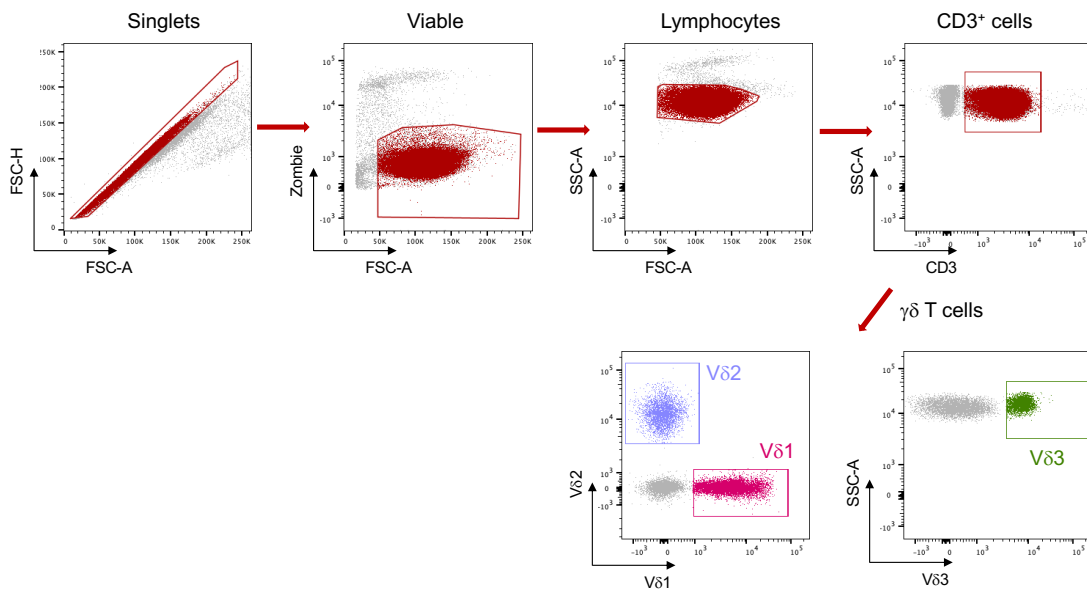


Figure 6 | Gating strategy for the identification of $\gamma\delta$ T cells in EC patients.

Representative flow cytometry dot plots showing V δ 1, V δ 2 and V δ 3 T cell subpopulations in a patient with EC. The subpopulations were identified among total viable CD3⁺ T lymphocytes after double events exclusion.

When we assessed the relative distribution of $\gamma\delta$ T cell subpopulations in the PB and the tumor of EC patients we observed that, similarly to HCs (106), V δ 2 T cells constitute the main population in the circulation (**Figure 7**, left panel). On the other hand, among tumor-infiltrating $\gamma\delta$ T cells, there is a predominance of V δ 1 T cells, known to predominantly localize in peripheral tissues (95), followed by V δ 2 and V δ 3 T subsets (**Figure 7**, right panel).

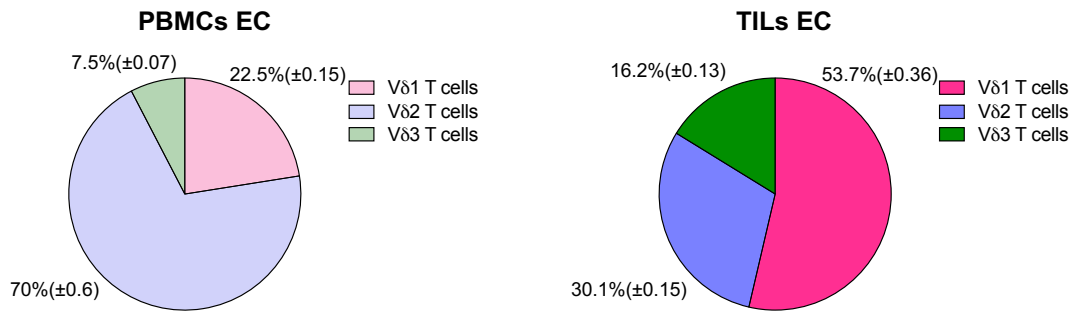
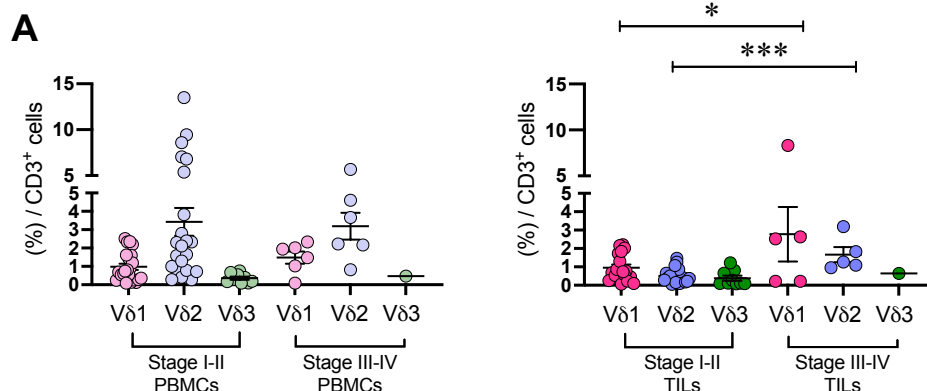


Figure 7 | Frequency of $\gamma\delta$ T cells in EC patients.

Pie charts showing the frequency of Vδ1, Vδ2 and Vδ3 subpopulations among total $\gamma\delta$ T cells in PB (left panel) and in the tumor (right panel) ($n=10-28$). Frequencies are represented as mean $\pm$ SEM.

Owing to the distinct clinical characteristics of the patients, as detailed in **Table 3** in the Materials and Methods section “Clinical features”, we were able to stratify them into various groups of comparison, despite the limited sample size in some groups. While in the PB there are no statistically significant differences in the frequencies of all $\gamma\delta$ T cell subpopulations between early (stage I-II) and advanced (stage III-IV) stages (**Figure 8A**, left panel), interestingly in the tumor there is a higher presence of both Vδ1 and Vδ2 T cells in patients with an advanced disease stage (**Figure 8A**, right panel), suggesting a potential role of these cells even in advanced stages of the disease. Instead, we did not observe differences in either circulating or tumor-infiltrating $\gamma\delta$ T cells when patients were classified based on the tumor grade (low grade, G1/G2, versus high grade, G3) or the histological type (endometrioid versus other histological types) (**Figure 8B-C**).



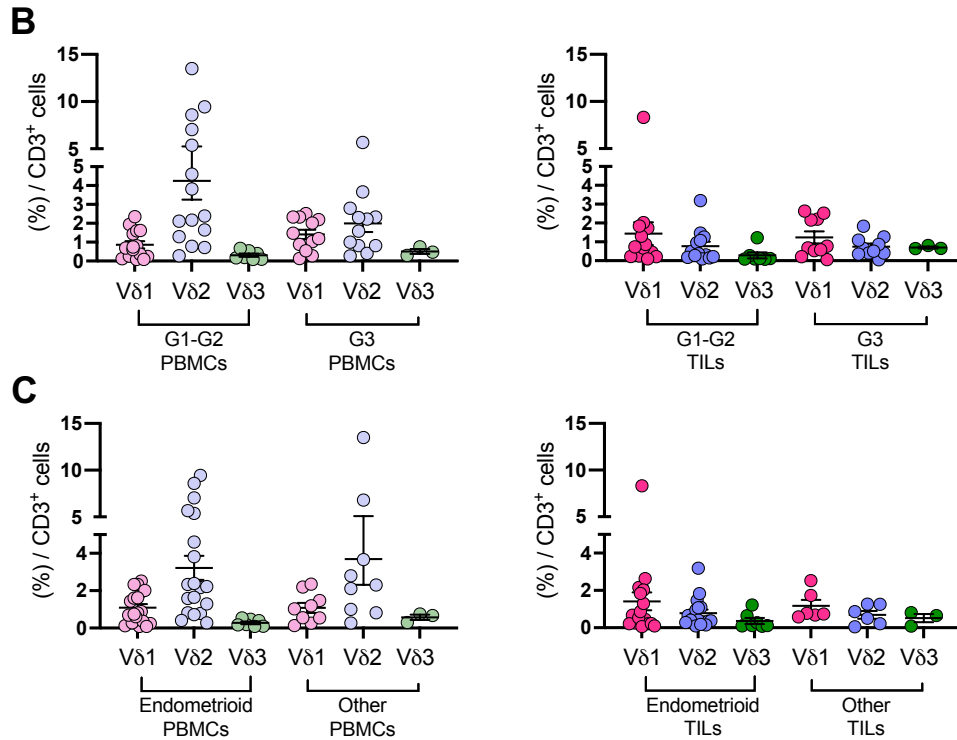


Figure 8 | Frequencies of $\gamma\delta$ T cells in EC patients according to disease stage, grading and histological type.

Frequencies of V δ 1, V δ 2 and V δ 3 subpopulations among CD3⁺ cells in PB (left panel) and in the tumor (right panel) stratifying patients based on disease stage (A), grading (B) and histological type (C) (n=1-22 per group). Frequencies are represented as mean $\pm$ SEM. Unpaired t-test: * $P\leq 0.05$; *** $P\leq 0.001$

One of the significant achievements in EC diagnosis and treatment has been the molecular classification proposed by TCGA which categorizes this tumor into four distinct subtypes: *POLE* ultramutated, MSI, copy number low and copy number high (14). In our cohort we assessed whether the patients exhibited microsatellite instability and subsequently classified them into MSI and microsatellite stability (MSS). MSI patients are known to be highly immunogenic, leading to increased tumor-infiltrating lymphocytes (57). We also investigated the frequency of tumor-infiltrating $\gamma\delta$ T cells, particularly V δ 1 and V δ 2 cell subsets. We noticed a higher trend in their frequency among MSI patients, although this difference was not statistically significant (Figure 9).

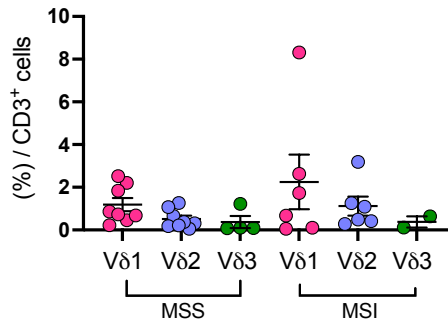


Figure 9 | Frequencies of $\gamma\delta$ T cells in EC patients subdivided in MSS and MSI.

Frequencies of Vδ1, Vδ2 and Vδ3 subpopulations among CD3⁺ cells in the tumor stratifying patients based on microsatellite instability (MSI) or not (MSS) (n=2-8 per group). Frequencies are represented as mean $\pm$ SEM.

Based on these data, we found that the EC tumor microenvironment harbors $\gamma\delta$ T cells, mainly the Vδ1 T subpopulation. Their infiltration appears to be unrelated of different clinical features, including the tumor grade and the histological type. However, they are enriched in more advanced stages of the disease, thus being a possible therapeutic target for this group of patients facing poor clinical outcomes and low responses to standard treatments.

Tumor-infiltrating $\gamma\delta$ T cells express immune checkpoints and exhibit functional activity

In order to define the role of $\gamma\delta$ T cells, we assessed their phenotype based on the markers included in the flow cytometry panel and we compared tumor-infiltrating $\gamma\delta$ T cells with their blood circulating counterpart.

First of all, we analyzed the differentiation stages of $\gamma\delta$ T subpopulations based on surface markers CD27 and CD45RA expression and distinguishing four maturation statuses from the less differentiated phenotype to the most terminally differentiated one: T_{NAIVE} (CD27⁺CD45RA⁺), T_{CM} (central memory, CD27⁺CD45RA⁻), T_{EM} (effector memory, CD27⁻CD45RA⁻) and T_{EMRA} (terminally differentiated effector memory, CD27⁻CD45RA⁺) (**Figure 10A**). In the PB we observed that the three subpopulations of $\gamma\delta$ T cells show a heterogeneous phenotype, in fact, while Vδ1 and Vδ3 T cells are characterized mainly by cells with a T_{EMRA} phenotype, followed by T_{NAIVE}, T_{CM} and T_{EM}, the majority of Vδ2 T cells are T_{EM} and T_{CM}, with less T_{EMRA} and T_{NAIVE} (**Figure 10B**). On the other hand, in the tumor all $\gamma\delta$ T cell subpopulations predominantly display a T_{EM} phenotype, with higher frequencies compared with the circulating counterpart, accompanied by a reduction of T_{EMRA}, T_{CM} and

T_{NAIVE} (**Figure 10B**). These findings suggest that the tumor microenvironment has an impact on the differentiation statuses of $\gamma\delta$ T cells.

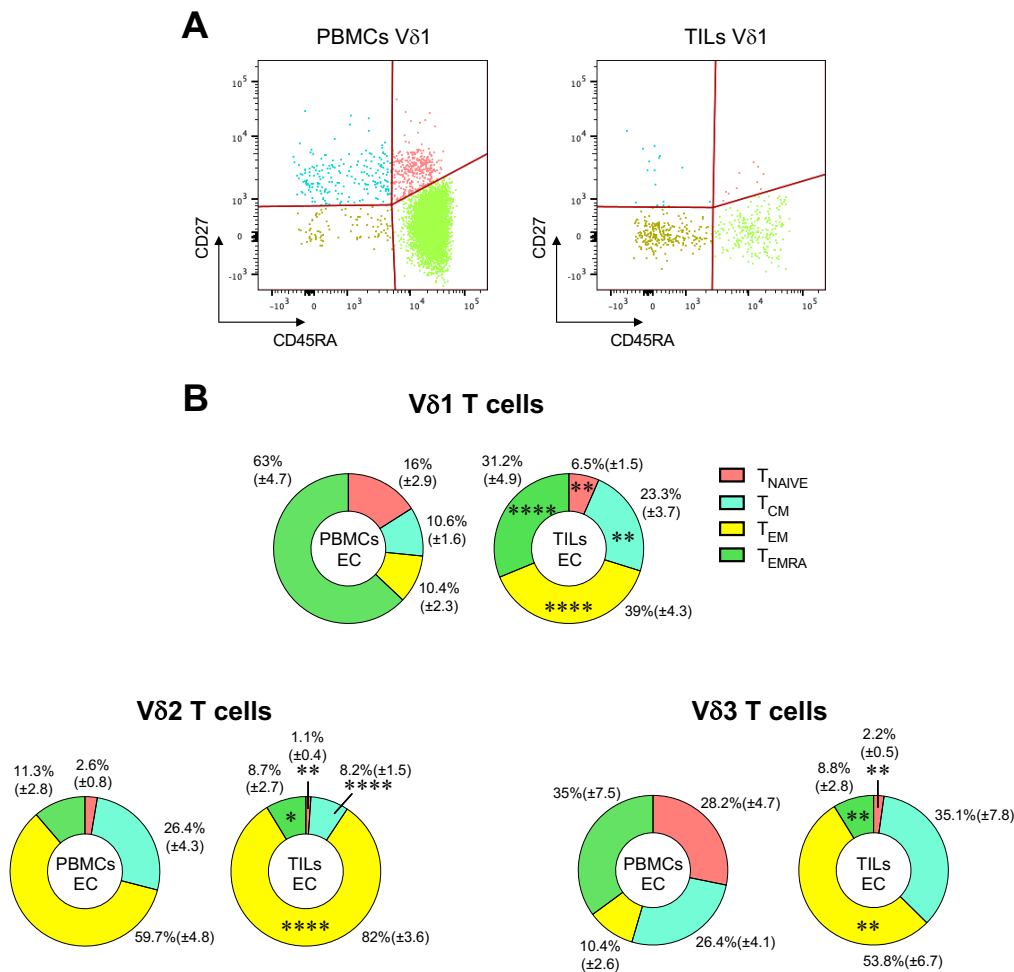
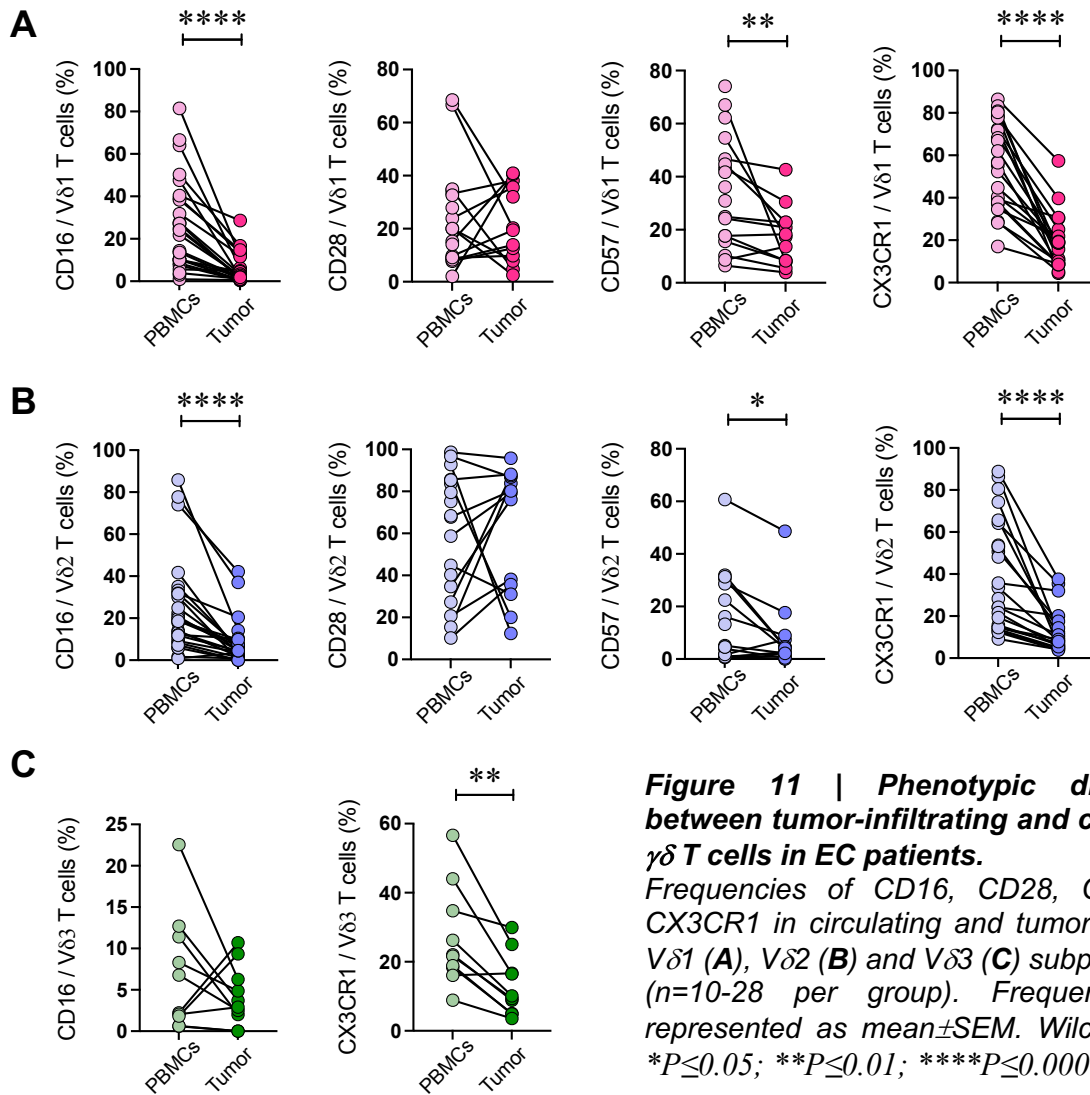


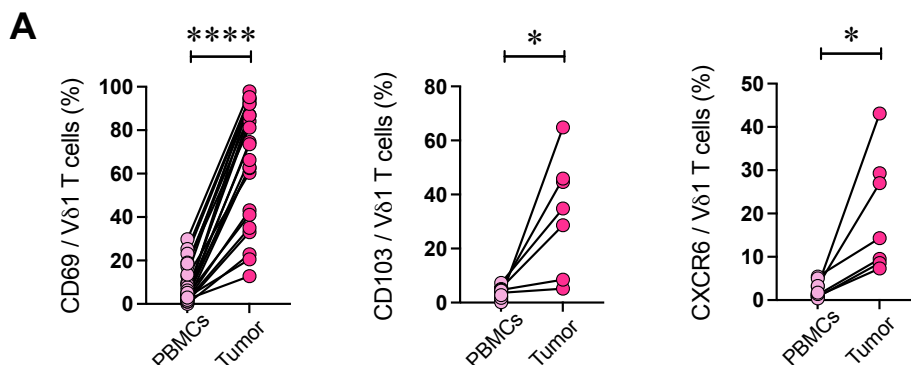
Figure 10 | Differentiation status of $\gamma\delta$ T cells in EC patients.

(A) Representative dot plots of circulating (left panel) and tumor-infiltrating (right panel) Vδ1 T cells showing the gating strategy used for the identification of the four subpopulations of $\gamma\delta$ T cells based on CD27 and CD45RA expression: T_{NAIVE} (CD27⁺CD45RA⁺), T_{CM} (CD27⁺CD45RA⁻), T_{EM} (CD27⁻CD45RA⁻) and T_{EMRA} (CD27⁻CD45RA⁺). (B) Pie charts showing T_{NAIVE} , T_{CM} , T_{EM} and T_{EMRA} mean distribution on total Vδ1, Vδ2 and Vδ3 T cells in PB (left panel) and in the tumor (right panel) of EC patients ($n=10-28$). Frequencies are represented as mean $\pm$ SEM. Wilcoxon test: * $P\leq 0.05$; ** $P\leq 0.01$; *** $P\leq 0.001$; **** $P\leq 0.0001$

Consistent with this observation, tumor-infiltrating Vδ1 and Vδ2 T cells are characterized by a lower expression of CD16, CD57 and CX3CR1, which are related to $\gamma\delta$ T cells' effector function and maturation (214–216), compared with circulating $\gamma\delta$ T cells. Conversely, Vδ3 T cells show reduced level of CX3CR1 only (**Figure 11A-C**).



To confirm the tissue-resident nature of tumor-infiltrating $\gamma\delta$ T cells we evaluated the expression of markers related to tissue residency and homing, particularly CD69, CD103 and CXCR6 (217). As expected, $\gamma\delta$ T cells isolated from the tumor exhibit higher expression levels of all the analyzed markers compared with those isolated from the PB (Figure 12A-C), confirming their tissue-resident tropism.



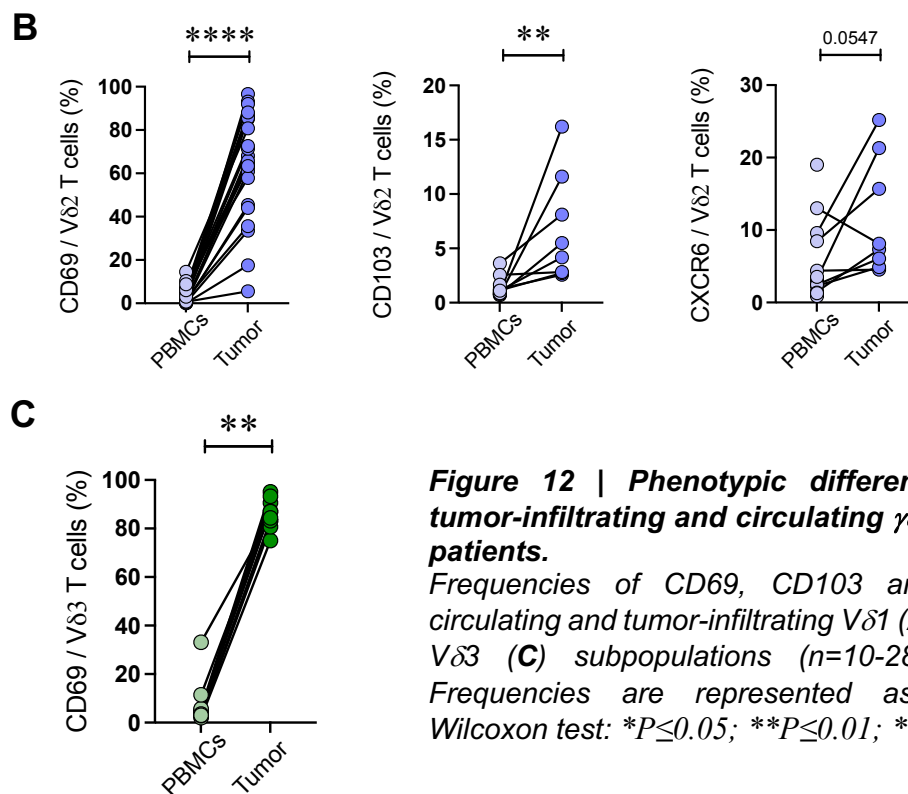
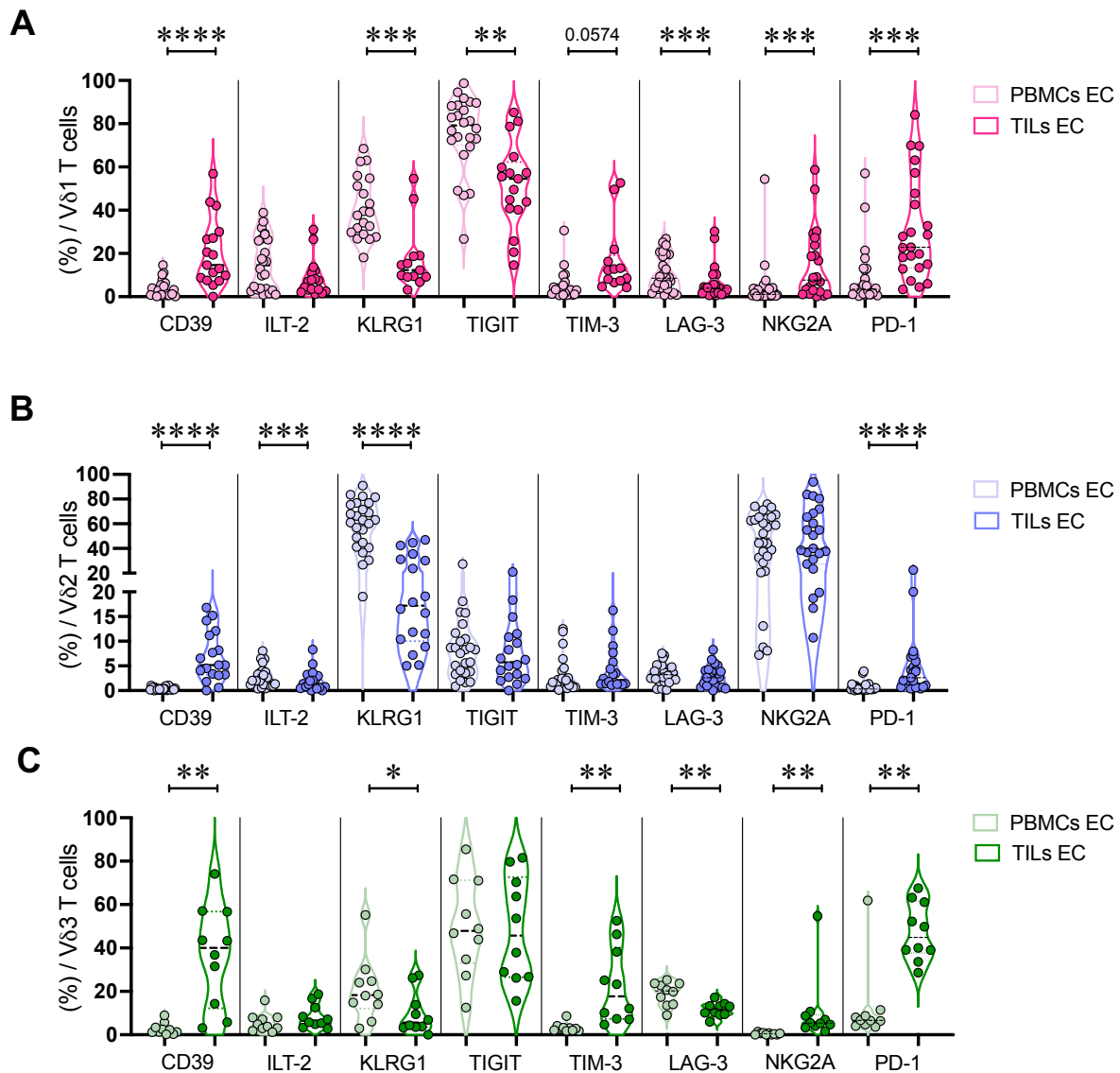


Figure 12 | Phenotypic differences between tumor-infiltrating and circulating $\gamma\delta$ T cells in EC patients.

Frequencies of CD69, CD103 and CXCR6 in circulating and tumor-infiltrating Vδ1 (A), Vδ2 (B) and Vδ3 (C) subpopulations (n=10-28 per group). Frequencies are represented as mean±SEM. Wilcoxon test: * $P\leq 0.05$; ** $P\leq 0.01$; **** $P\leq 0.0001$

Given the promising potential of immunotherapies, particularly immune checkpoint inhibitors, in treating EC (62), we assessed the expression of immune checkpoints on different $\gamma\delta$ T cell subsets (**Figure 13A-C**). Some immune checkpoints, such as LAG-3 and ILT-2, exhibit low expression in the PB and are further reduced in tumor-infiltrating $\gamma\delta$ T cells (**Figure 13A-C**). Others, such as KLRG1 in all $\gamma\delta$ T subpopulations and TIGIT in Vδ1 T cells, are highly expressed in the circulating cells, but show decreased expression in the tumor (**Figure 13A-C**). This can be linked to the differentiation status of $\gamma\delta$ T cells, as they are characterized by a more mature phenotype in the PB, whereas in the tumor they mainly display a T_{EM} phenotype, as shown in **Figure 10B**. Accordingly, KLRG1 is associated with a terminal effector phenotype in T cells (218). Although *KLRG1* gene expression has been associated with effector-like $\gamma\delta$ T cells in mice (219), its precise role in human $\gamma\delta$ T cells remains undefined. Our analysis of PBMCs from HCs supports the evidence observed in mice and suggests the association of KLRG1 expression with a more effector phenotype of $\gamma\delta$ T cells (data not shown). Indeed, KLRG1 expression on Vδ1 T cells correlates with the frequencies of T_{EMRA} Vδ1 T cells (**Figure 13D**), moreover, KLRG1⁺ Vδ1 T cells exhibit a more pronounced T_{EMRA} phenotype compared with their KLRG1-negative counterpart (**Figure 13E**). In conventional $\alpha\beta$ T cells TIGIT is absent in the naïve state, while its expression is upregulated in activated effector and memory cells (220). In $\gamma\delta$ T cells,

analogously to KLRG1, our analysis of HCs reveals a correlation between TIGIT expression and T_{EMRA} V δ 1 T cell frequencies (**Figure 13F**). Moreover, TIGIT⁺ V δ 1 T cells are enriched in T_{EMRA} phenotypic profile while TIGIT⁻ cells are constituted mainly by T_{NAIVE} cells (**Figure 13G**). This supports our hypothesis that the lower expression of KLRG1 and TIGIT in the tumor could be associated with a less differentiated phenotype of $\gamma\delta$ T cells. Interestingly, several immune checkpoints, particularly CD39, TIM-3, NKG2A and PD-1, show low expression levels in circulating $\gamma\delta$ T cells but are upregulated in the tumor across all $\gamma\delta$ T cell subsets (**Figure 13A-C**). This suggests that the expression of these immune checkpoints is induced in the tumor microenvironment. The only exception is NKG2A, which is constitutively expressed by V δ 2 T cells, as we previously reported (106).



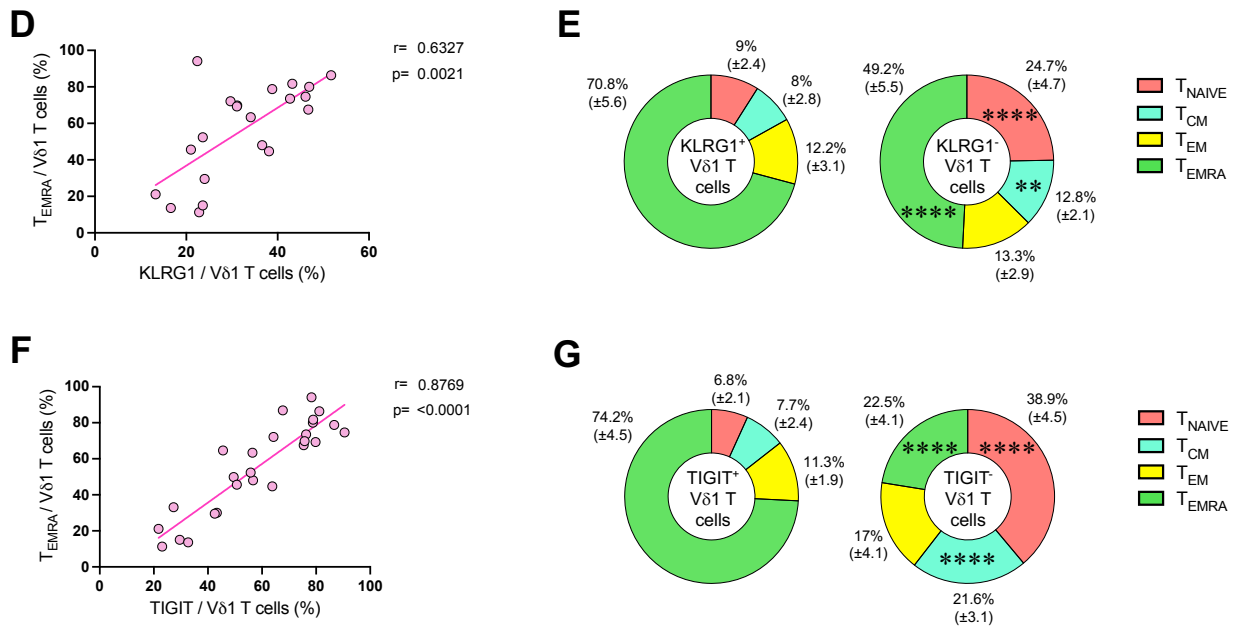


Figure 13 | Immune checkpoints expression on $\gamma\delta$ T cells in EC patients.

Violin plots showing the frequencies of different immune checkpoints (CD39, ILT-2, KLRG1, TIGIT, TIM-3, LAG-3, NKG2A and PD-1) in circulating and tumor-infiltrating Vδ1 (A), Vδ2 (B) and Vδ3 (C) subpopulations ($n=10-28$ per group). (D) Correlation (Spearman) between the expression of KLRG1 on Vδ1 T cells and the frequency of T_{EMRA} cells on Vδ1 T cells in PBMCs of HCs ($n=21$). (E) Pie charts showing T_{NAIVE}, T_{CM}, T_{EM} and T_{EMRA} mean distribution on KLRG1⁺ (left panel) and KLRG1⁻ (right panel) Vδ1 T cells in PBMCs of HCs ($n=21$). (F) Correlation (Spearman) between the expression of TIGIT on Vδ1 T cells and the frequency of T_{EMRA} cells on Vδ1 T cells in PBMCs of HCs ($n=21$). (G) Pie charts showing T_{NAIVE}, T_{CM}, T_{EM} and T_{EMRA} mean distribution on TIGIT⁺ (left panel) and TIGIT⁻ (right panel) Vδ1 T cells in PBMCs of HCs ($n=21$). Frequencies are represented as mean $\pm$ SEM. Wilcoxon test: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$

In light of the increased expression of several immune checkpoints in the EC microenvironment, we investigated potential correlations among these checkpoints' expression within the major population of tumor-infiltrating $\gamma\delta$ T cells, primarily composed of Vδ1 T cells. Interestingly, there was a positive correlation among PD-1, CD39 and TIM-3, which all are upregulated in the tumor, while no correlation was detected for NKG2A (Figure 14). On the other hand, KLRG1 and TIGIT, which showed reduced expression in the tumor, displayed positive correlation with each other. Notably, both KLRG1 and TIGIT negatively correlate with the other immune checkpoints, particularly TIGIT with NKG2A and KLRG1 with both CD39 and PD-1 (Figure 14). This suggests a relationship among the immune checkpoints' expression induced within the tumor microenvironment.

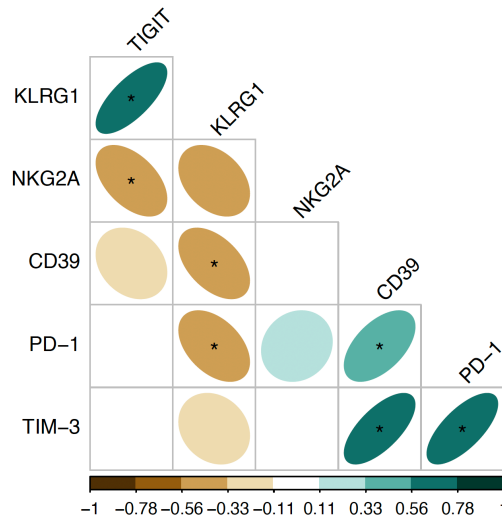


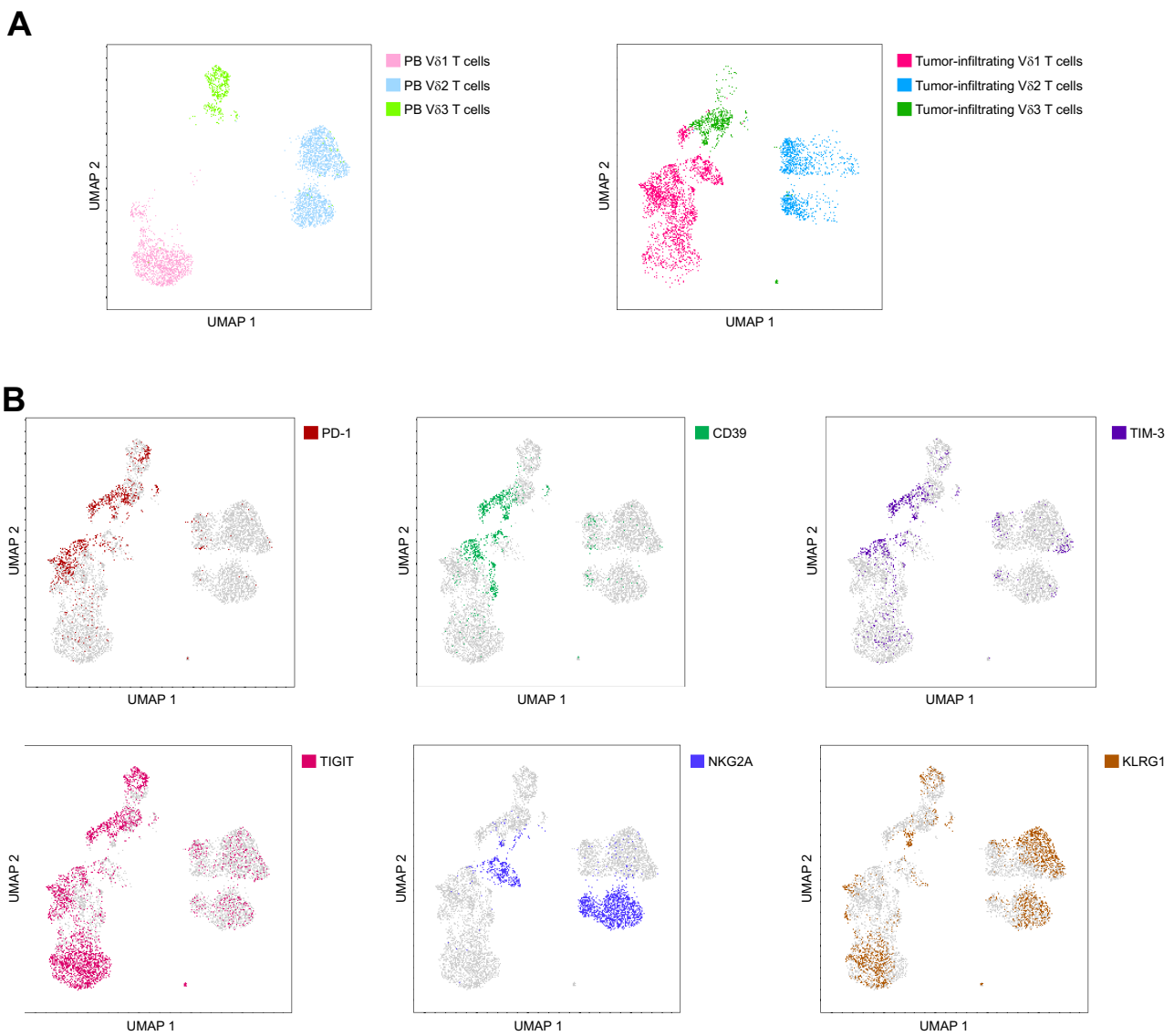
Figure 14 | Correlation of immune checkpoints expression on tumor-infiltrating Vδ1 T cells. Heatmap showing Pearson's correlation analysis between the frequencies (%) of immune checkpoints on tumor-infiltrating Vδ1 T cells (n=14). The green ellipses that go from the lower left to the upper right indicate a positive correlation, while the brown ellipses that go from the upper left to the lower right indicate a negative correlation. * $P \leq 0.05$

To further analyze the immune checkpoints' expression in the tumor, we performed an unbiased analysis through the UMAP algorithm on the flow cytometry data obtained for $\gamma\delta$ T cells of EC patients. This algorithm allows dimensionality reduction and enables the visualization of complex high-dimensional data into a two-dimensional map, in which cells with a similar expression of the analyzed markers are placed in the same region of the graph depending on the intensity of the fluorescence (221). We observed that, as expected, the three subsets of $\gamma\delta$ T cells clustered in different groups, moreover, circulating and tumor-infiltrating $\gamma\delta$ T cells localize in distinct areas of the UMAP (**Figure 15A**), confirming the phenotypic differences of $\gamma\delta$ T cells from the two compartments, consistent with our manual gating strategy. Of note, tumor-infiltrating $\gamma\delta$ T cells, particularly Vδ1 and Vδ3 T cells characterized by high immune checkpoints' expression, exhibit co-expression of multiple immune checkpoints, notably PD-1, CD39, TIM-3 and TIGIT (**Figure 15B**). Conversely, only a small subset of cells co-expressed NKG2A and KLRG1, indicating potential differences in their regulation. This multi-checkpoints' expression was further validated by gating on tumor-infiltrating $\gamma\delta$ T cells displaying co-expression of immune checkpoints (**Figure 15C**, left panel). In fact, the cells included in this gate show a higher expression of PD-1, CD39, TIM-3 and TIGIT, but not NKG2A and KLRG1 (**Figure 15C**, right panel). In addition, all gated cells express CD69, indicative of a tissue-resident phenotype.

Contrarily to Vδ1 and Vδ3 T cells, Vδ2 T cells are characterized by a lower expression of the analyzed immune checkpoints except for NKG2A, which moreover divides Vδ2 T cells

into two distinct subpopulations, NKG2A⁺ and NKG2A⁻, suggesting phenotypic differences between these two populations, as we have already described in HCs (106) (**Figure 15B**).

Thus, the tumor microenvironment appears to influence $\gamma\delta$ T cells prompting the expression of immune checkpoints that may modulate the antitumor efficacy of $\gamma\delta$ T cells.



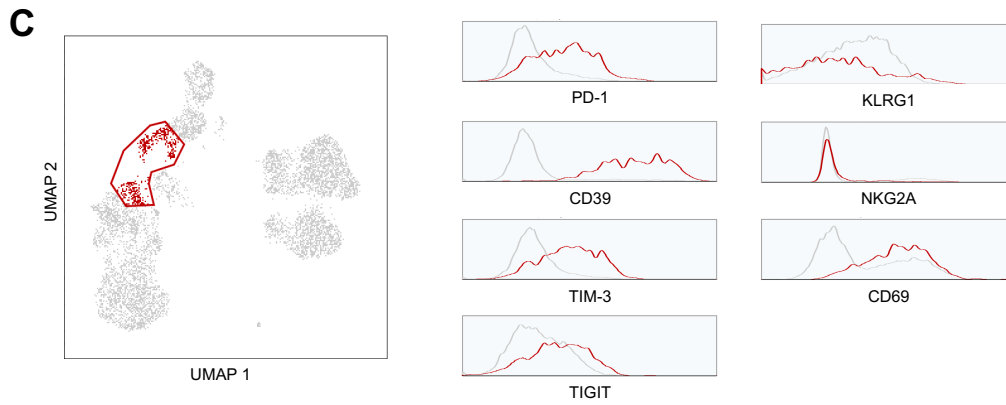


Figure 15 | Immune checkpoints co-expression on tumor-infiltrating $\gamma\delta$ T cells.

(A) UMAP graph showing the spatial distribution of circulating (left panel) and tumor-infiltrating (right panel) $V\delta 1$, $V\delta 2$ and $V\delta 3$ subpopulations. (B) UMAP graph showing the spatial distribution of PD-1, CD39, TIM-3, TIGIT, NKG2A and KLRG1. (C) UMAP graph with a manual gate made on tumor-infiltrating $\gamma\delta$ T cells expressing immune checkpoints (left panel) and histograms with the expression of PD-1, CD39, TIM-3, TIGIT, KLRG1, NKG2A and CD69 on the cells included in the gate (red histograms) compared with all other cells (grey histograms). UMAP was performed on concatenated $V\delta 1$, $V\delta 2$ and $V\delta 3$ T cells.

Considering that the immune checkpoint inhibitor therapies for EC treatment primarily target the PD-1 axis and given that $V\delta 1$ T cells are the main subset in the tumor, we focused our investigation on the role of PD-1 in this population. As we have observed the correlation and the co-expression of different immune checkpoints by tumor-infiltrating $\gamma\delta$ T cells through UMAP algorithm, we then compared the phenotype of tumor-infiltrating PD-1⁺ and PD-1⁻ $V\delta 1$ T cells by manual gating strategy. As expected, PD-1⁺ $V\delta 1$ T cells showed higher expression levels of the immune checkpoints CD39, ILT-2, TIGIT and TIM-3 (**Figure 16A**, upper panel). Moreover, PD-1⁺ $V\delta 1$ T cells express more CD28 and less CX3CR1, suggesting a less differentiated phenotype, however, they show more CD57 and a lower frequency of the activating marker CD56 (**Figure 16A**, lower panel). In accordance, PD-1⁺ $V\delta 1$ T cells are characterized mainly by cells with a T_{CM} and T_{EM} phenotype, while PD-1⁻ $V\delta 1$ T cells show a decrease of T_{CM} cells counterbalanced by an increase of T_{EMRA} cells, which is the main population (**Figure 16B**). So, in $\gamma\delta$ T cells PD-1 is associated with a T_{CM} phenotype. Accordingly, there is a positive correlation between the expression of PD-1 and the frequency of T_{CM} cells on $V\delta 1$ T cells (**Figure 16C**, left panel), while it negatively correlates with the frequency of T_{EMRA} cells (**Figure 16C**, right panel).

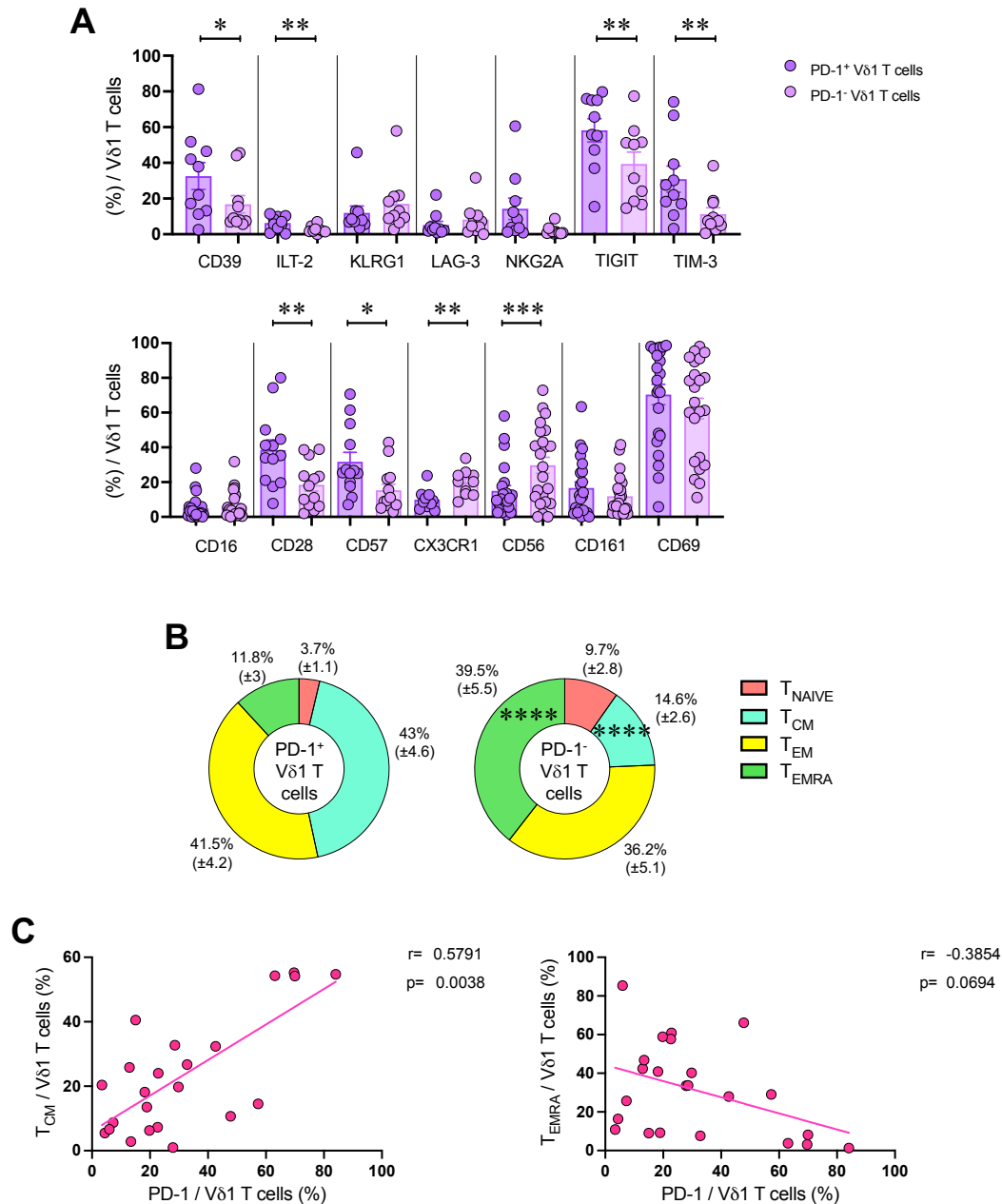


Figure 16 | Phenotype of PD-1⁺ and PD-1⁻ Vδ1 T cells.

(A) Frequencies of several immune checkpoints (upper panel) and other markers (lower panel) on PD-1⁺ and PD-1⁻ Vδ1 T cells (n=10-28 per group). (B) Pie charts showing T_{NAIVE} , T_{CM} , T_{EM} and T_{EMRA} mean distribution on PD-1⁺ (left panel) and PD-1⁻ (right panel) tumor-infiltrating Vδ1 T cells (n=23). (C) Correlation (Spearman) between the expression of PD-1 on Vδ1 T cells and the frequency of T_{CM} (left panel) T_{EMRA} cells (right panel) on tumor-infiltrating Vδ1 T cells (n=23). Frequencies are represented as mean $\pm$ SEM. Wilcoxon test: * $P\leq 0.05$; ** $P\leq 0.01$; *** $P\leq 0.001$; **** $P\leq 0.0001$

Given the high expression of immune checkpoints, notably PD-1, on tumor-infiltrating $\gamma\delta$ T cells, we questioned whether these cells retained functionality or had become dysfunctional. To explore this, we stimulated circulating $\gamma\delta$ T cells from HCs and EC patients as well as tumor-infiltrating $\gamma\delta$ T cells with PMA/ionomycin and assessed their cytokines

production capacity, specifically IFN- γ and TNF- α . Upon stimulation, $\gamma\delta$ T cells maintained their ability to produce cytokines, in fact, both stimulated V δ 1 and V δ 2 T subsets secreted IFN- γ and TNF- α (**Figure 17A**). While V δ 2 T cells exhibited no differences in cytokines production between circulating and tumor-infiltrating cells or when compared with cells isolated from HCs (**Figure 17A**, right panel), circulating V δ 1 T cells from EC patients showed a higher production of IFN- γ and TNF- α compared with both circulating V δ 1 T cells from HCs and tumor-infiltrating V δ 1 T cells (**Figure 17A**, left panel). This discrepancy in IFN- γ production between circulating and tumor-infiltrating $\gamma\delta$ T cells may stem from the enrichment of less differentiated cells in the tumor. To investigate this, we examined the production of IFN- γ by T_{NAIVE}, T_{CM}, T_{EM} and T_{EMRA} cells. As expected, in HCs there is a higher frequency of IFN- γ ⁺ cells with the maturation of $\gamma\delta$ T cells, indeed, T_{NAIVE} cells show less IFN- γ production while T_{EMRA} cells display the highest levels (**Figure 17B**). Circulating $\gamma\delta$ T cells from EC patients are generally characterized by a higher capacity to produce IFN- γ compared with HCs. On the other hand, tumor-infiltrating cells, especially T_{EMRA} cells, show reduced levels of IFN- γ compared with their circulating counterpart. Moreover, IFN- γ production was comparable in all the $\gamma\delta$ T subsets. This was confirmed by assessing the distribution of $\gamma\delta$ T subsets on IFN- γ ⁺ cells upon PMA/I stimulation. In line with this, while IFN- γ ⁺ cells in the circulation are predominantly T_{EMRA} cells, all the $\gamma\delta$ T subsets are equally distributed among tumor-infiltrating IFN- γ ⁺ cells (**Figure 17C**).

Therefore, $\gamma\delta$ T cells from EC patients retain the antitumor activity by producing cytokines upon stimulation; however, tumor-infiltrating cells exhibit reduced functionality compared with their circulating counterparts. This underscores the need to augment their effector response.

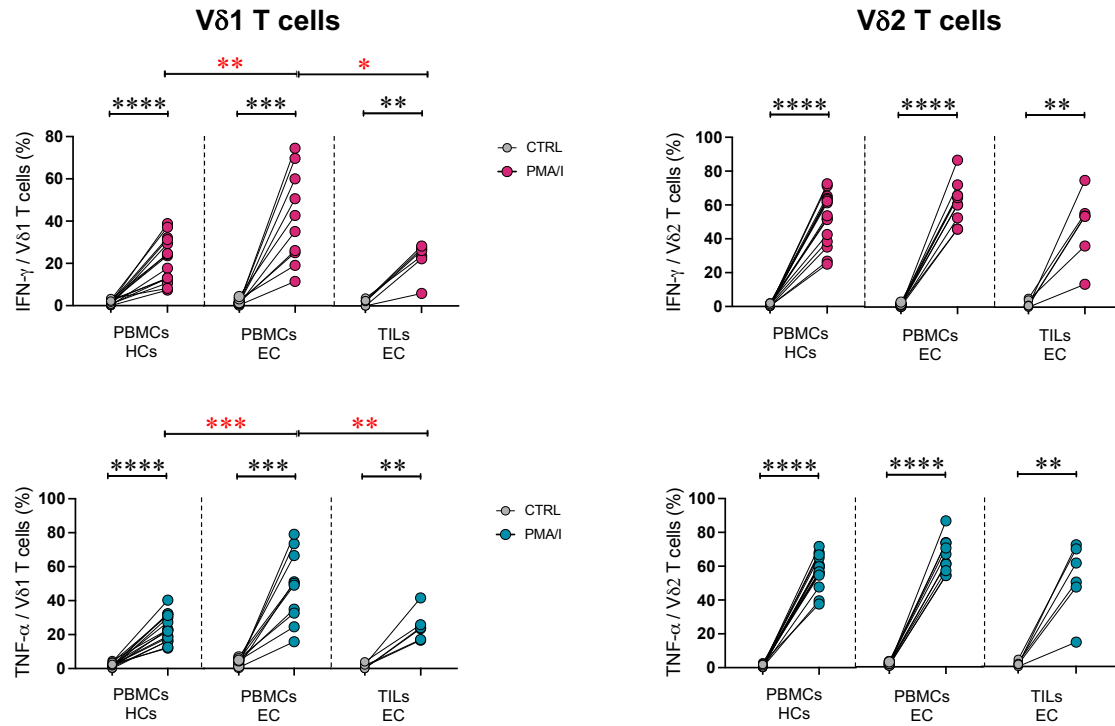
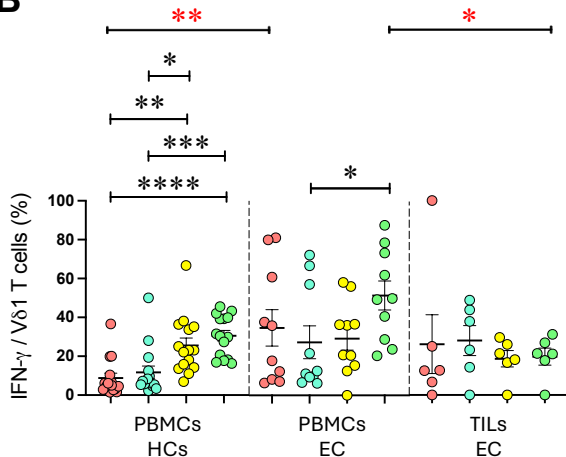
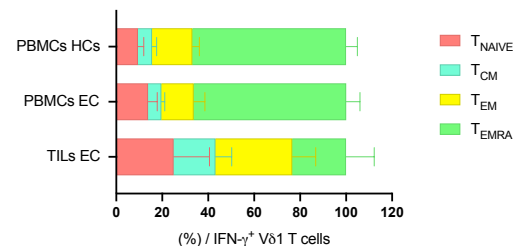
A**B****C**

Figure 17 | IFN- γ and TNF- α production by $\gamma\delta$ T cells upon PMA/I stimulation.

(A) Frequencies of IFN- γ^+ (upper panel) and TNF- α^+ cells (upper panel) on V δ 1 (left panel) and V δ 2 T cells (right panel) from PB of HCs and EC patients and the tumor of EC patients ($n=6-15$ per group). (B) Frequencies of IFN- γ^+ V δ 1 T cells on T_{NAIVE} , T_{CM} , T_{EM} and T_{EMRA} from PB of HCs and EC patients and the tumor of EC patients ($n=6-15$ per group). (C) Frequencies of T_{NAIVE} , T_{CM} , T_{EM} and T_{EMRA} from PB of HCs and EC patients and the tumor of EC patients on IFN- γ^+ V δ 1 T cells ($n=6-15$ per group). Frequencies are represented as mean $\pm$ SEM. In figure (A) black asterisks were calculated with Wilcoxon test and red asterisks with one-way ANOVA, in figure (B) black asterisks were calculated with Friedman test and red asterisks with Kruskal-Wallis test with Dunn's multiple comparison: * $P\leq 0.05$; ** $P\leq 0.01$; *** $P\leq 0.001$; **** $P\leq 0.0001$

Considering that immune checkpoints are expressed mainly by tumor-infiltrating $\gamma\delta$ T cells with a tissue-resident phenotype, we sought to evaluate the impact of immune checkpoint expression on the functional activity of these cells. To this end, we compared the

production of IFN- γ and TNF- α between cells expressing and not expressing the tissue-resident marker CD103. While there are no differences in the production of TNF- α between CD103⁺ and CD103⁻ V δ 1 T cells (**Figure 18**, right panel), IFN- γ production is higher for CD103⁺ V δ 1 T cells (**Figure 18**, left panel). This suggests that tissue-resident $\gamma\delta$ T cells are endowed with a higher antitumor potential due to a higher capacity to produce IFN- γ upon stimulation. However, whether these cells could specifically recognize tumor cells and could be unleashed by antibodies targeting PD-1 remains to be determined.

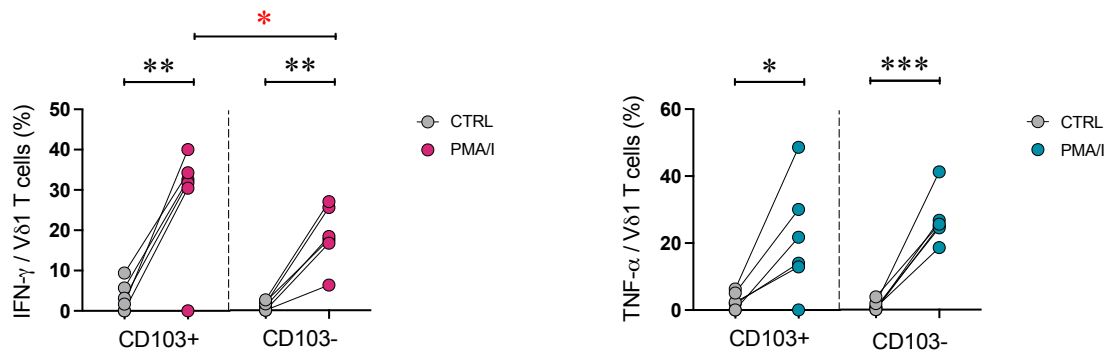


Figure 18 | IFN- γ and TNF- α production by tissue-resident $\gamma\delta$ T cells upon PMA/I stimulation. Frequencies of IFN- γ ⁺ (left panel) and TNF- α ⁺ cells (right panel) on CD103⁺ and CD103⁻ V δ 1 T cells from the tumor of EC patients (n=6). Paired t-test: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Single-cell transcriptional profiling of tumor-infiltrating $\gamma\delta$ T cells

Since our cohort lacked healthy tissue samples for comparison, we took advantage of two public datasets in which scRNA-seq was performed on healthy and tumor endometrial specimens (222,223). These datasets included a total of 10 EC patients, all of whom underwent sequencing of the tumor tissue, with peritumor sequencing performed for three patients. Additionally, normal tissue samples from five individuals unaffected by EC were included for reference. Details regarding the datasets and patients' information are reported in **Table 7**.

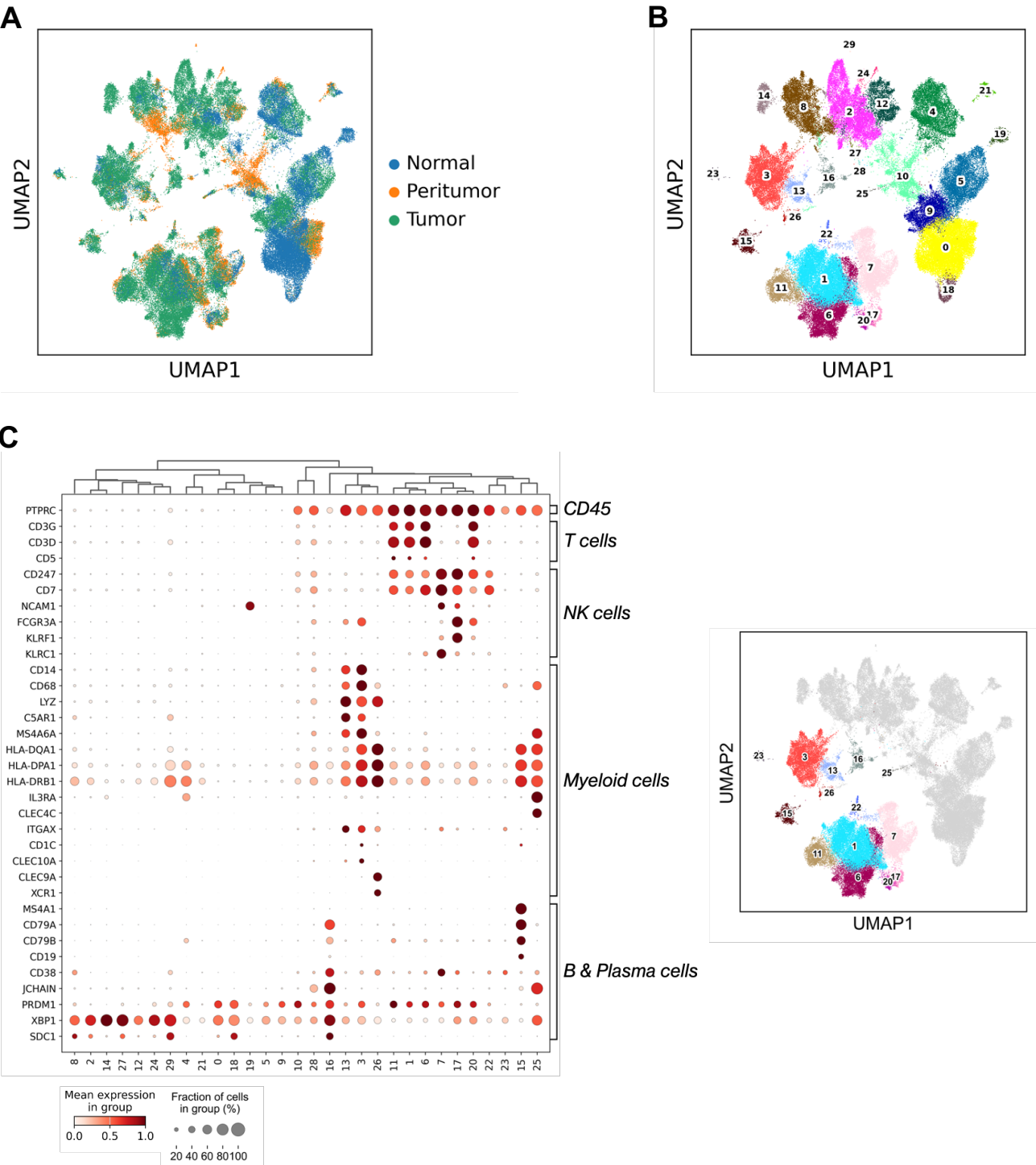
Sample ID	Dataset	Site	Patient	Age	Grade	Sequencing
EC1-Peri	1	Peritumor	EC1	51	Grade 1	3GEX
EC1-Tumor		Tumor				
EC2-Peri	1	Peritumor	EC2	68	Grade 1	3GEX
EC2-Tumor		Tumor				
EC3-Peri	1	Peritumor	EC3	42	Grade 1	3GEX
EC3-Tumor		Tumor				
EC4-Tumor	1	Tumor	EC4	55	Grade 1	3GEX
EC5-Tumor	1	Tumor	EC5	56	Grade 1	3GEX
EC6-Tumor	2	Tumor	EC6	50	Grade 1	3GEX
EC7-Tumor	2	Tumor	EC7	51	Grade 1	3GEX
EC8-Tumor	2	Tumor	EC8	44	Grade 1	3GEX
EC9-Tumor	2	Tumor	EC9	49	Grade 1	3GEX
EC10-Tumor	2	Tumor	EC10	52	Grade 1	3GEX
Norm1	2	Normal	Norm1	47	Normal endometrium	3GEX
Norm2	2	Normal	Norm2	47	Normal endometrium	3GEX
Norm3	2	Normal	Norm3	52	Normal endometrium	3GEX
Norm4	2	Normal	Norm4	50	Normal endometrium	3GEX
Norm5	2	Normal	Norm5	51	Normal endometrium	3GEX

Table 7 | scRNA-seq datasets used for the transcriptional analysis of $\gamma\delta$ T cells.

Information about the patients included in the two datasets of scRNA-seq analyzed: dataset of origin, 1 or 2; analyzed tissue (peritumor, tumor or normal tissue); number of the patient; age; grade of the tumor and type of sequencing (3' scRNA-seq).

Initially, we examined the spatial distribution of the three analyzed compartments - normal, peritumor and tumor - on UMAP graph (**Figure 19A**). Notably, while some overlap among the three tissues suggested similarities, distinct areas enriched in only one compartment hinted at specific features. Using UMAP graph-based clustering projection we obtained 30 clusters (**Figure 19B**) and we annotated the different cell populations using canonical cell markers. In particular, among the immune cells we identified T cells (based on the expression of *CD3G*, *CD3D*, *CD5* genes), NK cells (based on *NCAM1*, *FCGR3A*, *KLRF1*), myeloid cells including DCs and macrophages (based on *CD14*, *CD68*, *LYZ*, *CD1C*, *CLEC10A*, *CLEC9A*) and B and plasma cells (based on *CD19*, *MS4A1*, *XBP1*, *JCHAIN*, *PRDM1*) (**Figure 19C**). While among the non-immune compartment we identified

epithelial cells (based on *EPCAM*, *KRT18*, *CDH1*, *FOXJ1*), endothelial cells (based on *CDH5*, *ENG*, *PCDH17*) and fibroblasts (based on *COL3A1*, *COL6A2*, *COL6A3*, *DCN*), the presence of megakaryocytes and erythrocytes was instead excluded (**Figure 19D**). The populations identified from this macroscopic annotation are shown in the **Figure 19E**.



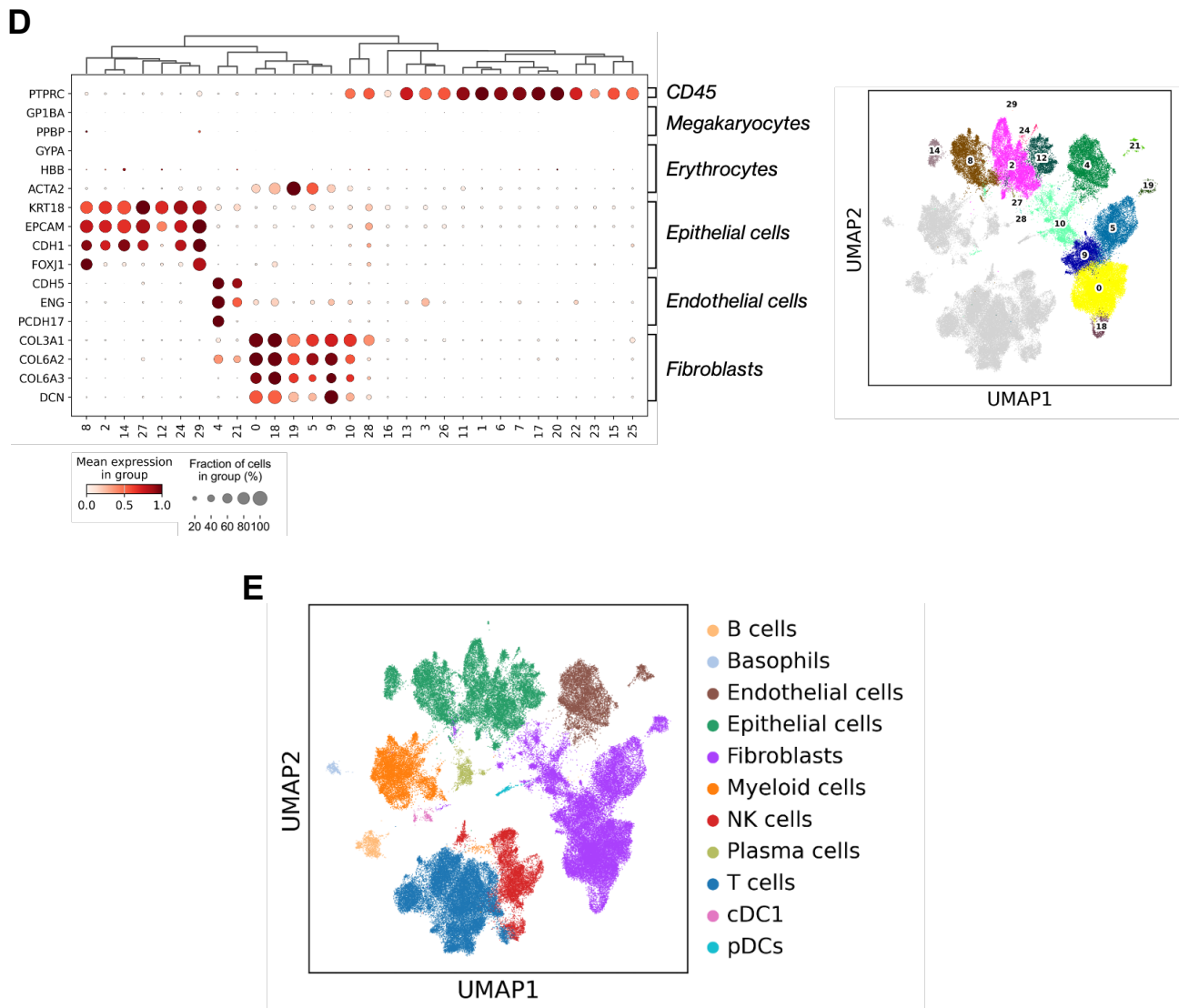
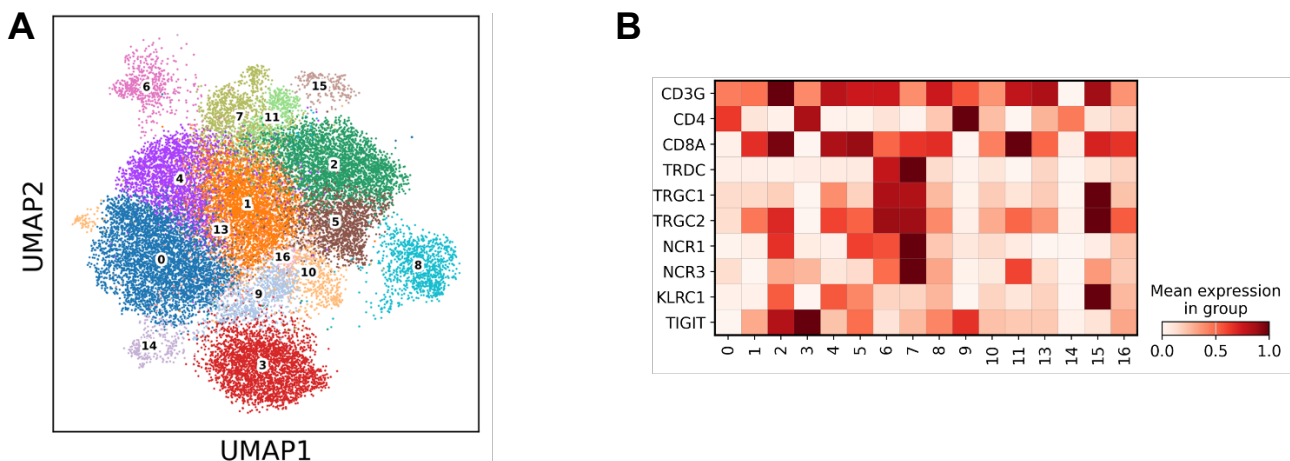


Figure 19 | Single-cell transcriptional annotation of cells from normal, peritumor and tumor tissue of EC patients.

(A) UMAP graph showing the distribution of normal, peritumor and tumor tissue. (B) UMAP graph-based clustering projection showing the formation of 30 clusters at a resolution of 0.5, each dot corresponds to a single cell, colored according to the cell cluster. (C). Dot plot (left panel) and UMAP graph-based clustering projections (right panel) showing canonical cell markers used for the annotation of immune cell populations, e.g. T cells, NK cells, myeloid cells including dendritic cells and B and plasma cells. (D). Dot plot (left panel) and UMAP graph-based clustering projections (right panel) showing canonical cell markers used for the annotation of non-immune cell populations, e.g. epithelial cells, endothelial cells and fibroblasts; the presence of megakaryocytes and erythrocytes was excluded. (E) UMAP graph showing the macroscopic annotation of the identified cell populations, each dot corresponds to a single cell, colored according to the cell type.

As the macroscopic annotation didn't reveal $\gamma\delta$ T cells, we re-clustered the T cell-enriched clusters, resulting in 17 clusters at a resolution of 1.0 (**Figure 20A**). Clusters 6 and 7 were annotated as $\gamma\delta$ T cell clusters based on the expression of genes related to the expression of the $\gamma\delta$ TCR, e.g. *TRDC*, *TRGC1* and *TRGC2* (**Figure 20B**). Due to the limitations of the 3' RNA-sequencing in discriminating among different $\gamma\delta$ T subpopulations,

we pooled clusters 6 and 7 together for subsequent analysis, considering them as total $\gamma\delta$ T cells. The comparison of $\gamma\delta$ T cells between normal, peritumor and tumor tissue (**Figure 20C-D**) confirmed our FACS analysis and showed the gradual changes in the phenotype of $\gamma\delta$ T cells from the normal to the tumor tissue. In particular, the less differentiated phenotype of tumor-infiltrating $\gamma\delta$ T cells was confirmed by the upregulation of the differentiation marker *CD27*. Moreover, they have a tissue-resident phenotype with the expression of *CXCR6* and they are characterized by the expression of immune checkpoints, e.g. *CD96*, *HAVCR2* which encodes for TIM-3, *KLRC1* which encodes for NKG2A, *TIGIT*, *CTLA4*, *LAG3* and importantly *PDCD1* encoding PD-1. Despite immune checkpoint expression, they display antitumor potential as shown by expression of several genes related to cytotoxicity, e.g. *GZMB*, *GZMK*, *GZMA*, *IFNG* and *NCR1* (encoding NKp46), which we have described being associated with high antitumor activity of $V\delta 1$ T cells in colorectal cancer (104). A tumor-specific nature of the tumor-infiltrating $\gamma\delta$ T cells has been suggested by the upregulation of *CXCL13* in the tumor setting. Comparative analysis of *PDCD1* gene expression on $\gamma\delta$ T cells among normal, peritumor and tumor tissues, revealed the highest expression in tumor-infiltrating $\gamma\delta$ T cells (**Figure 20D**). Moreover, we observed that *PDCD1*⁺ $\gamma\delta$ T cells are composed mainly of cells derived from the tumor, followed by the peritumor and the normal tissue (**Figure 20E**). To further investigate the transcriptional profile of $\gamma\delta$ T cells we performed Gene Ontology using the top 20 DEGs comparing the tumor with the normal tissue. This analysis revealed that tumor-infiltrating $\gamma\delta$ T cells upregulate pathways related to their antitumor potential, e.g. T cell receptor signaling, TNF signaling, type I IFN production, IFN- γ mediated signaling, in addition, as well as related to T cell proliferation, and PD-L1 expression and PD-1 checkpoint pathway in cancer, confirming our previous results (**Figure 20F**).



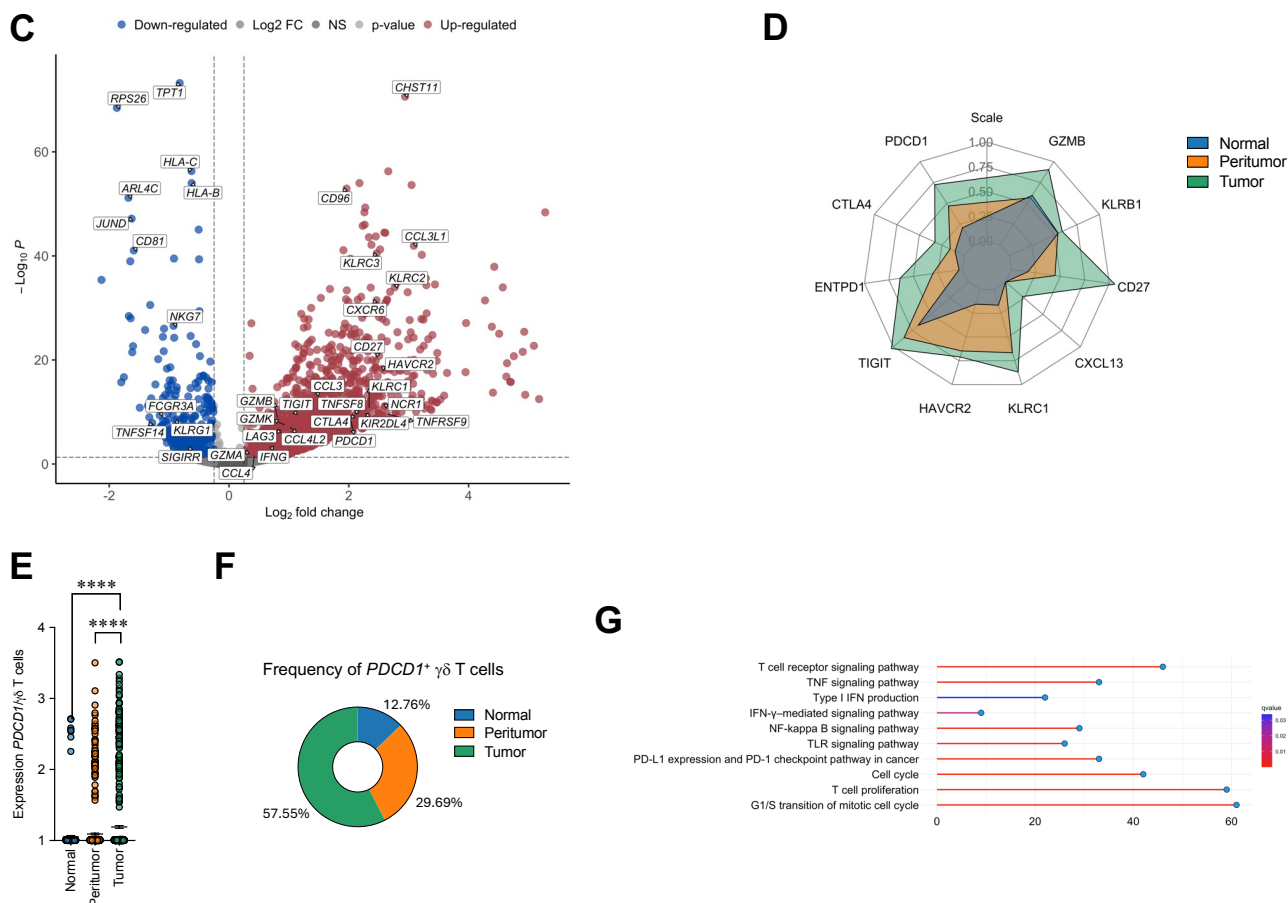


Figure 20 | Transcriptomic diversity of $\gamma\delta$ T cells in EC.

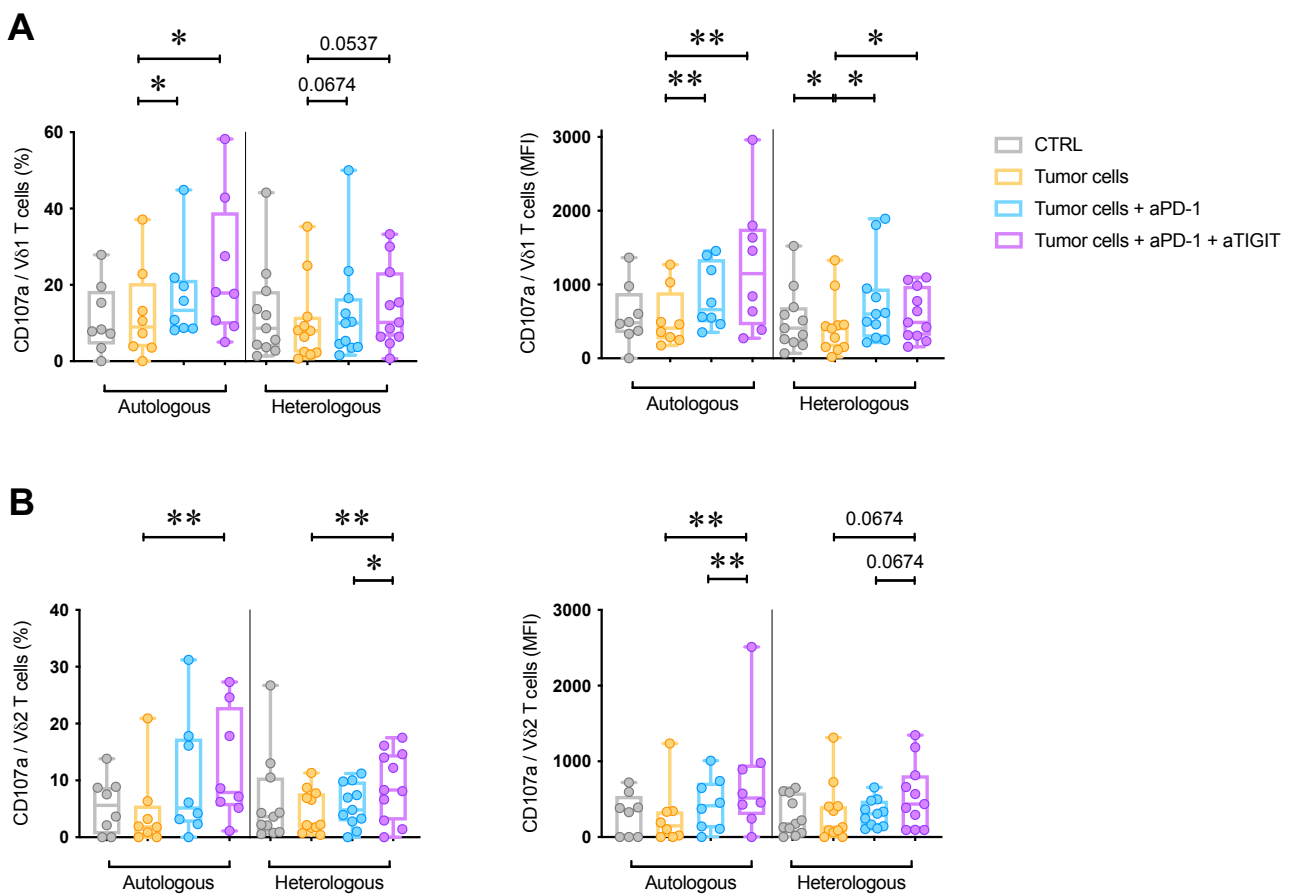
(A) UMAP graph-based clustering projection showing the formation of 17 clusters at a resolution of 1.0, each dot corresponds to a single cell, colored according to the cell cluster. (B). Heatmap showing the mean expression of the genes used to identify the clusters of $\gamma\delta$ T cells. (C). Volcano plot showing the DEGs between $\gamma\delta$ T cells from normal and tumor tissues. (D) Radial graphs showing the expression level of specific DEGs among $\gamma\delta$ T cells from normal, peritumor and tumor tissues. (E) Expression of PDCD1 gene in $\gamma\delta$ T cells from normal, peritumor and tumor tissue. (F) Pie chart showing the frequency of PDCD1⁺ $\gamma\delta$ T in the three compartments analyzed. (G) Pathways upregulated in $\gamma\delta$ T cells in the tumor compared with the normal tissue obtained with Gene Ontology using the top 20 DEGs. One-way ANOVA: **** $P \leq 0.0001$

Therefore, scRNA-seq analysis confirmed that tumor-infiltrating $\gamma\delta$ T cells retain their antitumor potential, despite upregulation of immune checkpoints, especially PD-1. However, the feasibility of targeting PD-1 on $\gamma\delta$ T cells remains to be determined.

PD-1 on $\gamma\delta$ T cells is a therapeutic target

To define the antitumor activity of $\gamma\delta$ T cells against tumor cells we performed co-culture experiments. PBMCs or TILs from EC patients were cultured with tumor cells isolated from the same EC patient (autologous setting) or a different patient (heterologous setting). We then evaluated the degranulation capacity of the effector cells by measuring the

degranulation marker CD107a. Moreover, we attempted to enhance $\gamma\delta$ T cells activity by using a blocking mAb targeting PD-1 alone or in combination with a blocking mAb targeting TIGIT. Interestingly, both V δ 1 and V δ 2 TILs did not show any response in the presence of autologous or heterologous target cells, suggesting their possible inhibition. However, the frequency of CD107a⁺ cells increased when a mAb against PD-1 alone or in combination with anti-TIGIT was added (**Figure 21A-B**, left panel). This was further confirmed by assessing not only the frequency but also the MFI of CD107a on tumor-infiltrating $\gamma\delta$ T cells (**Figure 21A-B**, right panel). Thus, targeting PD-1 increases the cytotoxic activity of tumor-infiltrating $\gamma\delta$ T cells against EC. Importantly, this increase is specific to tumor-infiltrating $\gamma\delta$ T cells. Indeed, their circulating counterpart did not show higher degranulation capacity (both frequency and MFI) in either autologous or heterologous settings when treated with anti-PD-1. This could be related to the lower expression of PD-1 by blood circulating $\gamma\delta$ T cells. Of note, no significant increase was observed when the mAb anti-PD-1 was combined with anti-TIGIT mAb (**Figure 21C-D**).



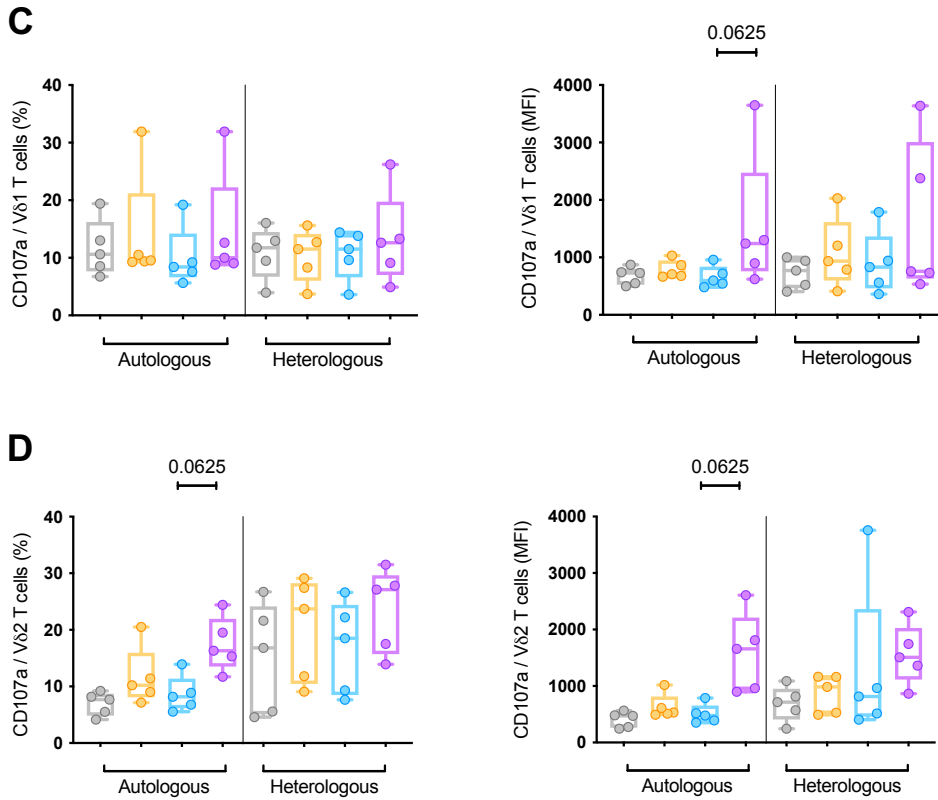


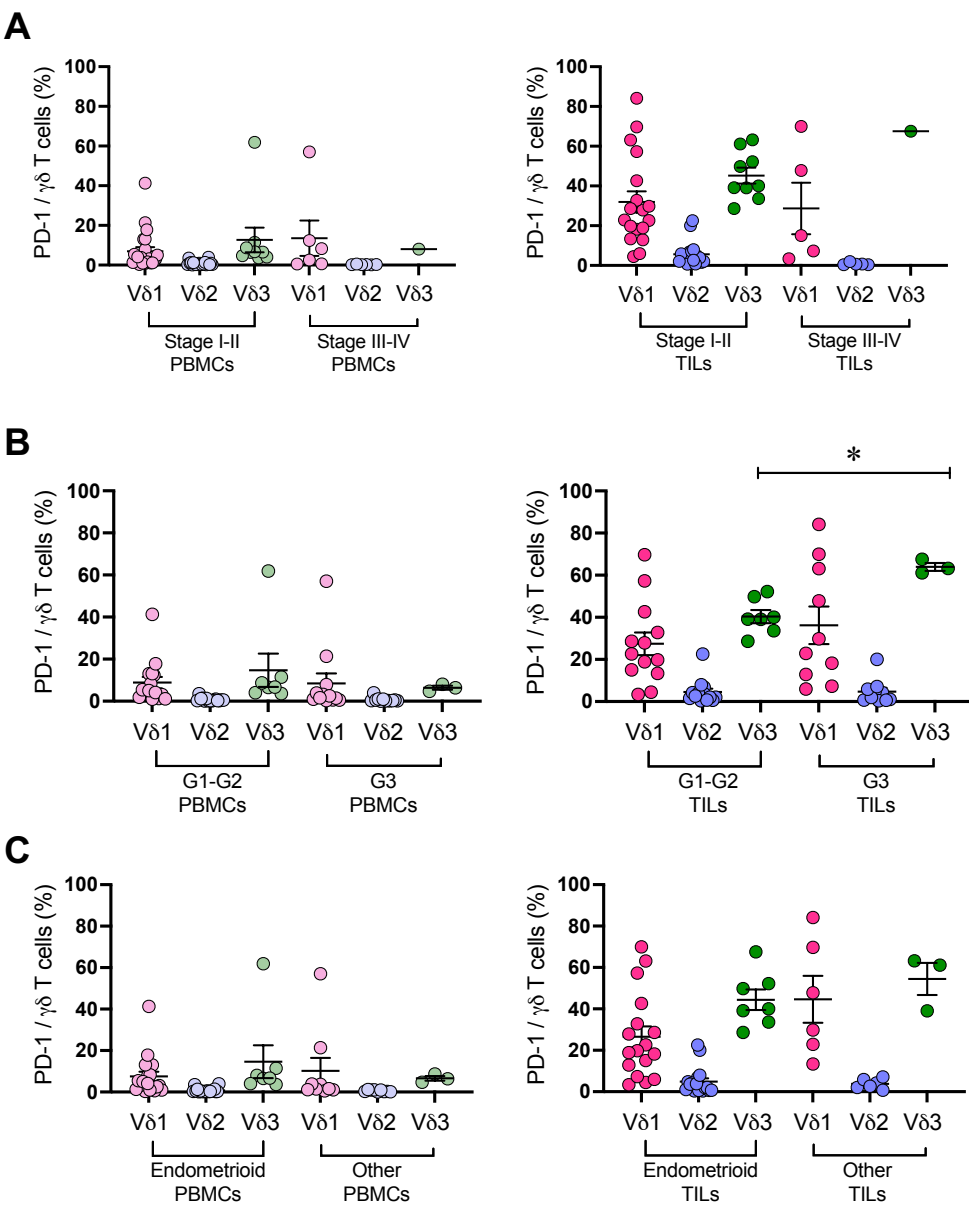
Figure 21 | Cytotoxic activity of $\gamma\delta$ T cells against autologous and heterologous tumor cells. Frequencies (left panel) and MFI (right panel) of CD107a⁺ cells on tumor-infiltrating Vδ1 (A) and Vδ2 T cells (B) against autologous and heterologous tumor cells blocking PD-1 alone or in combination with TIGIT (n=8). Frequencies (left panel) and MFI (right panel) of CD107a⁺ cells on circulating Vδ1 (C) and Vδ2 T cells (D) from EC patients against autologous and heterologous tumor cells blocking PD-1 alone or in combination with TIGIT (n=5). Frequencies are represented as mean±SEM. Wilcoxon test: * $P\leq0.05$; ** $P\leq0.01$

Thus, targeting PD-1 on $\gamma\delta$ T cells shows promising results in the context of EC, in fact, even in an autologous setting blocking PD-1 on tumor-infiltrating $\gamma\delta$ T cells increases their degranulation capacity. Importantly, this is relevant already in the early stages of the disease, indeed, the majority of the analyzed patients are characterized by an early stage of the disease (stage I-II), with only 2 patients having an advanced disease stage (stage III-IV). Considering this, we evaluated the clinical impact of $\gamma\delta$ T cells and PD-1 expressed in this population for EC.

Clinical relevance of $\gamma\delta$ T cells and PD-1 in EC

In the first section of the Results, we have already described how $\gamma\delta$ T cells tumor infiltration is more pronounced in patients with advanced-stages disease, and it remains independent of other analyzed clinical features, such as the tumor grade and the histological type. Considering the high expression of PD-1, we assessed whether its expression is related to

any patients' characteristics. Interestingly, PD-1 expression on all circulating and tumor-infiltrating $\gamma\delta$ T subsets appears to be unrelated to any clinical feature, including the disease stage (**Figure 22A**), the tumor grade (**Figure 22B**), the histological type (**Figure 22C**) and the microsatellite stability/instability (**Figure 22D**). Even if PD-1 is highly expressed on tumor-infiltrating V δ 3 T cells from EC patients with grade 3 (**Figure 22B**, right panel), suggesting a potential relationship with high tumor grade, however, confirmation of this relationship would necessitate analysis in a larger cohort.



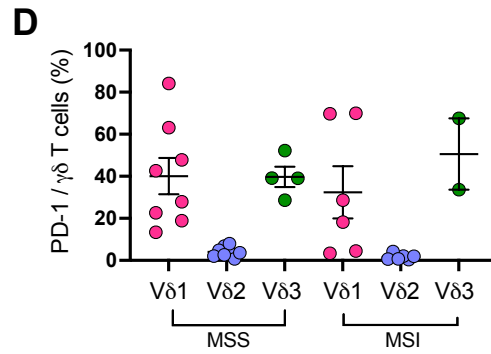


Figure 22 | Frequencies of PD-1 on $\gamma\delta$ T cells in EC patients according to disease stage, grading, histological type and microsatellite instability.

Frequencies of PD-1 on V δ 1, V δ 2 and V δ 3 subpopulations in PB (left panel) and in the tumor (right panel) stratifying patients based on disease stage (A), grading (B), histological type (C) and microsatellite instability (D) ($n=1-22$ per group). Frequencies are represented as mean $\pm$ SEM. Unpaired t -test: * $P\leq 0.05$

Thus, any patient with different clinical profiles might potentially benefit from anti-PD-1 therapy. In fact, targeting PD-1 could be considered even in early stage-cases due to its pronounced expression. Particularly, patients in advanced stages of the disease, typically with poor responses to standard treatments, may gain advantages, given their high PD-1 expression in the tumor-infiltrating $\gamma\delta$ T cells. In addition, targeting PD-1 on $\gamma\delta$ T cells could be relevant, as the expression of PD-1 on V δ 1 T cells positively correlates with the tumor size (Figure 23), thereby underlining its involvement in EC progression.

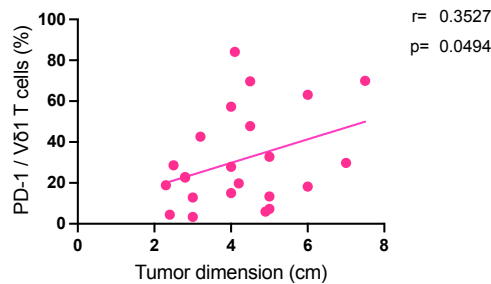


Figure 23 | Correlation of PD-1 with the tumor dimension.

Correlation (Pearson) between the expression of PD-1 on V δ 1 T cells and the tumor dimension (cm) ($n=22$).

In order to further clarify the effective role of $\gamma\delta$ T cells and PD-1 in EC progression, we assessed their impact on OS of EC patients. Due to the limited number of analyzed patients in our cohort for such analysis, we took advantage of the public dataset of Uterine Corpus Endometrial Cancer (UCEC) published by TCGA (224). Using the Survival Genie web platform (225), we observed that high expression of *TRDV1* gene, defining V δ 1 TCR-cell profile, is associated with better OS of EC patients (Figure 24A), whereas the

expression of *TRDV2* gene (identifying V δ 2 T cells) does not have any impact on patients' OS (**Figure 24B**). Importantly, when we assessed the impact of the co-expression *TRDV1-PDCD1* and *TRDV2-PDCD1* genes, we obtained that a high frequency of both $\gamma\delta$ T subsets expressing PD-1 is associated with a better OS (**Figure 24C**). This highlights the hypothesis of tumor-reactive $\gamma\delta$ T cells expressing PD-1 and their potential role in controlling tumor growth in the absence of inhibitory ligands.

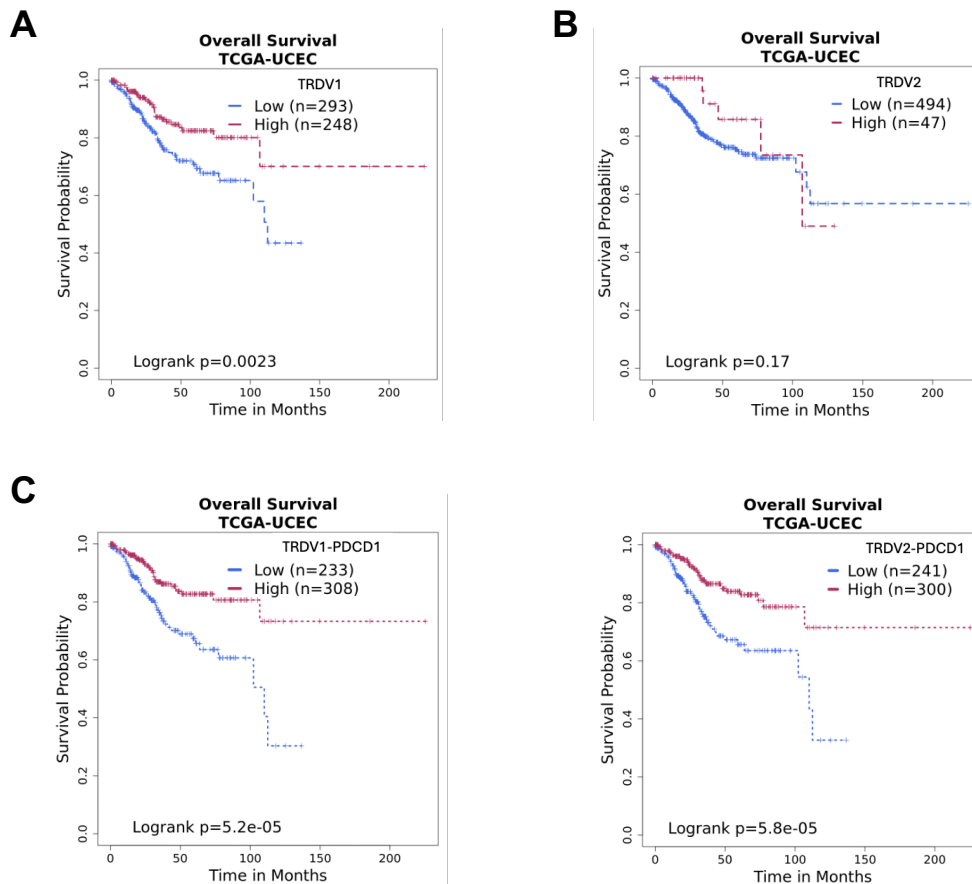


Figure 24 | Prognostic relevance of $\gamma\delta$ T cells and PD-1 in EC.

Kaplan-Meier survival analysis of EC patients from UCEC cohort of TCGA by Survival Genie web platform according to *TRDV1* (A) and *TRDV2* (B) gene expression and *TRDV1-PDCD1* (left panel) and *TRDV2-PDCD1* (right panel) gene sets (C).

As the results obtained so far are mainly based on tumor-infiltrating $\gamma\delta$ T cells, we wondered if any of them could be reflected in circulating $\gamma\delta$ T cells, identifying possible biomarkers in the PB. In this context, we previously reported that intrahepatic CD69⁺ V δ 1 T cells could recirculate in the blood of patients with colorectal liver metastasis and that their frequency correlated with a better clinical outcome (109). Interestingly, in EC there was a correlation between tumor-infiltrating and circulating CD69⁺ V δ 1 T cells (**Figure 25A**). Moreover, we observed a correlation of both the frequency of V δ 1 T cells on CD3⁺ cells

(**Figure 25B**) and the expression of PD-1 on V δ 1 T cells (**Figure 25C**) between cells from the tumor and the circulation. This suggests that, similar to what we have observed in liver metastasis from colorectal cancer, there is a relationship between tumor-infiltrating and circulating $\gamma\delta$ T cells, suggesting their prognostic role in EC.

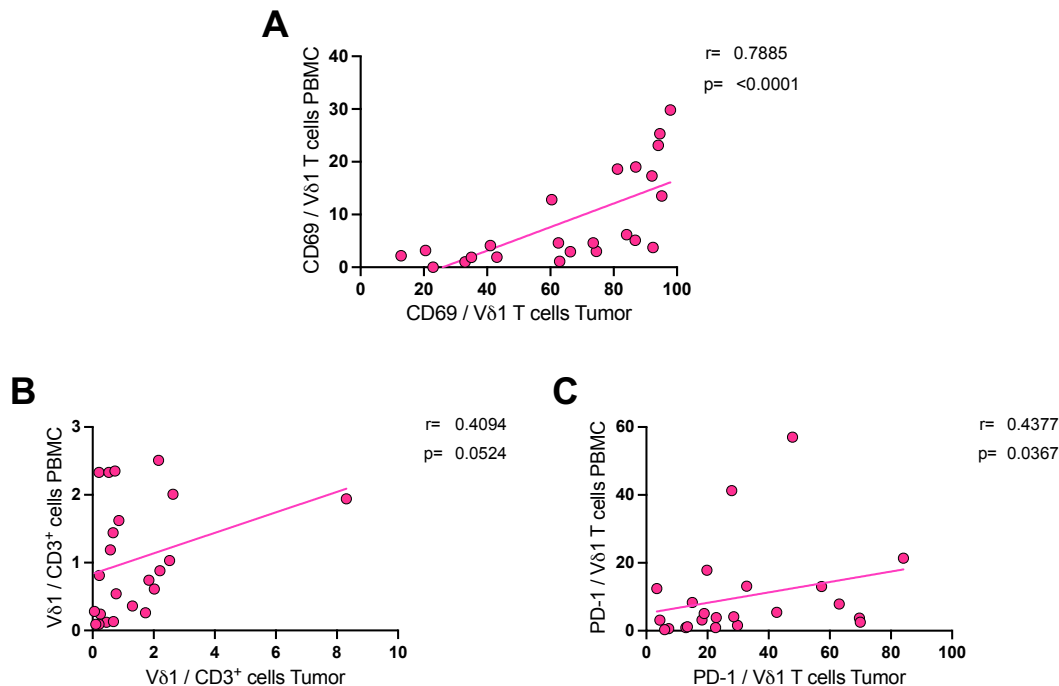


Figure 25 | Correlations between tumor-infiltrating and circulating V δ 1 T cells.

(**A**) Correlation (Spearman) between tumor-infiltrating and circulating CD69⁺ V δ 1 T cells ($n=22$). (**B**) Correlation (Spearman) of the frequency of V δ 1 T cells between the tumor and the circulation ($n=22$). (**C**) Correlation (Spearman) of the expression of PD-1 on V δ 1 T cells between the tumor and the circulation ($n=22$).

$\gamma\delta$ T cells predict the response to anti-PD-1 therapy in EC

We evidenced the possibility of targeting PD-1 on $\gamma\delta$ T cells through *in vitro* experiments only, so we wondered which is the effect of ICIs against PD-1 *in vivo*. To answer this question, we took advantage of a public dataset of scRNA-seq performed on EC patients treated with pembrolizumab (anti-PD-1 drug) in a phase II trial (NCT02899793) (226). Longitudinal analysis of PBMCs was performed before anti-PD-1 therapy and post-treatment (at day 21 and 42) (**Figure 26**). The response to pembrolizumab was assessed by RECIST 1.1 criteria (227) and patients with a $\geq 30\%$ decrease in tumor size were defined as responders (226). For a total of 24 analyzed EC patients, 10 were classified as nonresponders and 14 as responders.

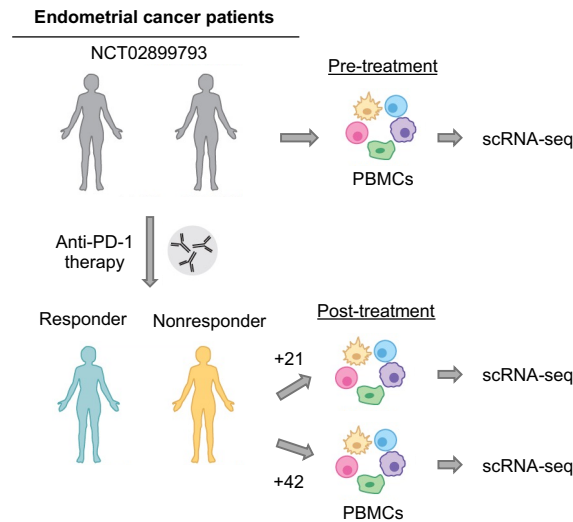
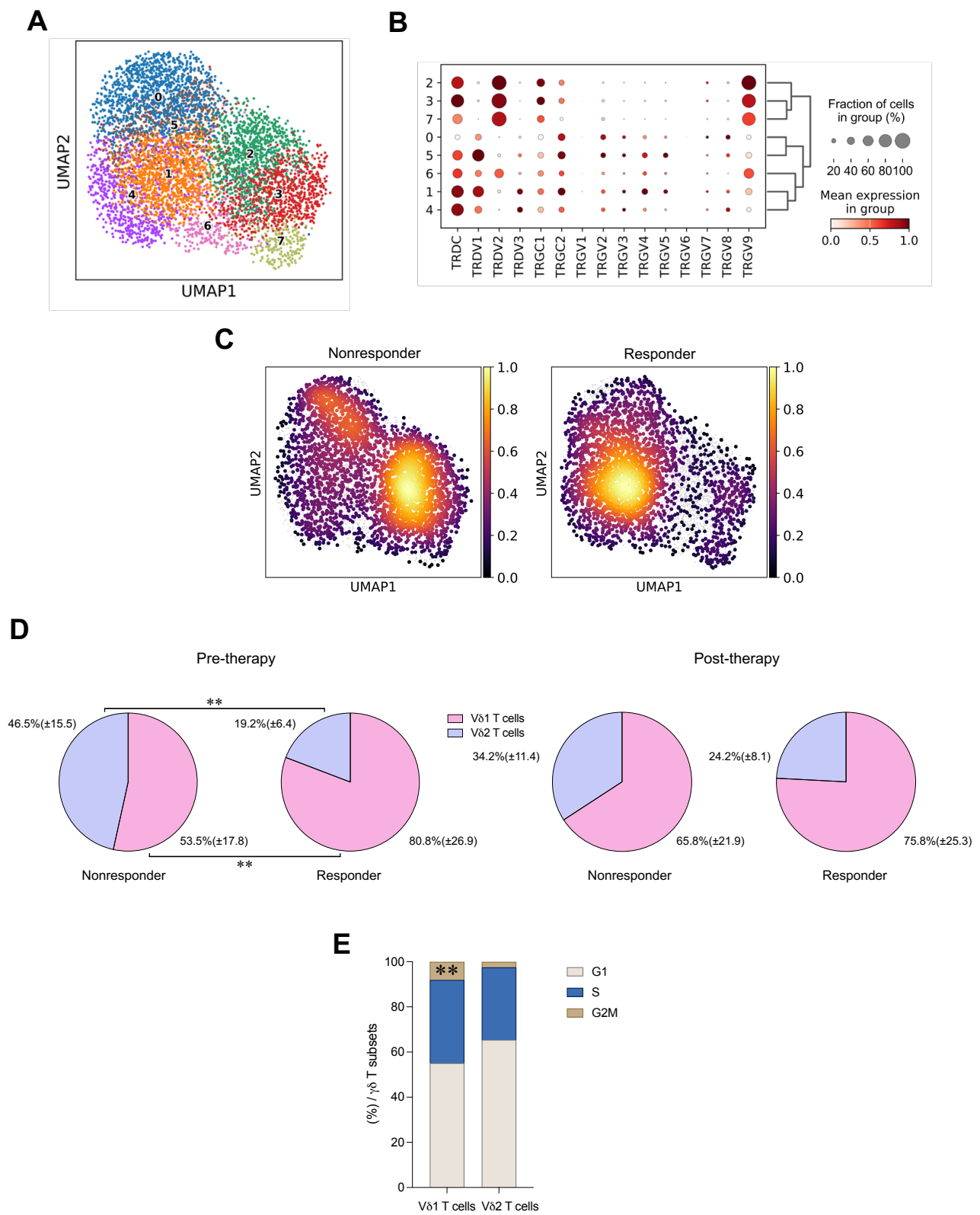


Figure 26 | Schematic representation of samples collection for scRNA-seq.

PB from EC patients enrolled in the clinical trial NCT02899793 was collected before and after the therapy (at day 21 and day 42) with pembrolizumab (anti-PD-1). Based on the response to the therapy patients were classified as responder or nonresponder. Figure adapted from (226).

As the type of scRNA-seq performed was a 5' sequencing, we were able to identify specific $\gamma\delta$ TCR expressing cells, which were subsequently re-clustered (**Figure 27A**). Based on the canonical genes expressed by the $\gamma\delta$ TCR we distinguished among the main subpopulations of $\gamma\delta$ T cells (**Figure 27B**). So, V δ 1, V δ 2 and V δ 3 T subsets were annotated based on the expression of *TRDV1*, *TRDV2* and *TRDV3* genes, respectively. As expected, *TRDV2* was found mainly expressed with the TCR γ -chain *TRGV9*, while *TRDV1* and *TRDV3* showed variable TCR γ -chains (**Figure 27B**). As V δ 3 T cells constitute only a minority among $\gamma\delta$ T cells, we focused on the main V δ 1 and V δ 2 T subsets. Interestingly, assessing the distribution of nonresponder and responder patients on $\gamma\delta$ T cells we observed a distinct separation (**Figure 27C**), suggesting that $\gamma\delta$ T cells are different in these two groups of patients. In accordance, already before the therapy nonresponders show that $\gamma\delta$ T subsets are equally distributed between V δ 1 and V δ 2 T cells, while responders show a predominance of V δ 1 T cells (**Figure 27D**, left panel). This difference was lost after the treatment, as $\gamma\delta$ T cells from both groups of patients are composed mainly of V δ 1 T cells (**Figure 27D**, right panel). The higher frequency of V δ 1 T cells in responder patients is associated with a higher proliferative capacity of V δ 1 T cells compared with V δ 2 T cells, as they show a higher frequency of cells in the G2M phase (**Figure 27E**). When we compared the different time points, we observed that the frequency changes were due to the first time point after therapy (at day 21). In fact, V δ 1 T cells increased in nonresponder patients at day 21 and then their frequency reduced at levels similar to the pre-therapy (**Figure 27F**, upper

panel). Conversely, only a not significant decrease of Vδ1 T cells was observed at day 21 in responder patients (**Figure 27F**, lower panel). Thus, indicating that PD-1 blockade affects $\gamma\delta$ T subsets inducing changes in their distribution and that the features of $\gamma\delta$ T cells already before the therapy are important in determining the response to the therapy or not.



F

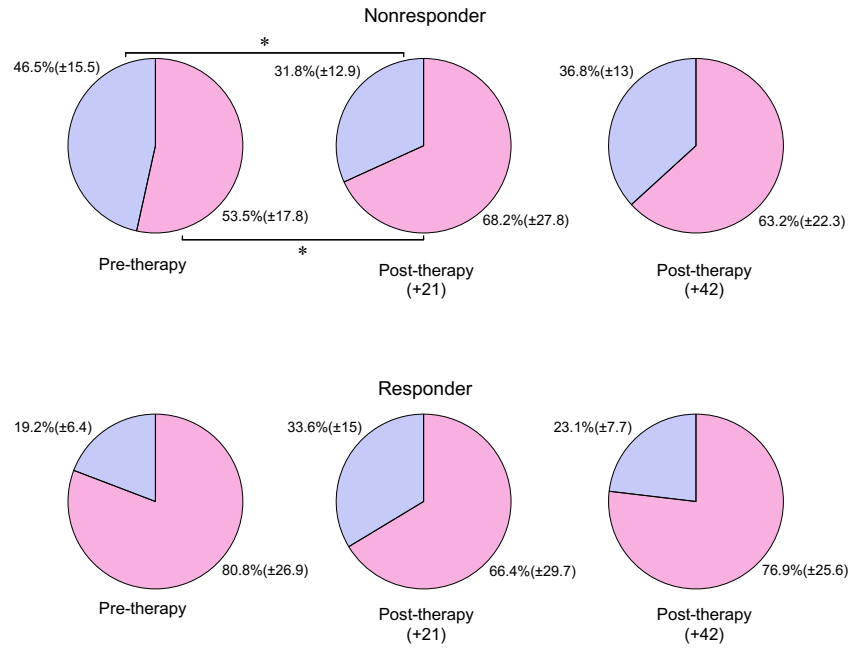


Figure 27 | Vδ1 T cells enrichment in responder EC patients before anti-PD-1 therapy.

(A) UMAP graph-based clustering projection showing the formation of 8 clusters at a resolution of 0.8, each dot corresponds to a single cell, colored according to the cell cluster. (B) Dot plot showing canonical cell markers used for the annotation of γδ T cells. (C) Density plot on UMAP graph showing the distribution of nonresponder (left panel) and responder (right panel) patients. (D) Pie charts showing the frequency of Vδ1 and Vδ2 subpopulations among total γδ T cells in PB of nonresponder and responder patients before (left panel) and after PD-1 blockade (right panel) ($n=9$). (E) Bar plot illustrating the relative frequencies of specific cell cycle phase G1, G2M, and S distribution in Vδ1 and Vδ2 T subsets before the therapy. (F) Pie charts showing the frequency of Vδ1 and Vδ2 subpopulations among total γδ T cells in PB of nonresponder (upper panel) and responder (lower panel) patients before (pre-therapy) and after PD-1 blockade (post-therapy) differentiating the two time points analyzed (day 21 and day 42 after treatment) ($n=5-9$). Frequencies are represented as mean±SEM. Unpaired t-test and Wilcoxon test: * $P \leq 0.05$; ** $P \leq 0.01$

The differences in the frequency of Vδ1 T cells induced by anti-PD-1 therapy are reflected also in their activation. Accordingly, Vδ1 T cells from responder patients at the first time point after therapy (at day 21) show the highest number of DEGs, with few DEGs shared with Vδ1 T cells before the therapy and at day 42 after the therapy, while Vδ1 T cells at the second time point are more similar to the cells before the treatment, suggesting that the effect of anti-PD-1 therapy is transient (**Figure 28A**). Vδ1 T cells upon PD-1 blockade showed the upregulation of different pathways related to their activation, including T cell receptor signaling pathway, NK mediated cytotoxicity and PD-L1 expression and PD-1 checkpoint pathway in cancer, in addition to cell adhesion molecules (**Figure 28B**). Comparing Vδ1 T cells between responder and nonresponder patients, before and at the first time point after therapy, we observed the upregulation of DEGs which indicates an activation in responder patients (**Figure 28C**). Specifically, before anti-PD-1 therapy, Vδ1 T

cells from responder patients already show the expression of genes related to cytotoxicity (*CD160*, *GZMM*, *PRF1*, *KLRB1*), interferon-induced signaling pathway (*IFITM1*, *IFITM2*) and T cell receptor signaling (*CD3D*, *CD3G*, *PTPRC*, *TRDV1*) (**Figure 28C**, left panel). The activation upon PD-1 blockade is observed with the upregulation of *CD69*, *KLRF1* (Nkp80), *PTPRC*, *IFITM2*, *GNLY*, *GZMK*, *KLRC2* (NKG2C) and *KLRB1* (CD161), in addition to genes related to cell adhesion (*CCL5*, *CCL3L1*) (**Figure 28C**, right panel).

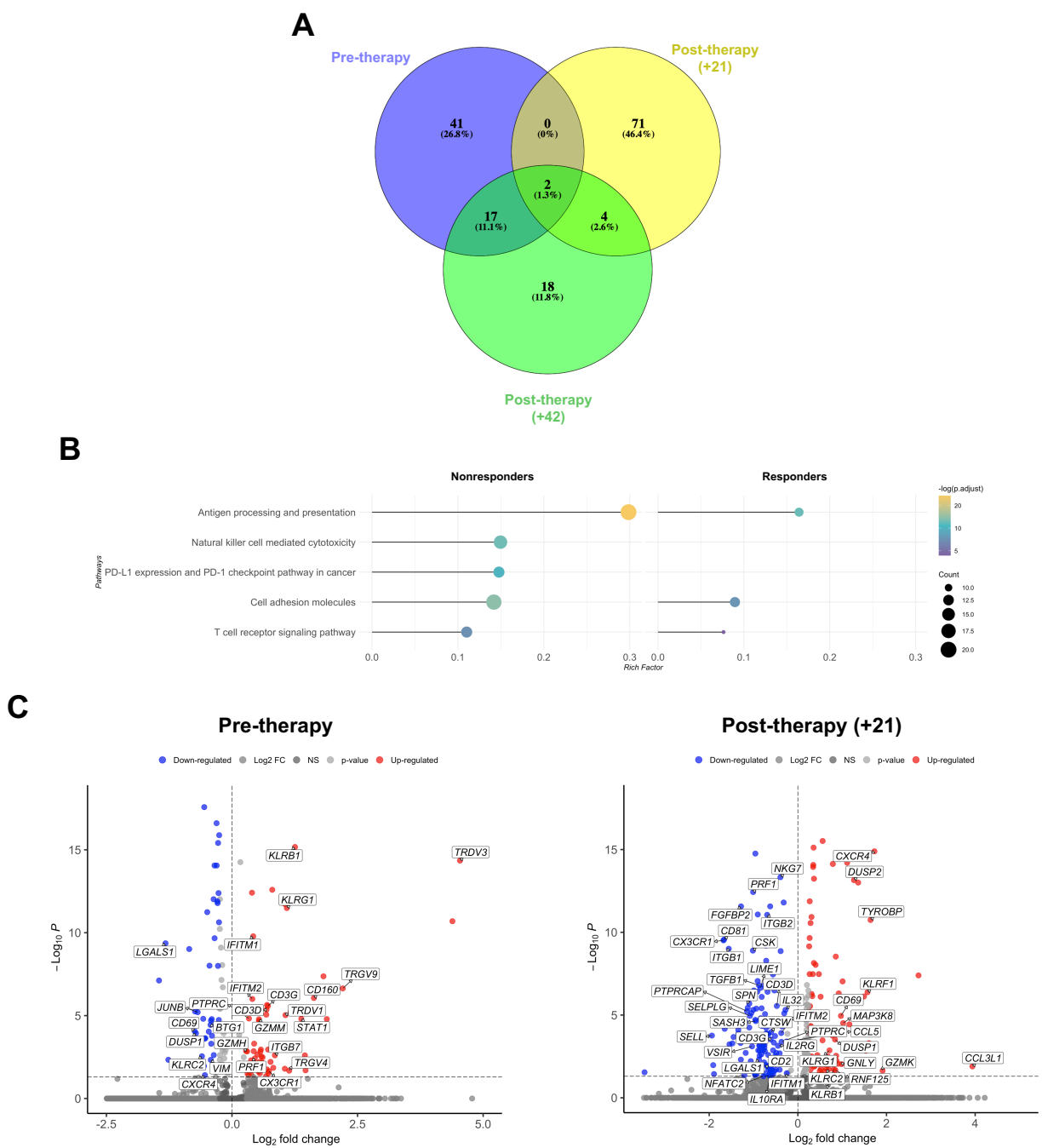


Figure 28 | Activation of $\gamma\delta$ T cells upon PD-1 blockade.

(A) Venn diagram illustrating the DEGs upregulated in V δ 1 T cells from responder patients detected among the different time points. **(B)** Pathways upregulated in V δ 1 T cells after PD-1 therapy (at day 21) compared before the treatment obtained with Gene Ontology. **(C)** Volcano plot showing the DEGs between V δ 1 T cells from responder and nonresponder patients before (left panel) and after therapy at day 21 (right panel). Venn diagram created through “Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams.” <https://bioinfogp.cnb.csic.es/tools/venny/index.html>.

These data highlighted the significance of $\gamma\delta$ T cells, specifically V δ 1 T cells, in the context of PD-1 blockade therapy in EC patients. Their presence in the PB, even prior to the treatment, may serve as a predictive indicator of the treatment response to the therapy. Moreover, the activation of V δ 1 T cells following treatment highlights the impact of anti-PD-1 therapy on these cells.

Stage I HGSOC features an immunosuppressive microenvironment dominated by regulatory T cells

A scRNA-seq atlas of the stage I HGSOC TME

To comprehensively characterize the TME of early-stage HGSOC, considering that it is a rare disease so we could not reach a huge cohort to perform a flow cytometric immunological characterization, we conducted a 3' scRNA-seq profiling on cells isolated from matched tumor samples and PBMCs obtained from two early-stage (stage I) patients. Flow cytometry analysis revealed that the frequency of CD45⁺ immune cells in the tumor is 40% for patient 1 (P1) and 17% for patient 2 (P2), as shown in the gating strategy in **Figure 29**. So, due to a relatively low immune infiltrate, scRNA-seq analysis was performed on FACS-sorted viable tumor-associated CD45⁺ immune cells and their CD45⁻ non-immune/tumor counterpart.

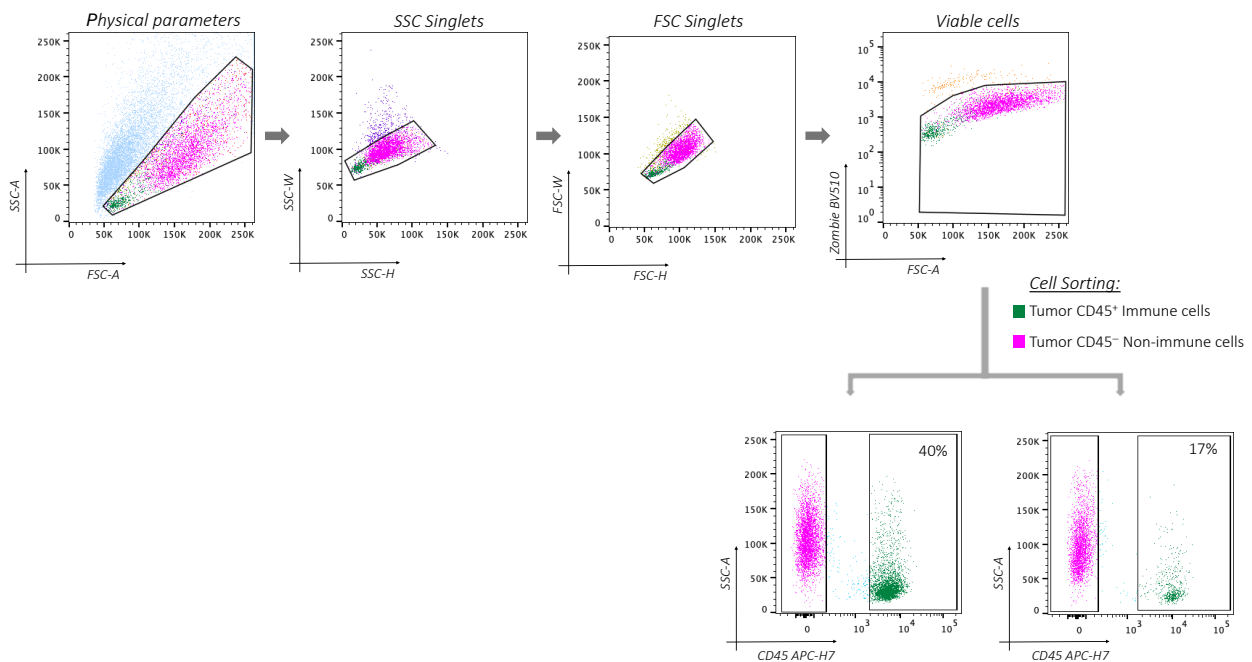


Figure 29 | Gating strategy for the FACS-sorting of viable CD45⁺ and CD45⁻ cells from the tumor of stage I HGSOC patients.

Representative flow cytometry dot plots showing the frequency of CD45⁺ cells in the two stage I HGSOC patients. The FACS-sorted CD45⁺ and CD45⁻ cells were identified among total viable cells after double events exclusion.

For the subsequent analysis we merged the expression profiles across all tissues (blood, tumor CD45⁺ cells and tumor CD45⁻ cells), and patients (**Figure 30A**). Through a macroscopic annotation, we annotated 10 major populations of immune, stromal, and epithelial cell types consistently distinguished across different tissue compartments (**Figure 30B-D**). Specifically, in HGSOC lesions, among CD45⁻ cells, there was a notable percentage

(84%) of cells with an epithelial profile compared to stromal fibroblasts and endothelial cells (**Figure 30C**). Furthermore, in the tumor, we identified CD45⁺EPCAM⁺ cells, previously reported in OC patients with aggressive tumors (228). Among immune cells, we detected CD4 and CD8 T cells, NK cells, B and plasma cells, and myeloid-originated cells including monocytes, macrophages and DCs (**Figure 30D**). Assessing the distribution of the different immune compartments we observed that HGSOC lesions exhibited a higher percentage of TILs compared to the myeloid counterpart (**Figure 30C**), with CD4 TILs being the dominant population (40%). To verify if the distribution of the various populations is due to one of the two patients, we checked the frequency of the identified populations and we observed that nearly every cell type was found in the two patients (**Figure 30E**), confirming a similar TME composition.

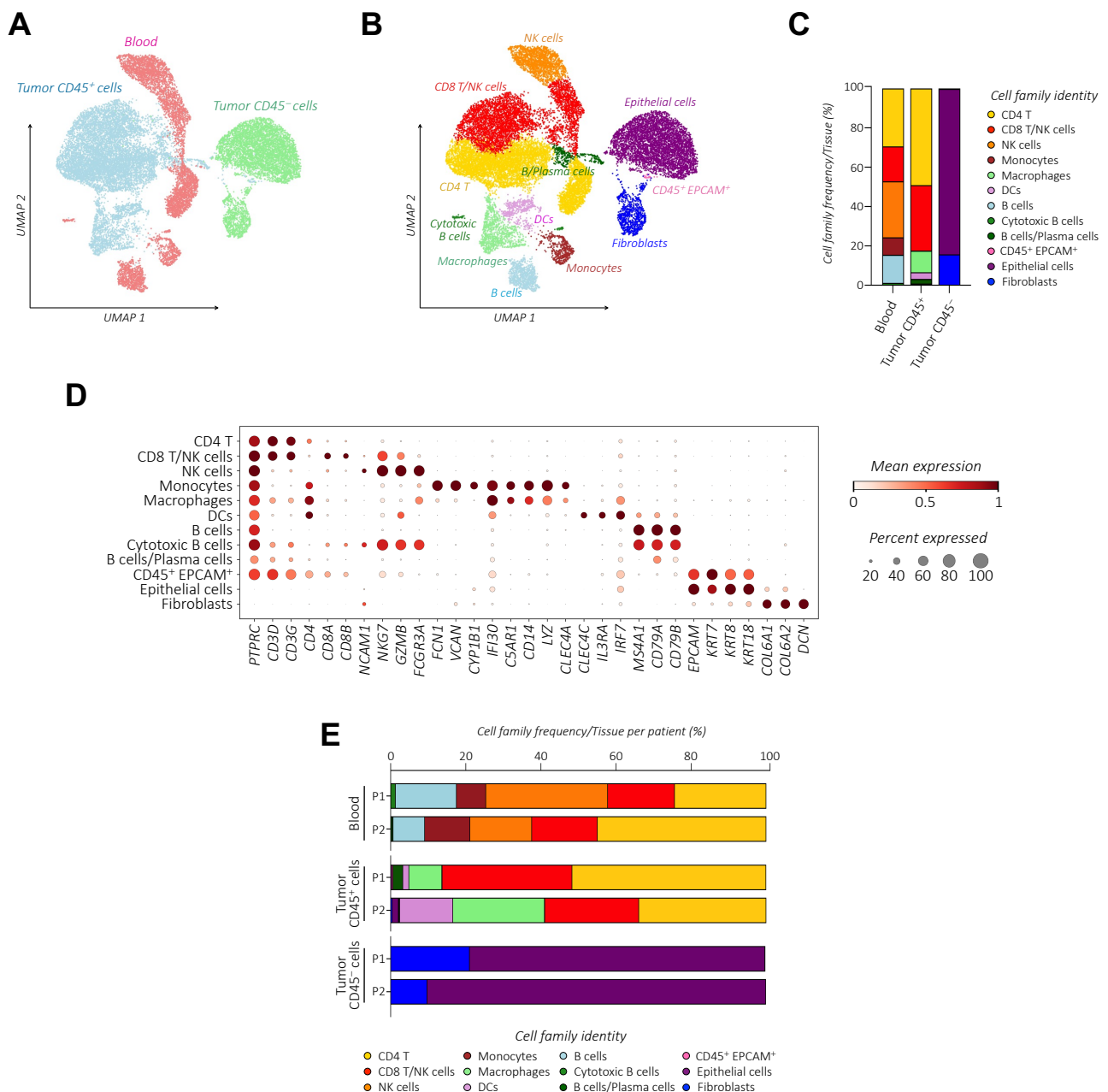


Figure 30 | scRNA-seq analysis reveals heterogeneous composition in stage I HGSOE TME. (A-B) UMAP graph showing the visualization of all cells derived from tumor-associated CD45⁺ immune cells, their CD45⁻ tumor counterpart and matched PBMCs (blood). Cells are colored by tissue origin (A) or cell type (B). (C) Bar plot showing the frequency of each cell type detected among blood and tumor-derived CD45⁺ and CD45⁻ cells. Cell numbers were normalized to the total loaded cell number for each tissue-origin. (D) Dot plot displaying the expression of canonical gene markers used for annotation of blood and tumor-associated CD45⁺ and CD45⁻ cell types. (E) Bar plot showing the frequency of different cell type distributions in blood and tumor-derived CD45⁺ and CD45⁻ cells for each patient (P1 and P2). Cell numbers were normalized to the total loaded cell number for each patient and each tissue origin.

Characterization of malignant epithelial cells

To better characterize tumor cells, CD45⁻ cells from the two patients were re-clustered and divided into 12 distinct cell clusters related to the specific cell type phenotypes (c_T0-c_T11) based on canonical markers for the identification of epithelial cells, fibroblasts and endothelial cells (Figure 31A-C).

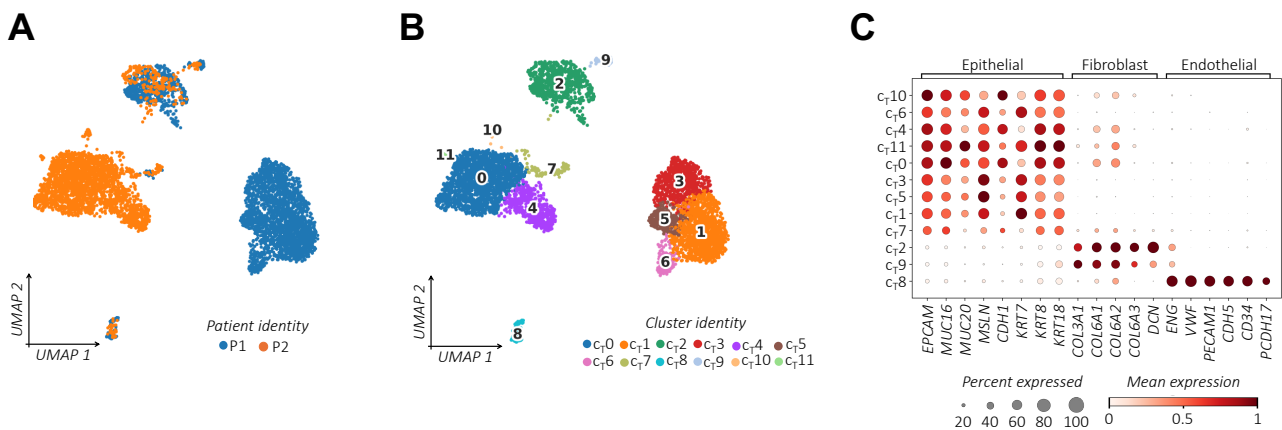
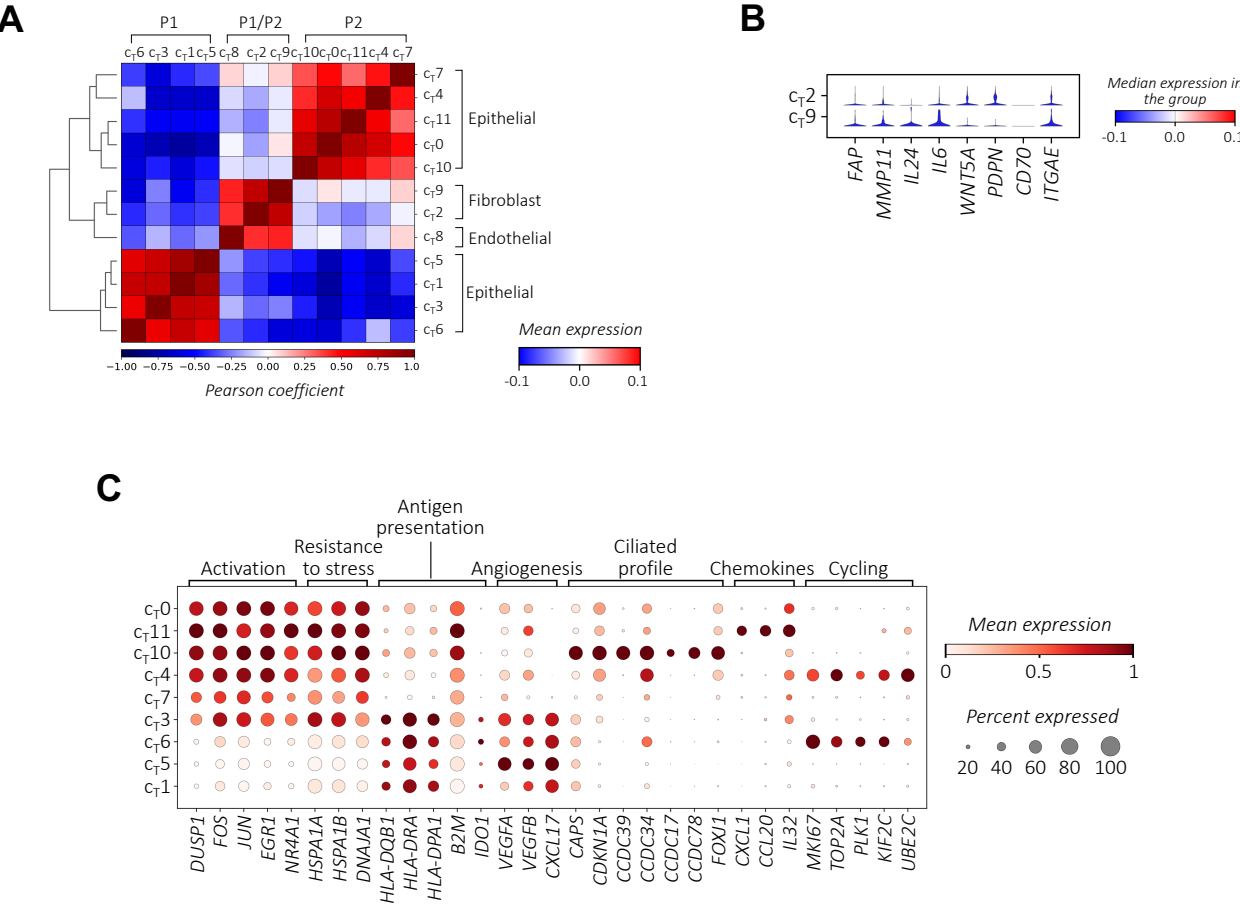


Figure 31 | Dissecting the heterogeneity of tumor cells in stage I HGSOE at single cell resolution.

(A-B) UMAP graph showing the visualization of tumor-derived CD45⁻ cells analyzed in the two stage I HGSOE lesions. Cells are colored by patient (A) or cluster identity (c_T0-c_T11) (B). (C) Dot plot displaying the expression of canonical markers used for the annotation of the different tumor-associated cell types.

The correlation analysis of CD45⁻ cells evidenced on one hand the transcriptional similarities of fibroblasts (c_T2 and c_T9) and endothelial cells (c_T8) between the two patients, and, on the other hand, patient-specific profiles for malignant epithelial cells (Figure 32A). In particular, fibroblasts exhibit modest levels of cancer-associated fibroblasts (CAFs) markers such as *FAP*, *MMP11*, *WNT5*, and the inflammatory cytokines *IL24* and *IL6* (Figure 32B). While malignant cells express many secretory markers such as *EPCAM*, cytokeratins (*KRT8/10/18*), *MUC16*, the carrier of the CA125 tumor marker, and mesothelin (*MSLN*), an OC differentiation antigen (Figure 32C). After evaluating the proliferation rates of tumor cells based on the expression of cycling genes, including *MKI67*, *TOP2A* and *UBE2C*, and

calculating a proliferation score based on these genes, c_T4 and c_T6 emerged as the most actively cycling cells in P1 and P2, respectively (**Figure 32C-D**). While the status of activation (*DUSPs*, *AP-1s*, *EGRs*, *NR4A1/2/3/4*), stress-resistance response (e.g., *HSPA1A/1B*, *DNAJA1*), and expression of antigen presentation-associated molecules (*HLA-DQB1*, *HLA-DRA*) varied among tumor cells, we found that these features were common across the different tumor cell clusters (**Figure 32C**). Nevertheless, some clusters reported specific features. Specifically, c_T5 showed signs of angiogenesis (*VEGFA/B* and *CXCL17*). Remarkably, c_T10 expressing *FOXJ1*, *CAPS*, *CDKN1A* and several coiled-coil domain containing (CCDC) proteins, essential for cilia functioning (e.g., *CCDC17/34/39/78*), showed features of ciliated cells, reminiscent of the fallopian epithelium (229). Higher expression of cytokines like *CXCL1*, *CCL20*, *IL32* and *CXCL10/11*, which support the processes of inflammation and immunoregulation, was observed in cells in c_T11 and c_T3. Finally, in c_T0 and c_T4, we found the expression of several epithelial-mesenchymal transformation (EMT)-associated genes (e.g., *MYH14*, *CDH1*, *CDH6*, *HOXD1*, *SOX5/11/17*), but not the canonical EMT markers (e.g., *TWIST1/2*, *SNAI1/2*) (**Figure 32E**). Thus, while several features are common for all tumor cell clusters with different degrees, others are unique to singular clusters, indicating distinctive roles in the HGSOC TME.



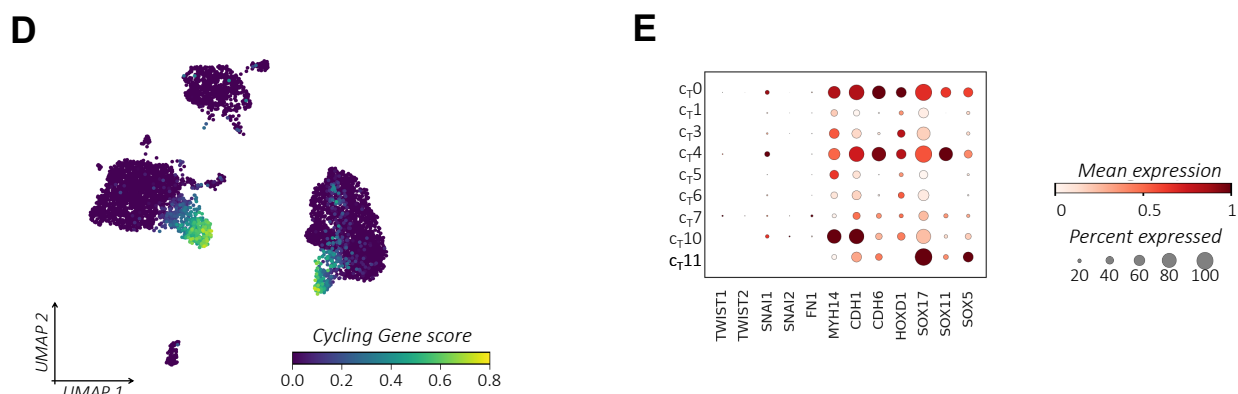


Figure 32 | Transcriptomic profiling of tumor cell subtypes in stage I HGSOV.

(A) Heatmap displaying the correlation matrix (Pearson correlation) for different tumor-associated CD45⁺ cell clusters (c_T0-c_T11). (B) Violin plot showing the expression of key selected gene markers depicting the fibroblast malignant transformation in c_T2 and c_T9. (C) Dot plot showing the expression of selected gene markers for each malignant epithelial cell cluster. (D) Feature plot showing the expression of the Cycling gene score, calculated based on the proliferation signature (expression of MKI67, TOP2A and UBE2C cycling genes). (E) Dot plot showing the expression of selected gene markers depicting the mesenchymal transformation profile among malignant epithelial cell clusters c_T0-1/3-7/10-11.

To further characterize the epithelial tumor cells, we performed pseudotime trajectory analysis for each patient. This revealed distinct branches for proliferative cells of both patients, in c_T4 and c_T6, compared to other tumor cell clusters (**Figure 33A-B**). To assess whether these separations indicate the malignant progression of tumor cells, we explored the correlation between specific tumor cell transcriptomic profiles and the prognostic prediction of OS in patients with OC. To achieve this, we used the TCGA datasets of primary advanced disease stage, employing the Survival Genie web platform. For each analysis, a Kaplan–Meier curve was plotted using the specific tumor cell cluster-associated signature based on the top 35 up-regulated DEGs. Strikingly, our findings revealed that tumor cells in c_T0 and c_T5, originating from the two different patients, showed a significant association with a worse prognosis for the disease (**Figure 33C-D**).

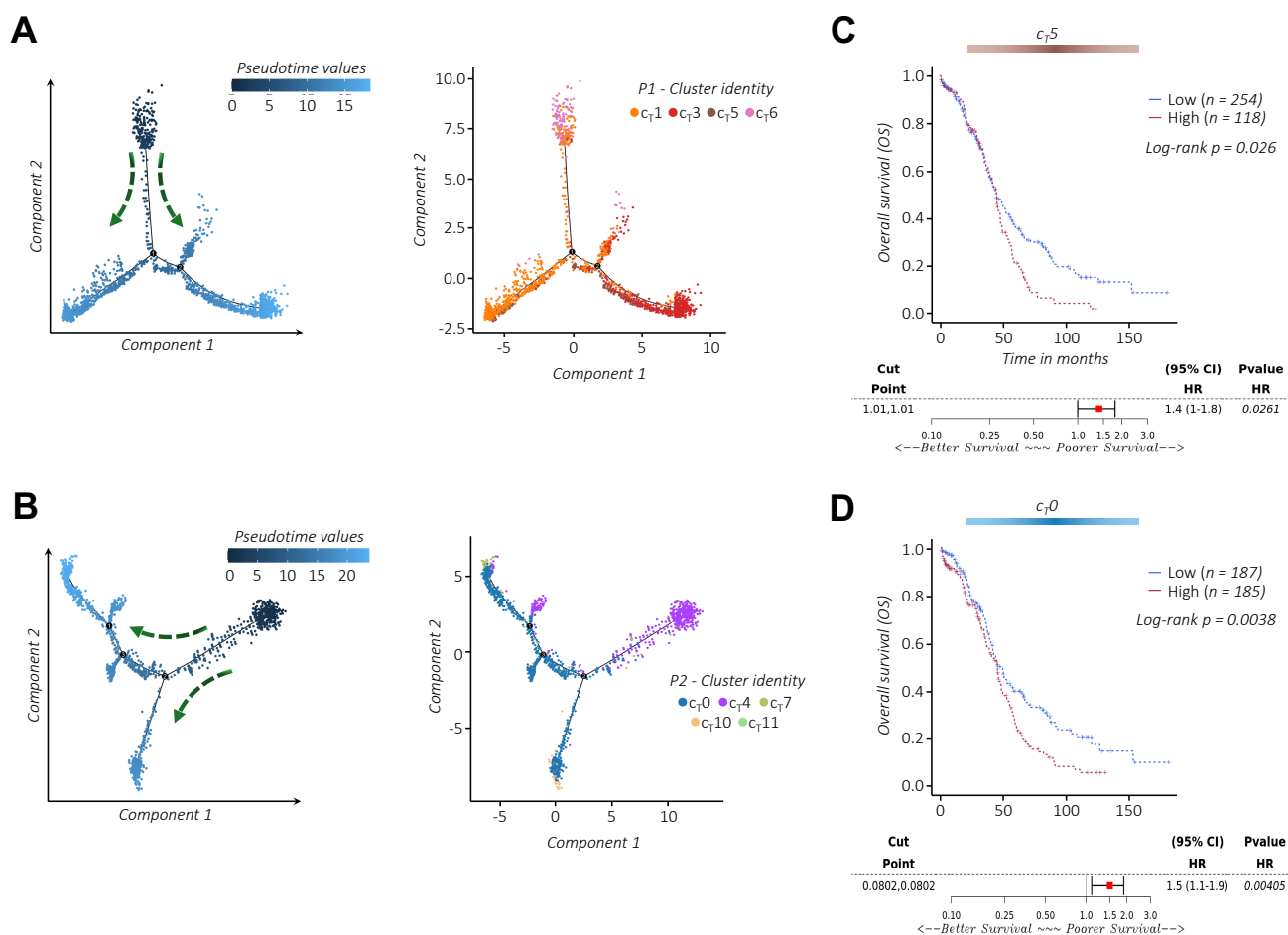


Figure 33 | Pseudotime analysis and clinical impact of tumor cell subtypes in HGSOc.

(A-B) Pseudotime trajectory of malignant epithelial cells for each patient (A, P1; B, P2). Each cell is colored by its pseudotime values from dark to light blue (left panel) or by cluster identity (right panel). The arrows indicate the starting point of the trajectory, represented by the proliferative cells in cT6 and cT4 for P1 and P2 respectively. (C-D) Kaplan-Meier curves with corresponding Forest plots for patients with advanced OC (n=372) (TCGA dataset), showing OS differences between high-risk and low-risk of cT5 (C) and cT0 (D).

Therefore, we observed that stage I HGSOc tumors in the two examined patients share cycling epithelial-secretory and ciliated profiles that exhibit a unique patient-dependent progressive transformation accompanied by concurrent features of cell activation, resistance to the stress, and the worst clinical outcome.

Enrichment of CD4 cells in the TME of stage I HGSOc

To dissect the diversity within CD4 cells, which is the dominant population among TILs, we re-clustered all the CD4 T cells derived from both blood and tissue (tumor CD45⁺ cells), resulting in 16 distinct cell clusters (c0-c15) (Figure 34A-B). c11 was excluded from further analysis due to the negative expression of both CD3 and CD4 (CD3⁻CD4⁻ cells) (Figure 34C). A hierarchical clustering analysis reflects high dissimilarities between blood and tissue

cells (**Figure 34D-E**). In fact, in the blood, CD4 T cells were predominantly composed of T_{NAIVE} cells in c0 and T_{CM} cells in c2. A small subset in c15 exhibited a $TCF7^{high}$ T_{NAIVE} profile, reminiscent of the stem CM (T_{scm}) precursors previously described in CD8 T cells (230). While c13 comprised blood resting Tregs expressing the canonical markers *FOXP3*, *CTLA4*, *TIGIT* and *IL2RA*, along with the T_{NAIVE} profile (*SELL*, *LEF1*).

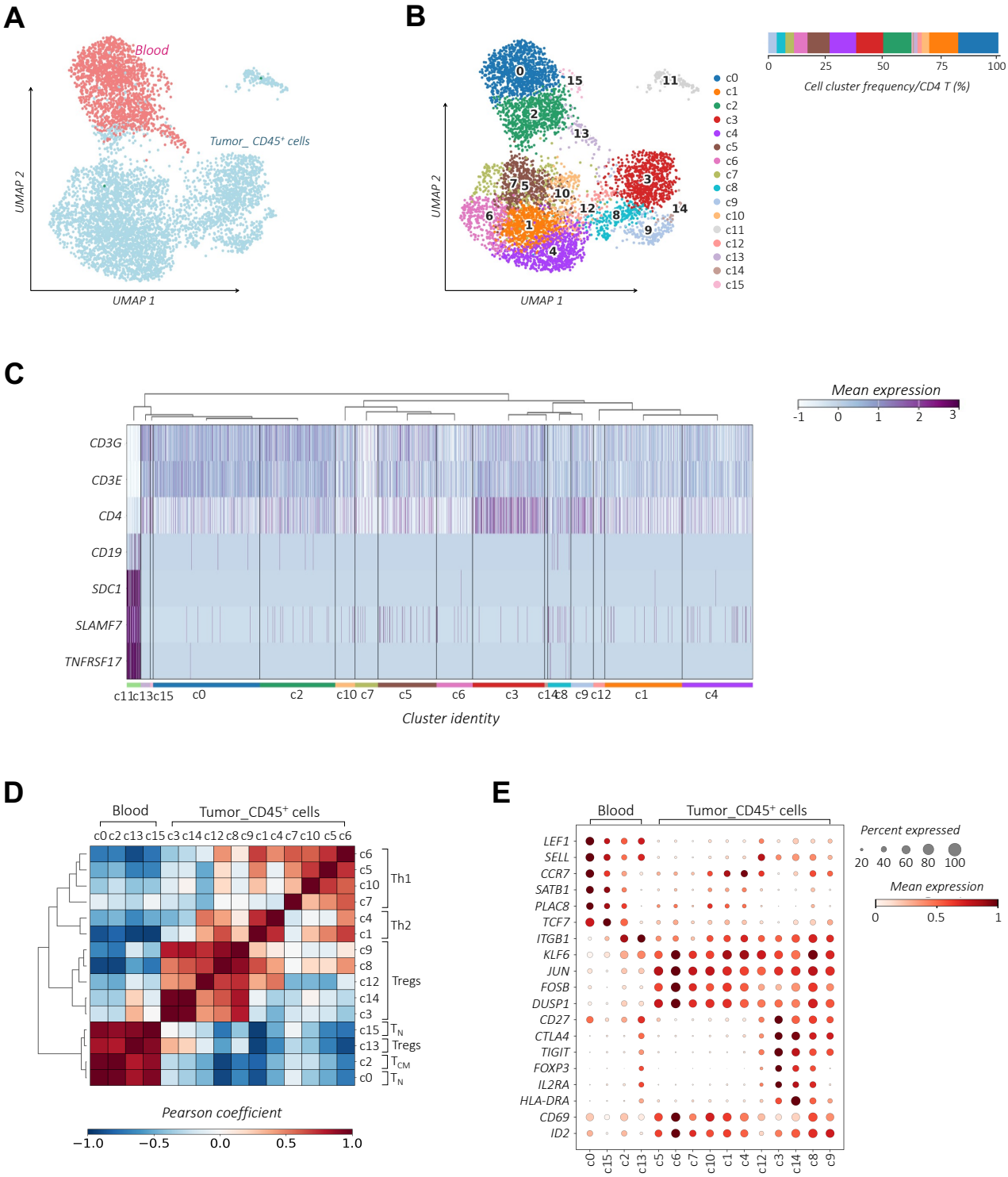


Figure 34 | Dissecting the complexity of tumor-infiltrating CD4 T lymphocytes in stage I HGSOC.

(A) UMAP graph showing the visualization of total re-clustered CD4 T cell subsets dissected by blood (red) and tumor (blue) origin. (B) UMAP graph showing the visualization (left panel) with the relative frequency distribution (right panel) of re-clustered total blood and tumor-infiltrating CD4 T cell subsets. (C) Heatmap displaying the expression of key selected gene markers used for the exclusion of CD4⁺ contaminating cells in c11, detected among re-clustered CD4 T lymphocytes. (D) Heatmap showing the correlation matrix (Pearson correlation) for different CD4 T cell subsets. (E) Dot plot showing the expression of key selected differentiation and activating gene markers distinguishing between circulating and tumor-infiltrating CD4 T cells.

In the tumor, CD4 TILs, which display a tissue-associated activating profile (*CD69*, *JUN*, *FOSB*, *DUSP1*, *ID2*, *ITGB1*), showed patterns of T helper (Th) and Treg subtypes (**Figure 34E**), with Th1 and Th2 exhibiting different effector states (**Figure 35A**). On one hand, Th1 CD4 TILs (c5-7, c10) were characterized by a higher expression of *IFNG* and *TNF* cytokines. While, on the other hand, CD4 Th2 TILs, comprising c1 and c4, were characterized by the *GATA3*⁺*CCR7*⁺*CREM*⁺ gene signature, among which cells in c4 exhibited an immunosuppressive *TGFB1*⁺ profile. Different Th1 and Th2 subsets, most notably in c1/c5/c6, share a high heat-shock protein (*HSP*^{high}) stress-responsive pattern (i.e., *HSPA1A/B*, *DNAJB1*). Moreover, both Th1 and Th2 cells in c6 and c7 displayed activated T effector (*T_{EFF}*) features with increased respiratory (*MT-CO1/3*, *MT-ND1/2/4-5*) and apoptotic regulation (*MTRNR2L1/6/8/10*) profile. In particular, Th1 cells in c6 present an early activated pattern (*EGR1/3*, *ID2*, *IER2/5*) (**Figure 35A**). Similarly, c10 manifested the IFN-induced profile (*ISG*^{high}), evidenced by the expression of *ISG15*, *IFIT13/6*, *IRF7* and *STAT1*. The increased cytotoxic profile detected in c4-c5 reflects highly differentiated *T_{EMRA}* cells (*GZMK*, *GZMA*, *CCL5*, *CST7*). The different activation status of Th CD4 TILs was also confirmed by conducted pathway analysis (Reactome, KEGG), reflecting a possible degree of immunogenicity, revealing TCR and cytokine-regulating signaling, proliferation, and multiple RNA/protein and respiratory metabolism stress-induced adaptations (**Figure 35B**).

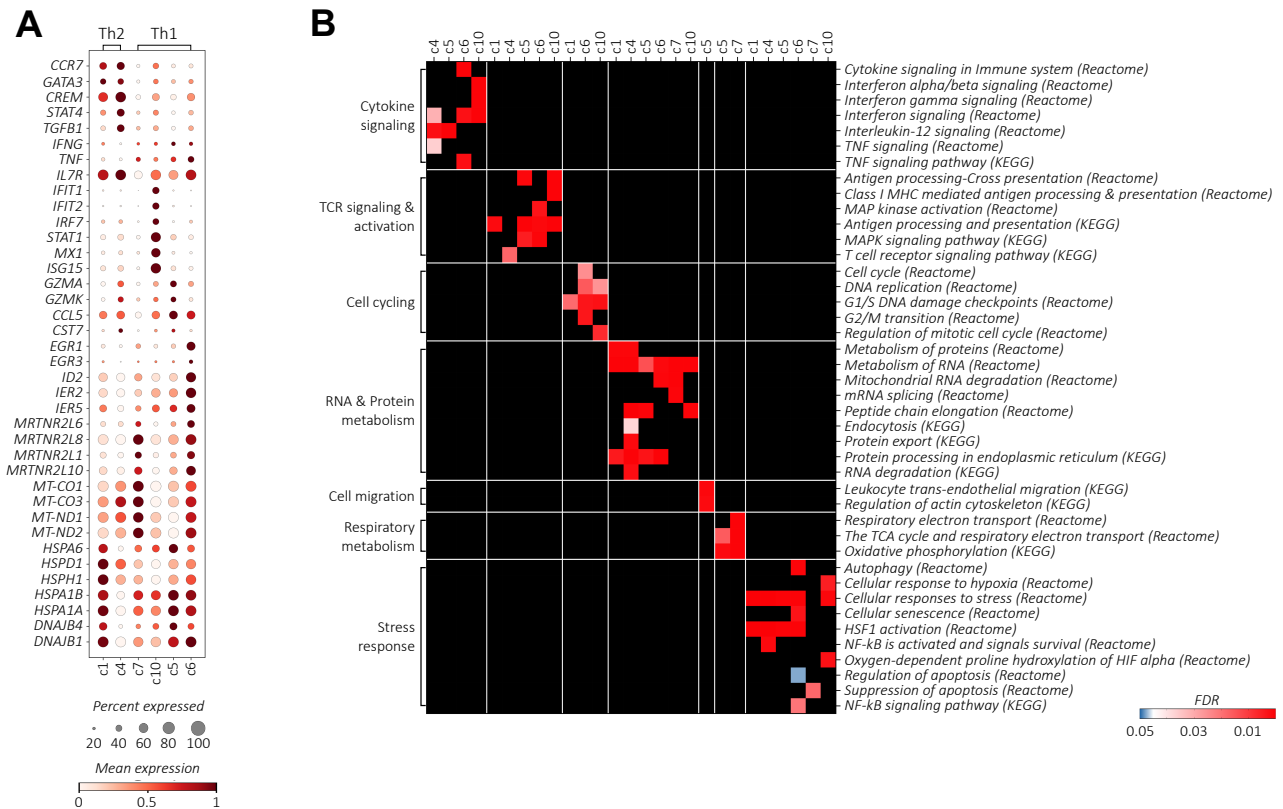


Figure 35 | Transcriptional heterogeneity of tumor-infiltrating CD4 T lymphocytes in stage I HGSOV.

(A) Dot plot showing the expression of key selected differentiation and activating gene markers distinguishing between CD4 T cells with a Th1 and Th2 profile. (B) Heatmap displaying the selection of significantly enriched Reactome and KEGG pathways with FDR values <0.05 (Reactome) or qvalue (KEGG) <0.05, identified among DEGs (Method section) in different CD4 T cell subtypes.

Tregs were identified across multiple clusters, including c3, c8-c9, c12, and c14, characterized by high transcriptional affinity based on the expression of canonical markers and correlation analysis across total CD4 TILs (Figure 34D-E). The frequency of Tregs was consistent in the two patients, reaching 30% and 40%, among total CD4 TILs, in P1 and P2, respectively (Figure 36). This highlights the presence of an immunosuppressive TME already in the early stages of HGSOV.

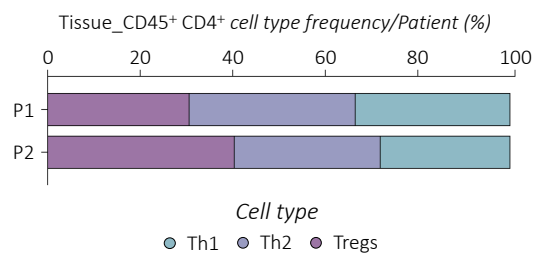
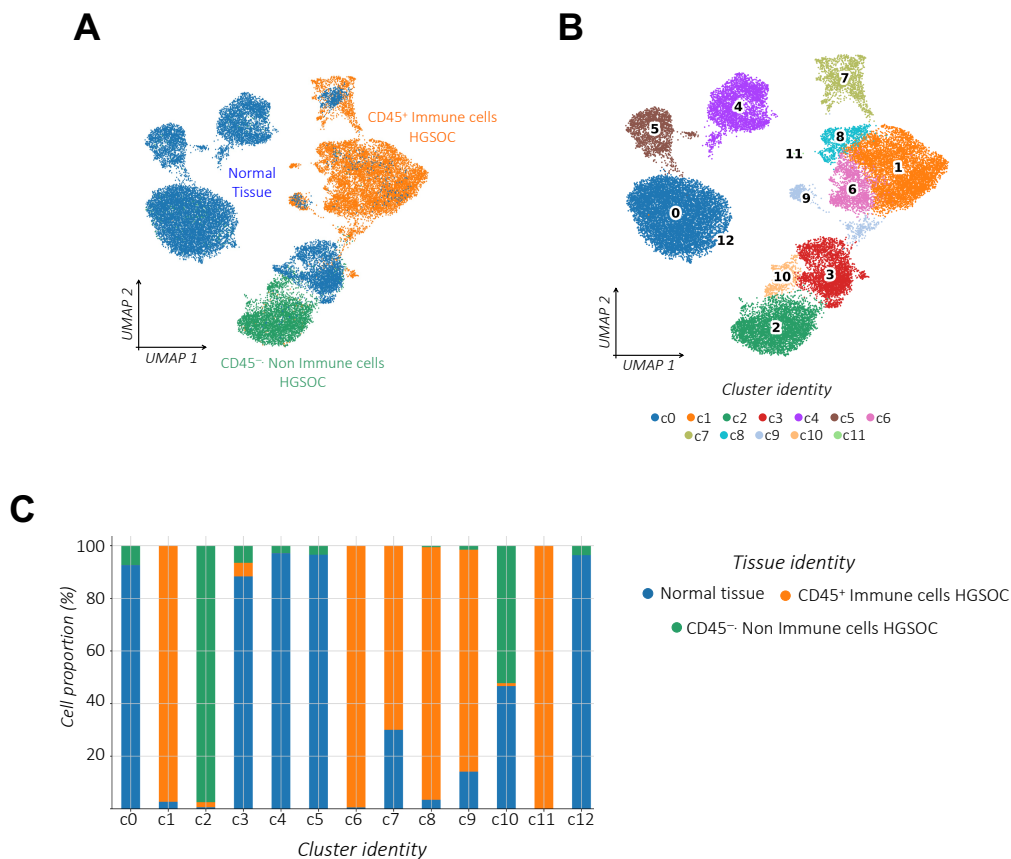


Figure 36 | Enrichment of Tregs among tumor-infiltrating CD4 T lymphocytes in stage I HGSOC.

Bar plot showing the relative frequencies of Th1, Th2, and Treg subtypes among tumor-infiltrating CD4 T lymphocytes analyzed for each patient, P1 and P2. Cell numbers were normalized to the total number of tumor-infiltrating CD4 T lymphocytes for each patient.

Dynamic remodeling of Treg cells

To deepen the role of Tregs in HGSOC TME, we initially investigated the possibility that the elevated percentage of Tregs in stage I could arise from tissue-resident Tregs already present in the healthy ovarian stroma. To address this, we integrated our dataset with a public dataset (GSE118127) of 5 healthy ovaries analyzed using the 3' 10X Genomics approach (231). From this integration, we observed as the distinct compartments (normal tissue, CD45⁺ HGSOC and CD45⁻ HGSOC) seem to be transcriptionally different (**Figure 37A**). Through the clustering of all cells, we obtained 12 clusters (**Figure 37B**), that, as already hypothesized by the UMAP visualization, were derived almost uniquely by one of the three compartments analyzed (**Figure 37C**). Using canonical markers, we identified a CD4 Treg subset (c6) exclusively originating from the tumor tissue (**Figure 37C-D**). This confirms that in stage I HGSOC, the TME favors the infiltration and enrichment of CD4 Tregs. This factor must be considered in the early stages of the disease.



D

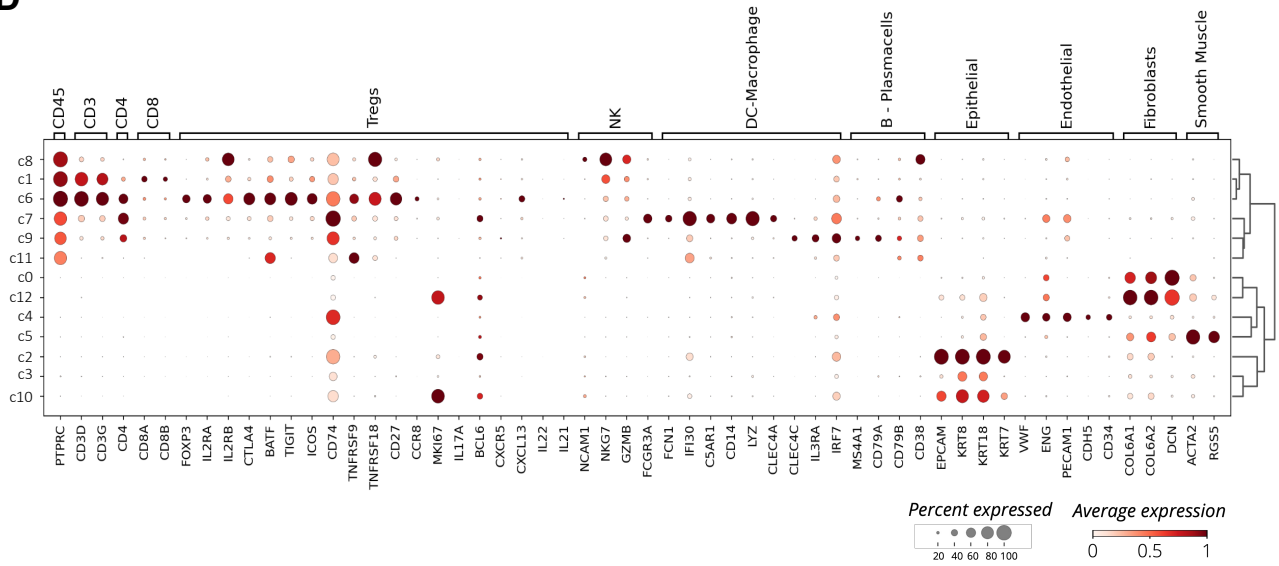


Figure 37 | Comparative assessment of immune cell profiles in healthy and stage I HGSOC ovarian tissues.

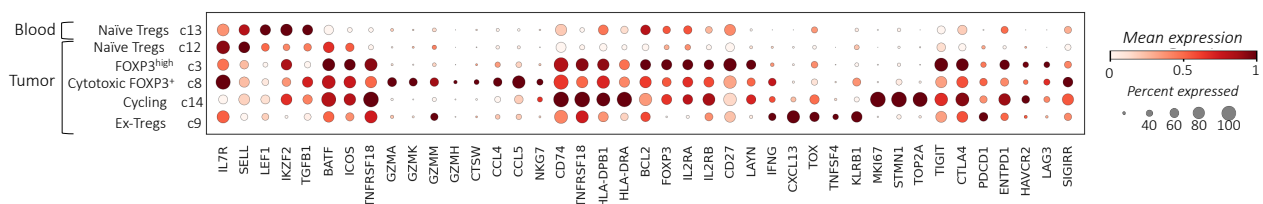
(A) UMAP graph showing the visualization of integrated healthy and stage I HGSOC scRNA-seq dataset, with cells colored by tissue origin. (B) UMAP graph showing the visualization of integrated healthy and stage I HGSOC scRNA-seq datasets with cells colored by clusters. (C) Relative frequency of the specific tissue originated cells for each cell cluster. (D) Dot plot displaying the expression of selected gene markers used for annotation of different cell types.

Going deeper into Tregs characterization, different Treg subsets in stage I HSGOC show distinct transcriptomic profiles (**Figure 38A**). Cells in c12 exhibit a $T_{NAIVE} SELL^{high}FOXP3^{low}CD25^{low}CTLA4^{low}$ phenotype, similar to blood Tregs, indicating c12 as the most naïve Treg status in the tumor. Notably, we found an increased expression of the adhesion molecule *ITGB1* in the circulating T_{NAIVE} Tregs (c13) (**Figure 34E**), implying their potential ability to migrate toward the tumor site. On the other hand, cells in c3 stand out for a hyperactive immunosuppressive $FOXP3^{high}CD25^{high}CTLA4^{high}$ profile, similar to that described in other solid tumors (232) (**Figure 38A**). High expression of the transcription factor (TF) *IKZF2* (Helios), a marker of the thymic-derived Tregs, indicates their thymic origin (233). Following observations in other tumors (232), Tregs in c3 expressed high levels of *LAYN* (Layilin), a protein linked to the motility and suppressive function of Tregs, which is related to poor prognosis in advanced stages OC within the TCGA dataset (**Figure 38B**). Moreover, cells in c3 show synergistically higher levels of ICs such as *HAVCR2* (TIM-3), *LAG3*, and *ENTPD1* (CD39), whose expression in Tregs, like *CTLA4*, associates with increased immunosuppressive activity (234) (**Figure 38A**). Conversely, they show low expression of *PDCD1* (PD-1), which, similarly to its role in conventional T cells, inhibits Tregs activity (235,236). Remarkably, the $IKZF2^{+}FOXP3^{+}CD25^{+}CTLA4^{high}$ Tregs in c14 present proliferative $MKI67^{high}$ signature with the maximal proportion of cells in the G2M phase

(**Figure 38C**). While the *IKZF2⁺FOXP3⁺CD25⁺CTLA4⁺* Treg profile in c8 was associated with increased cytotoxicity (i.e., *GZMA*, *GZMM*, *GZMK*, *GZMH*, *CTSW*, *NKG7*) and secretion of cytokines including *CCL4/5* and *TGFB1* (**Figure 38A**). Interestingly, cells in c9 show a *KLRB1^{high}FOXP3⁺CD25⁺CTLA4⁺* profile, presenting features of Treg instability and conversion to ex-Tregs. In fact, Tregs require tight control, as tipping the balance from a tolerogenic to a proinflammatory transcriptional program can lead to the loss of the Tregs fate, marked by the loss of *FOXP3* expression and functional reprogramming already observed in autoimmune and inflammatory diseases (237,238). However, there is limited understanding regarding the factors that prompt the differentiation of ex-Tregs, both generally and specifically in the context of cancer. The high transcriptional affinity of ex-Tregs cells with other detected Treg subsets in our dataset was indicated by hierarchical analysis (**Figure 35D**), and the expression of several Treg-associated genes such as *BATF*, *TIGIT*, *CTLA4*, *ICOS*, *BCL2*, *TNFRSF18* (GITR), *IL2RB*, *CD27*, *CD74*, and *HLA-DPB1* (**Figure 38A**). These cells express elevated levels of PD-1, which, in the tumor setting, has been linked to the loss of suppressive functions, and in line to our observation, increased secretion of IFN- γ . Furthermore, cells in c9 display high levels of *CXCL13*, a chemokine strongly associated with better OS in stage-III/IV OC patients, based on TCGA dataset analysis (**Figure 38D**). This suggests that Tregs in c9 not only lose their *FOXP3⁺* immunosuppressive profile, but also acquire effector functions that can control tumor aggressiveness. Notably, the prognostic benefits of *CXCL13* expression in HGSOC were linked to the PD-1 blockade therapy and organization of the tumor-associated tertiary lymphoid structures (239,240).

The Treg cytokine receptor-pattern analysis within the tumor identified the expression of *CXCR3*, *IL2RB* and *CCR4* in all differentiated Treg subsets (c3, c8, c9 and c14) (**Figure 38E**). Moreover, mature *FOXP3⁺* Tregs in the tumor express *CXCR6*, *IL21R*, *IL1R1*, *IL18R1*, and, to a lesser extent, *CCR8*. These findings are pertinent for potential therapeutic applications, as anti-CD25, anti-CCR4, and anti-CCR8 antibodies are currently being considered for Treg depletion in various tumors (234).

A



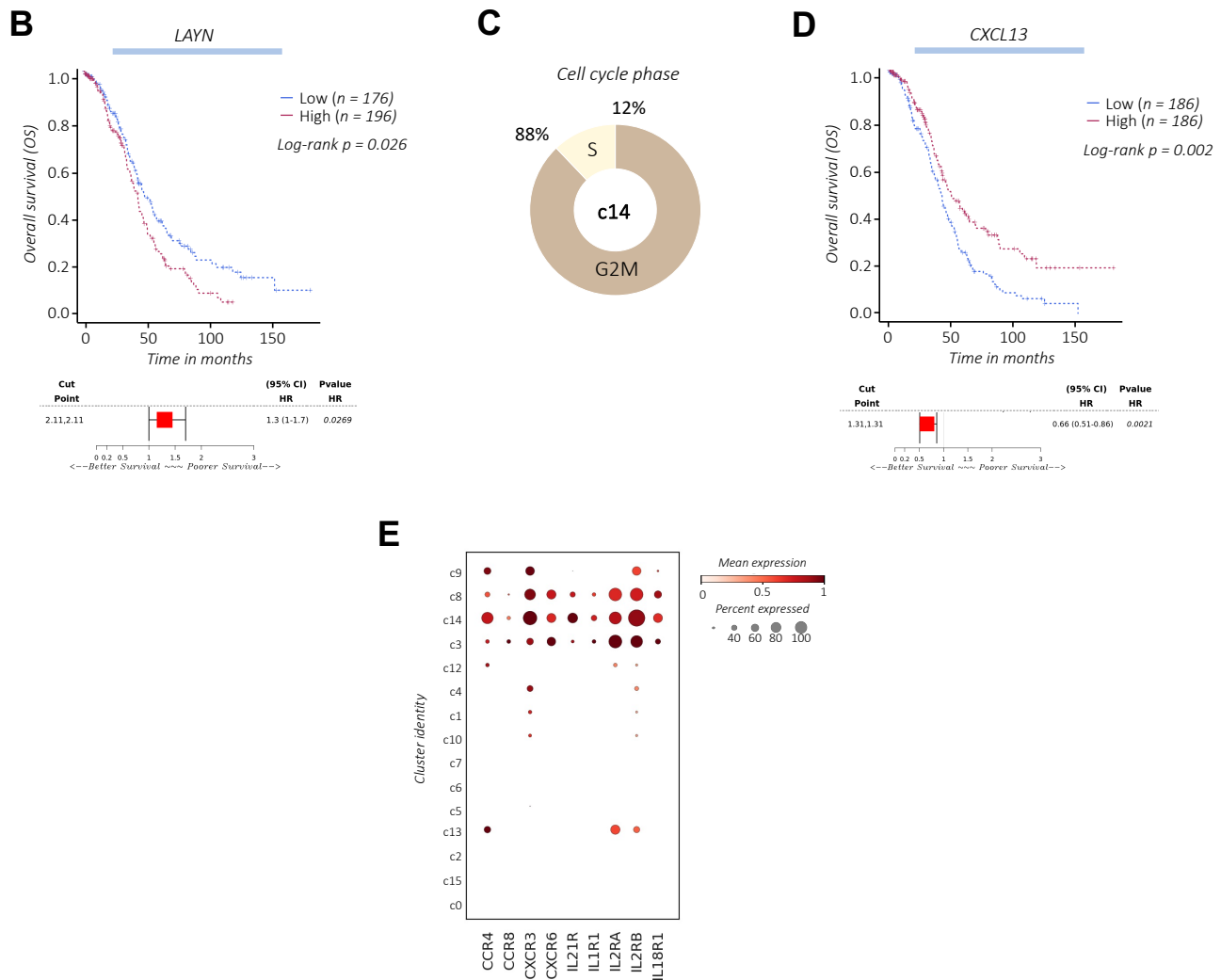


Figure 38 | Transcriptomic diversity of Tregs in stage I HGSOV.

(A) Dot plot displaying the expression of selected gene markers used for the annotation of different Treg cell subsets. (B) Kaplan-Meier curve with corresponding Forest plot for patients with advanced OC ($n=372$) (TCGA dataset), showing OS differences between high-risk and low-risk expression of *LAYN*. (C) Pie chart illustrating the relative frequency of cell cycle phase G2M and S distribution among tumor-associated proliferating Tregs in c14. (D) Kaplan-Meier curve with corresponding Forest plot for patients with advanced OC ($n=372$) (TCGA dataset), showing OS differences between high-risk and low-risk expression of *CXCL13*. (E) Dot plot displaying the expression of cytokine receptors among all CD4 T cell clusters (c0-c15), selected based on detectable gene expression in tumor-associated Tregs.

To further analyze the diversity in the transcriptional regulation of Tregs, we used the *SCENIC* tool. The top 10 active regulons (TFs), including activating and repressor gene programs detected for each Treg subset, are shown in **Figure 39**. The expression of transcriptional repressors including *ETS1* and the members of Krüppel-like (KLF) family (e.g., *KLF2*, *KLF3*) in c13 and c12 Tregs indicates naïve/repressed phenotype of Tregs. Contrarily, the elevated expression of *FOXP3* and the STAT-family in c3 confirmed the genetic stability of the Treg profile (241,242). Likewise, the enriched expression of the AP-1 TF family, exhibiting transcriptional activity in the *FOXP3* promoter, indicates the

transcriptional stability of Tregs in c8. In addition, c8 expresses *EGR1* which in turn promotes *TGFB1* expression (243), and *IRF8* which modulates the immunosuppressive activity of Tregs (244). The TF-pattern in c9 stands out for its ambiguity, which, on one hand, stabilizes the suppressor activity of Tregs (*NR3C1*, *IRF8*) and, on the other hand, supports their de-differentiation and re-specialization indicated by the expression of *TCF1* (*TCF7*), a repressor of *FOXP3*, and of *BHLHE40*, a positive regulator of *CXCL13* expression in Tregs (242,245). Overall, in stage-I HGSOc, the overplay activity of multiple TFs, interacting with *FOXP3*, the master TF of the Treg lineage, results in the activation of distinct Treg subsets.

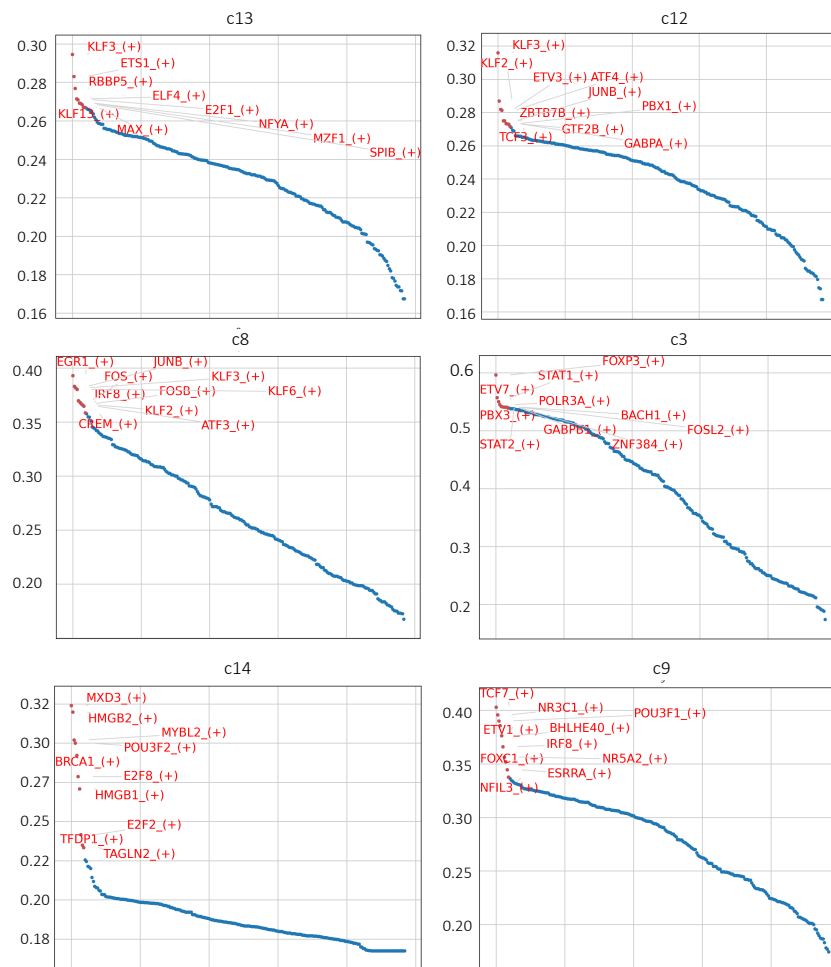


Figure 39 | TFs driving activation of Tregs in stage I HGSOc.

Top 10 predicted regulons (TFs) driving the activation of different tumor-infiltrating Treg subsets in c3/8/9/12/13/14. The regulons are ranked by the regulon specificity score shown on the y-axis ranging from 0 to 1, with 1 indicating complete specificity of the regulon for the cell type.

We then analyzed the differentiation trajectories across tumor-associated Tregs through pseudotime analysis (**Figure 40A**). Placing the most T_{NAIVE} Treg cells (c12) at the beginning of the trajectory, we observed that the initial state in c12 bifurcates into two main branches dissected by the specific gene changes along the Pre-branch T_{NAIVE} Tregs, Path-

I and Path-II (**Figure 40A-B**). Path-I consists of hyperactive *FOXP3*^{high}*CD25*^{high}*CTLA4*^{high} cells in c3, terminating with *FOXP3*⁺*MIK67*^{high} Tregs in c14. On the other hand, Path-II consists of cytotoxic *FOXP3*⁺*CD25*⁺*CTLA4*⁺ cells in c8, and the *CXCL13*^{high}*KRB1*^{high}*FOXP3*⁻ ex-Tregs in c9.

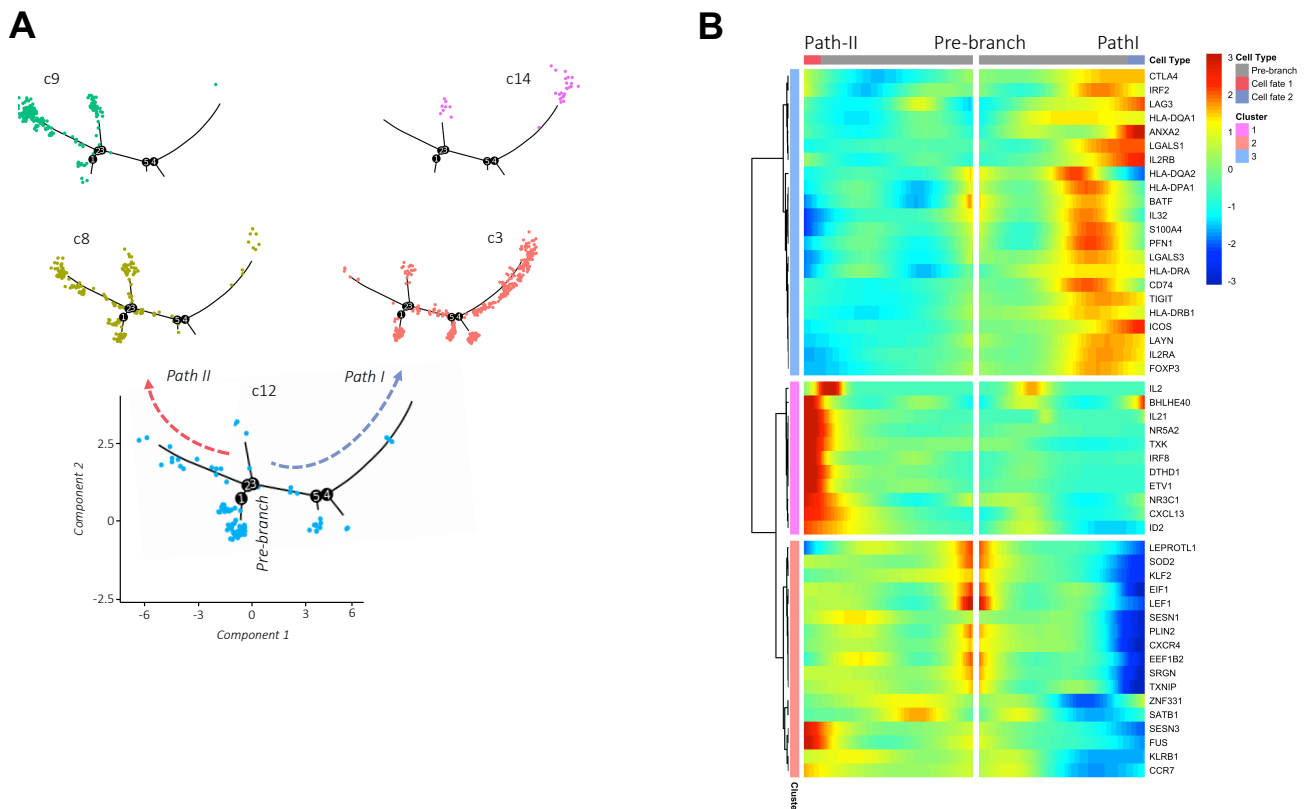


Figure 40 | Pseudotime trajectory of Tregs in stage I HGSOc.

(A) Pseudotime trajectory of distinct tumor-infiltrating Treg subtypes colored by clusters c3/8/9/12/14. The arrows represent the two trajectory paths starting from *T*_{NAIVE} Tregs in c12: Path I leading to *FOXP3*^{high} Tregs in c3 and proliferative Tregs in c14, and Path II leading to *FOXP3*⁺ Tregs in c8 and to ex-Tregs in c9. (B) Heatmap of top 50 significantly branch-dependent genes (*q*-value < 0.01), excluding ribosomal and mitochondrial genes, variable along the two pseudotime trajectory paths starting from *T*_{NAIVE} Tregs in c12. The x-axis represents cells ordered by pseudotime values along Path-I (from middle to right), and along Path-II (from middle to left), and different colors correspond to the scaled (*Z*-scored) expression of each gene in each cell.

In summary, our analyses have revealed the diverse adaptability of Tregs in stage I HGSOc, characterized by multiple transcriptional profiles that may indicate immunosuppressive activity, expansion, and transcriptional instability. This versatility of Tregs may allow their contribution to both tumor-promoting and tumor-inhibiting growth depending on the specific surrounding conditions. Moreover, mechanistic destabilization of effector Tregs may represent a means of eliciting antitumor immunity.

Cytotoxic lymphocyte profiling

In addition to Tregs, we further identified and characterized the other immune cell populations present in stage I HGSOC. So, starting from the cytotoxic lymphocytes we re-clustered both circulating and tumor-infiltrating NK and CD8 T cells (**Figure 41A**). From this analysis, we observed that tumor-infiltrating NK cells reach 25% of total cytotoxic cells and exhibit a distinctive profile compared to their counterparts circulating in the bloodstream (**Figure 41B-D**). While the $CD56^{dim}$ ($CD56^{dim}CD16^{+}$) subset predominates in the blood, two diverse NK cell subsets have been identified within the tumor, both displaying an activated profile (i.e., *JUN*, *JUNB*, *IER2/3/5*). Among them, the $CD56^{dim}$ subset, constituting a minority, exhibits the potential for a cytotoxic response (*PRF1*, *GZMB/A/M*, *GNLY*, *TBX21*), cytokine secretion (*IFNG*, *CCL3/4*), along with the expression of the activating receptors *KLRD1* (CD94), *KLRF1*, *CD226* (DNAM1) and *NKG7*. On the other hand, the predominant intratumoral NK cells display low cytotoxicity along with the expression of high levels of *XCL1/2*, *LTB*, *CCL5*, *CD2*, and the inhibitory receptor *KLRC1* (NKG2A). Despite the low detection of CD56, this profile closely resembles that of canonical NK^{bright} ($CD56^{bright}CD16^{-}$) cells, commonly enriched in both healthy and neoplastic peripheral tissues. Similar to findings in advanced stages of HGSOC, they express the tissue-resident markers *ITGA1* (CD49a) and *ITGAE* (CD103) and resemble decidual (d)NK-like cell phenotype (182,186,246). Indeed, they express multiple tetraspanins (*CD9*, *CD151*, *CD63*, *TSPAN5*), galectin-1 (*LGALS1*), *KLRC3*, the adhesion molecule *VIM*, and the angiogenic-promoting factors *VEGFA* and *CSF1* (M-CSF). Notably, we observed increased expression of dNK-like markers (CD103, VIM, galectin-1) also in proliferating blood NKs, indicating their inclination for blood-tumor migration.

When analyzing CD8 TILs, we examined the expression of *ENTPD1* (CD39), an indicator of activation in response to tumor-associated antigens (247). A substantial amount of $CD39^{+}$ CD8 T cells presented a highly exhausted profile, as evidenced by the expression of *PDCD1* (PD-1), *CTLA4*, *LAG3*, *TIGIT*, and *HAVCR2* (TIM-3) (**Figure 41B-E**). Although to a lesser extent, CD39 expression was also detected on cycling $MKI67^{high}$ CD8 TILs, providing in CD8 TILs evidence of tumor-specific activation, proliferation, and exhaustion. Conversely, the absence of CD39 was observed in two already identified CD8 TIL subsets, $GZMK^{high}$ and $GZMB^{high}$, suggesting their less relevant tumor-antigen specific activation (248,249). Indeed, all CD8 TILs display signs of activation (e.g., *JUN*, *FOS*, *IERs*, *HLA-DRA*, *HLA-DRB5*, *IFNG*, *TNF*), extended also to the blood CD8 T_{NAIVE} cells (e.g., *GZMB*, *PRF1*, *GNLY*, *FOS*, *JUNB*, *IL2RB*) (**Figure 41E**). The $GZMK^{high}$ CD8 T cells constitute the most

abundant subset among all CD8 TILs and exhibit low lytic potential, as evidenced by low levels of canonical cytotoxic markers such as *PRF1*, *GLNY*, and *GZMB*. Notably, several evidences have linked this specific *GZMK*^{high} phenotype of CD8 T cells to tumor progression, rather than to anti-tumor function (250).

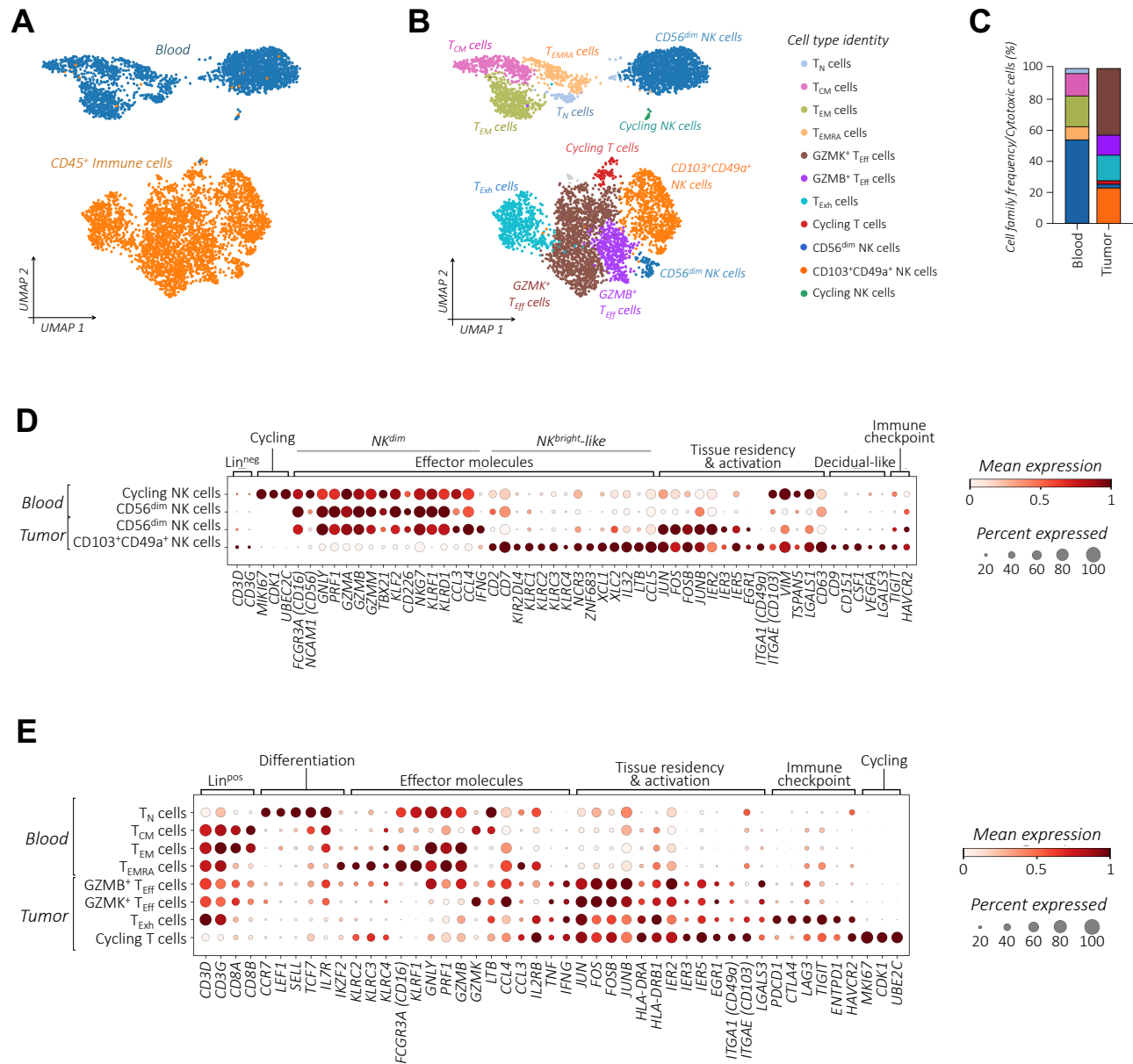


Figure 41 | Profiling of cytotoxic lymphocytes in stage I HGSOc at single cell level.

(A-B) UMAP graph showing the visualization of re-clustered blood and tumor-associated cytotoxic CD8 T and NK cells, colored by tissue origin (A) or by cell types (B). (C) Relative frequency distribution of each cell subtype for tissue origin. (D-E) Dot plots displaying the profiling of blood and tumor-infiltrating NK cell (D) and CD8 T (E) subsets by analyzing the expression of key selected differentiation and effector genes depicting various cell patterns: NK CD56^{dim} (CD56^{dim}CD16⁺), NK decidual-like (CD9⁺CD151⁺CSF1⁺VEGFA⁺LGALS3⁺), NK tissue-resident (CD49a⁺CD103⁺), cycling, exhausted T (T_{Exh}), T_{NAIVE}, T_{EM}, T_{CM}, T_{EMRA}, T_{Eff}.

Overall, the elevated cytotoxic potential in stage I HGSOC undergoes attenuation characterized by cellular polarization through compromised and pro-tumoral effector responses.

Myeloid cells analysis

Moving to the myeloid populations, we re-clustered circulating monocytes and tumor-infiltrating macrophages, DCs, and mast cells (**Figure 42A**). The blood comprises subsets of classical CD14⁺CD16⁻, cytotoxic classical CD14⁺CD16^{low}, and non-classical CD14^{low}CD16^{high} monocytes (**Figure 42B-D**). Considering TAMs complexity within HGSOC lesions, we applied specific gene-score signatures, in particular lipid-associated (n=19 genes, e.g., *ACP5*, *APOE*, *APOC1*), pro-angiogenesis (n=20 genes, e.g., *CCL2*, *CCL4*, *VEGFA*), and inflammatory (n=21 genes, e.g., *IL1B*, *IL1RN*, *IL6*) gene module scores, to define their functional diversity (251). The prevailing profile detected in TAMs exhibited a lipid-associated (LA)-pattern (e.g., *APOE*, *TREM2*, *APOC1*, *C1QA*, *C1QB*, *GPNMB*) (**Figure 42D-E**). Combining RNA-velocity and gene proliferation analysis we observed that the LA-TAM subset, boosting a relatively higher proliferation score (**Figure 42F**), undergoes further polarization, giving rise to the inflammatory (Inflam)_LA-TAM (e.g., *TNF*, *IL1B*, *IL18*, *IL32*, *CCL3/4*), and a pro-angiogenic (Angio)/Inflam_LA-TAM (e.g., *VEGFA*, *CXCL8*, *CSFR1*) subsets (**Figure 42G**). TAMs lacking the LA-profile, and manifesting highly Inflam/Angio-profile, constitute a minority within the TAMs population (**Figure 42B-C**). In summary, we support the notion that dysregulation in lipid-associated functions is a hallmark of TAMs in stage I HGSOC.

In the tumor, among DCs, we distinguished conventional cDC1 and cDC2, plasmacytoid (p)DCs and *LAMP3*^{high} DCs (**Figure 42B-C**). The latter, together with the high expression of *LAMP3* gene, expresses the maturation markers *CD80* and *CD83*, indicating a more mature profile, as well as *CCR7* gene, pointing to their ability to migrate towards lymph nodes and demonstrating diverse capabilities in recruiting and activating immune cells (*CCL16/19/22*, *IL15*) (252). Mature *LAMP3*⁺ DCs may originate from cDC1 and cDC2. In our dataset, maturation dynamics in the tumor-associated DCs, through RNA velocity analysis, indicate a predominant maturation from the cDC1 subset, which accordingly constitutes the most proliferative DC subset (**Figure 42F-G**).

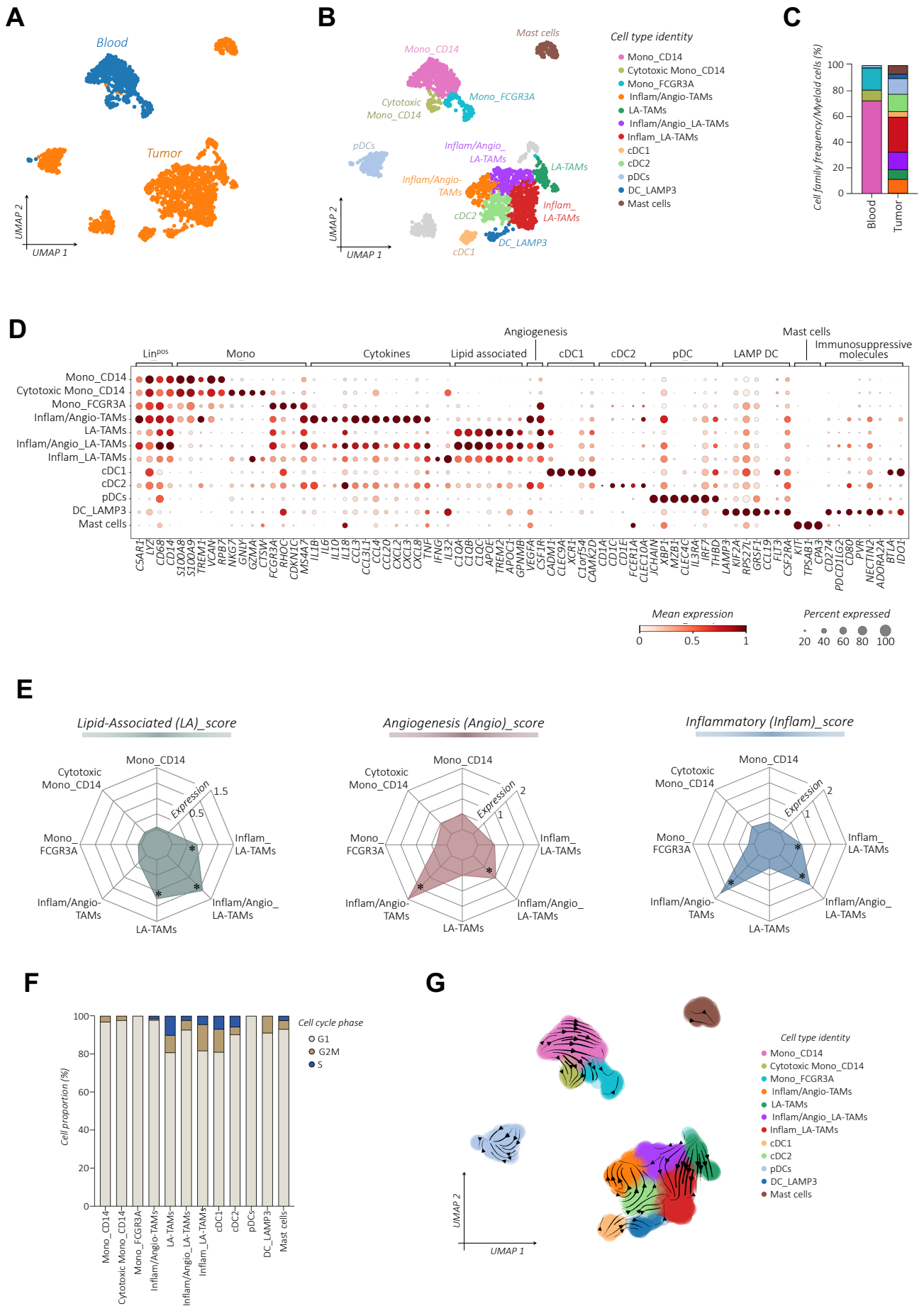


Figure 42 | Profiling of myeloid cell subsets in stage I HGSOC at single cell level.

(A-B) UMAP graph showing the visualization of re-clustered blood monocytes (Mono) and tumor-associated macrophages (TAMs), dendritic cells (DCs), and mast cells, colored by tissue origin (A) or by cell types (B). (C) Relative frequency distribution of each cell subtype for tissue origin. (D) Dot plot showing profiling of blood and tumor-associated myeloid cells analyzed by the expression of key selected differentiation and effector gene markers depicting specific cell patterns: CD14⁺ (CD14), CD16⁺ (FCGR3A), inflammatory (Inflam), pro-angiogenesis (Angio), lipid-associated (LA), IFNG⁺ (IFNG), conventional (c)DCs, plasmacytoid (p)DCs and LAMP3⁺ (LAMP3). (E) Radial graphs showing the expression level of the specific lipid-associated, pro-angiogenesis, and inflammatory gene module scores calculated for each myeloid cell group. (F) Bar plot illustrating the relative frequencies of specific cell cycle phase G1, G2M, and S distribution in all detected myeloid cell subsets. (G) UMAP graph showing the inferred development dynamics of myeloid cell subsets by RNA velocity. Kruskal-Wallis test with Dunn's multiple comparison:

* $P \leq 0.05$

Dynamic remodeling of Treg cell interactions

To predict cell communications via differentially expressed ligand-receptor pairs implicated in both the Treg enrichment and their inhibitory potential in stage I HGSOC TME, we employed the computational NicheNet algorithm (253). Focusing our analysis on the top 25 ranked ligand-receptor pairs, we identified multiple connections with T and myeloid cell subsets shared between the two main *FOXP3*-expressing Treg subsets (c3 and c8, representing the *FOXP3*^{high} and *FOXP3*⁺ clusters, respectively) (**Figure 43A-C**). Importantly, the interaction of *CTLA4* expressed on *FOXP3*^{high/+} Tregs with *CD80*, which is important for Treg-mediated inhibition of immune responses, was detected in TAMs, DCs, B cells, and exhausted CD8 T cells. Moreover, the release of *TGFB1* by *FOXP3*⁺ Tregs can broadly inhibit immune cell activation (234,241). The *IL2RA/RB-IL2* connection was enriched between *FOXP3*^{high/+} Tregs and the identified CD45⁺EPCAM⁺ cells, providing evidence of the IL-2 deprivation within the TME by Tregs. Several additional ligand-receptor engagements between *FOXP3*^{high/+} Tregs and various T and myeloid cell subsets play pivotal roles in Treg immunosuppressive activity, transcriptomic stability, maturation, and expansion. Among them we detected: *ICOS-ICOSLG*, *IL18R1-IL18*, *ILR1-IL1B*, *Notch1-DLL1*, *IL15R/IL2RA-IL15*, *IL27RA-IL27/EBI3*, *IL1R1-IL33*, *IL12RB1-IL23A* and *TGFBR1/2-TGFB1*, and interactions with members of the TNF Receptor Superfamily including *TNFRSF1B-TNF* and *TNFRSF9-TNFSF9* (241,254–259). Furthermore, the possible mobilization of Tregs in the TME was evidenced by the binding of *CXCR3-CXCL10/CXCL11*, *CXCR4-CXCL12*, and the expression of several adhesion molecules such as *ICAMs* and *integrins*, among which in particular β 2-integrins (*ITGB2*), playing an important role in Treg function regulation (241,260,261). It is worth mentioning the potential role of *IL32* on Treg differentiation within the tumor (262), a cytokine that we detected in myeloid cells including

A



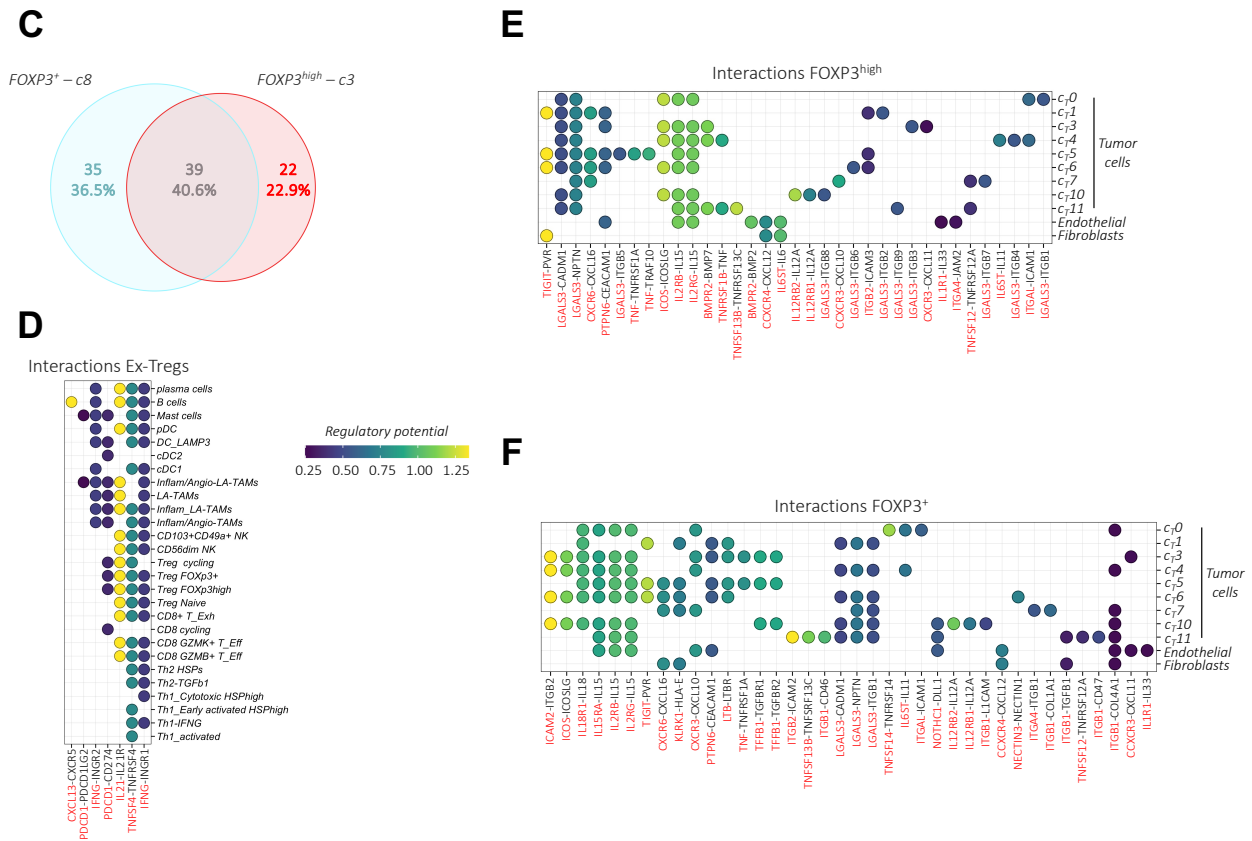


Figure 43 | Treg cells are key mediators of the complex cellular interaction in stage I HGSOC.

(A-B) Dot plots displaying ligand-receptor interactions on the x-axis, showcasing the top 25 prioritized (by Pearson correlation coefficient) paired ligand-receptor engagements detected in $FOXP3^{high}$ Tregs (A) and $FOXP3^{+}$ Tregs (B) with the other immune cell populations. (C) Venn diagram illustrating receptor-ligand paired interactions commonly or specifically detected among different cell types and $FOXP3^{high}$ and $FOXP3^{+}$ Tregs. (D) Dot plot displaying ligand-receptor interactions on the x-axis, showcasing the top 25 prioritized (by Pearson correlation coefficient) paired ligand-receptor engagements detected in ex-Tregs with other immune cell populations. (E-F) Dot plots displaying ligand-receptor interactions on the x-axis, showcasing the top 25 prioritized (by Pearson correlation coefficient) paired ligand-receptor engagements detected in $FOXP3^{high}$ Tregs (E) and in $FOXP3^{+}$ Tregs (F) with the CD45⁻ cell populations. Ligands or receptors expressed in Tregs (in red) are matched with their corresponding receptors or ligands (in black) expressed on different cell subsets shown on the x-axis.

Many of the detected ligand-receptor axes involving $FOXP3$ -expressing Tregs, including $TNF-TNFRSF1A/1B$, $TGFB1-TGFB1R1/R2$, $IL1A/1B-IL1R1/R2$ and $LGALS3$ -all ligand, correlate with the worst OS outcomes in advanced OC patients when analyzed in the TCGA dataset (Figure 44). Hence, the establishment of a Treg-permissive TME in stage I likely involves a complex interplay between Tregs, immune cells, and tumor cells.

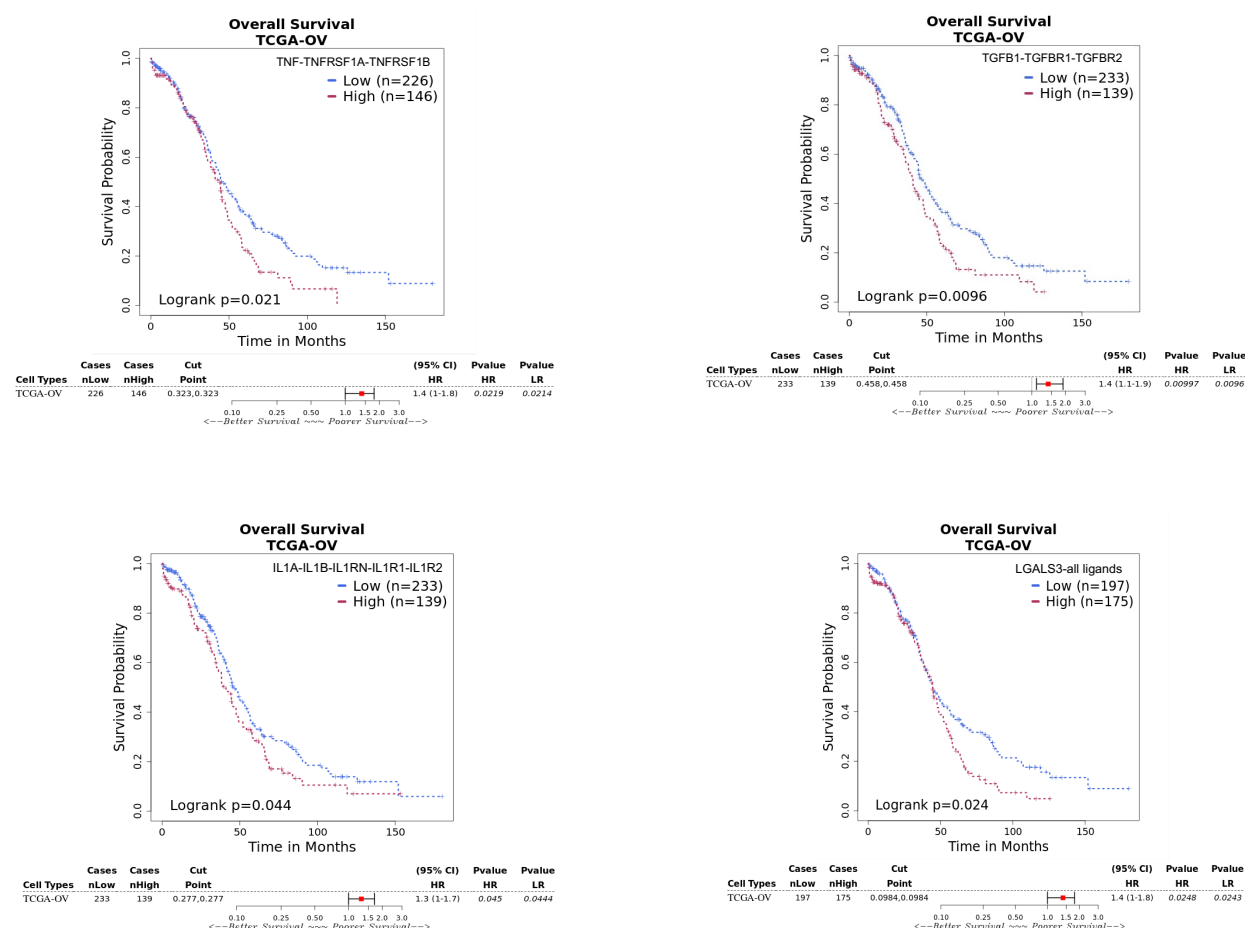


Figure 44 | Predicting $FOXP3^{high}$ and $FOXP3^+$ Treg cell interaction network in tumor progression.

Kaplan-Meier curves with corresponding Forest plots for patients with advanced OC (n=372) (TCGA dataset), showing significantly worse OS prognosis between high-risk and low-risk expression levels of specific receptor-ligand interaction axes detected in $FOXP3^{high}$ and $FOXP3^+$ Tregs: TNF-TNFRSF1A/1B, TGFB1-TGFB1/R2, IL1A/1B-IL1R1/R2 and LGALS3-all ligands.

Regarding ex-Tregs (c9), the interaction via *CXCL13-CXCR5* was specifically identified with B cells (**Figure 43D**). The most prevalent interaction of ex-Tregs was associated with their elevated expression of *IFNG*, leading to predictable binding with various T and myeloid cells, including *FOXP3*-expressing Tregs. In this context, recent observations indicate *IFN- γ* as a driver of conventional Tregs fragility (266). Of note, the expression of *IFNG* is associated with a better OS in TCGA-OV patients (**Figure 45A**). Similarly, the specific secretion of *IL21* by ex-Tregs, that could react with different immune cells targeting *IL21R*, is associated with better survival rates in OC patients analyzed from the TCGA dataset (**Figure 45B**). Conversely, PD-1 expression in ex-Tregs predicted enriched interactions with its ligands PD-L1/L2 (*CD274/PDCD1LG2*) within myeloid and *FOXP3*-expressing Treg cell subsets, possibly suggesting their inhibition (**Figure 43D**).

Therefore, specific interventions could be developed to counteract the differentiation fate, and the pro-tumoral role of Tregs by targeting these activities.

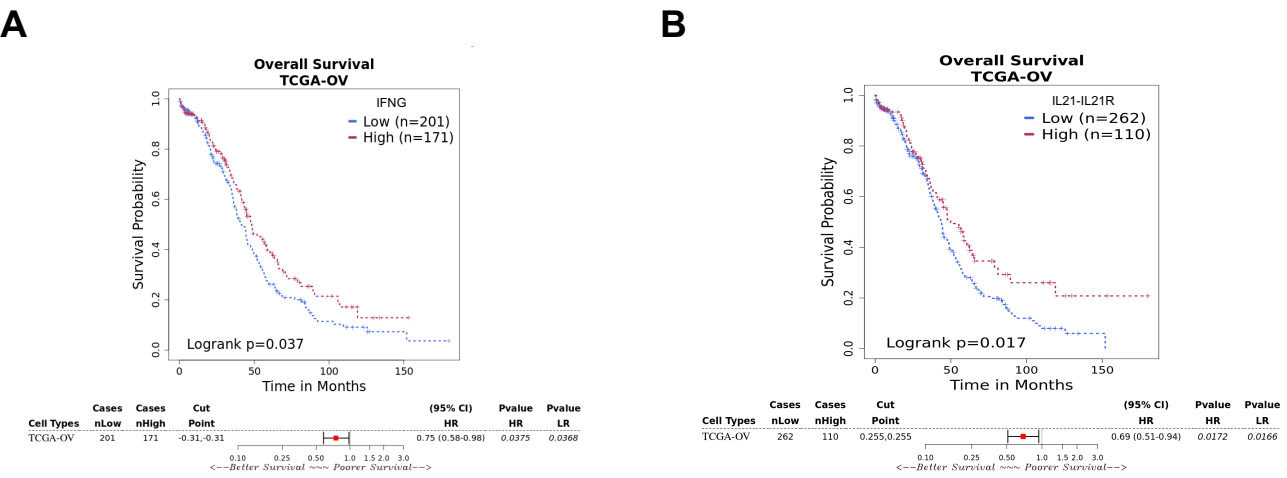


Figure 45 | Predicting ex-Treg cell interaction network in tumor progression.
Kaplan-Meier curves with corresponding Forest plots for patients with advanced OC (n=372) (TCGA dataset), showing significantly better OS prognosis between high-risk and low-risk expression levels of specific genes and receptor-ligand interaction axes detected in ex-Tregs: IFNG and IL21-IL21R.

Discussion and Conclusions

Exploring PD-1 targeting on $\gamma\delta$ T cells in EC

EC patients with advanced/recurrent disease have poor outcomes and respond poorly to the current management treatments based on surgery and chemotherapy or radiotherapy. In this context, remarkable progresses have been made in immunotherapy. In particular, clinical trials are exploring different ICIs for the treatment of EC. To date, the efficacy is not satisfactory, thus a detailed understanding of the biology and interactions between the cancer and the immune system is essential. In addition, it is important to identify biomarkers for the response to immunotherapy. Interestingly, highly activated tissue-resident $\gamma\delta$ T cells have been identified in patients who benefited from the combination of nivolumab and cabozantinib (83). However, the role of $\gamma\delta$ T cells in EC has not been defined yet.

Our work provides a detailed characterization of $\gamma\delta$ T cells in the context of EC. In EC TME V δ 1 T cells constitute the main subset of tumor-infiltrating $\gamma\delta$ T cells, confirming what has been described recently in the TCGA cohort (111), while V δ 2 and V δ 3 T cells are less abundant. Their infiltration is independent of the grade and the histologic subtype, however, an enrichment of both V δ 1 and V δ 2 T cells is observed in advanced-stage patients. Their presence in the advanced stages of the disease has been associated with a favorable response to immunotherapies (83), highlighting the important antitumor role that they could exert in EC. Although a previous study reported that V δ 1 and V δ 3 T cells are enriched in MMRd UCEC patients from the TCGA dataset (111), in our cohort we did not find statistically significant differences stratifying the patients according to the microsatellite instability. Nevertheless, more patients should be analyzed to confirm this.

A phenotypic characterization through FACS revealed that tumor-infiltrating $\gamma\delta$ T cells showed alteration in the maturation status and in the expression of several surface markers compared with their circulating counterpart, suggesting an impact of the TME. In particular, these cells resemble a less differentiated phenotype displaying mainly a T_{EM} and T_{CM} status accompanied by a reduced expression of CD16, CD57 and CX3CR1. Moreover, they comprise tissue-resident cells that exhibit an increased expression of CD69, CD103 and CXCR6. Considering the increased interest in immunotherapies for EC treatment, we observed a high expression of ICs by tumor-infiltrating $\gamma\delta$ T cells. Confirming our data in other solid tumors, including glioblastoma, hepatocellular carcinoma and non-small cell lung cancer (106), V δ 2 T cells constitutively express the inhibitory receptor NKG2A, with no differences between the PB and the tumor. Moreover, its expression, which is low on

circulating V δ 1 and V δ 3 T cells, is induced in the TME, suggesting that NKG2A expression could be regulated in the context of tumors, although little is known about its regulation (116). On the other hand, other immune checkpoints, such as TIGIT and KLRG1, are highly expressed by circulating $\gamma\delta$ T cells and their expression is even lower in the tumor setting. Their expression has been related to the maturation status of T cells (218–220), and our data support this notion also for $\gamma\delta$ T cells, thus indicating that their lower expression is linked to the less differentiated phenotype of $\gamma\delta$ T cells. Nevertheless, their role in the context of the TME needs further investigation. In fact, TIGIT is a new promising immunotherapy target that is gaining interest in gynecological cancers too (77). Notably, in the tumor we observed the upregulation of ICs that are lowly expressed in the circulation, including CD39, TIM-3 and PD-1. These are relevant in the context of tumors, as CD39 and PD-1 have been associated with tumor-reactive T cells (267,268). PD-1 has been investigated also in $\gamma\delta$ T cells, considering their important antitumor role. Historically, PD-1 has been associated with V δ 2 T subsets due to their interest as effector cells in adoptive cellular immunotherapy. Accordingly, in pathological settings and upon activation and expansion, they upregulate PD-1 and its blockade increases their effector functions (269–273). However, in the TME V δ 1 T cells constitute the predominant $\gamma\delta$ T subset, so, recently, PD-1 has been investigated in this subpopulation. In accordance, PD-1⁺ V δ 1 T cells have been identified in colorectal cancer, kidney cancer, melanoma and Merkel cell carcinoma (111,274–276).

The investigation of PD-1 on V δ 1 T cells in EC revealed the co-expression of PD-1 with CD39, TIM-3 and TIGIT by correlation analysis, unbiased analysis through UMAP algorithm and manual gating strategy. Phenotypic analysis and functional experiments confirmed that these cells are characterized by a T_{CM} phenotype and retain their ability to produce IFN- γ and TNF- α upon PMA/I stimulation. Moreover, tissue-resident cells, which are associated with the expression of ICs, showed higher cytokines production. The analysis of a public dataset of scRNA-seq conducted on healthy endometrial tissue, peritumor and tumor tissue confirmed that the EC TME induces the expression of *PDCD1* (PD-1), as its upregulation on $\gamma\delta$ T cells is observed in the tumor tissue compared with both the normal one and the peritumor. Furthermore, the comparison of tumor-infiltrating $\gamma\delta$ T cells with the cells from the healthy endometrium confirmed the less differentiated phenotype due to the expression of *CD27* and the upregulation not only of PD-1 but also of other ICs, such as *HAVCR2* (TIM-3), *TIGIT* and *KLRC1* (NKG2A). The antitumor potential was evidenced by the expression of cytotoxic genes, as *GZMB*, *GZMK*, *GZMA*, *IFNG* and *NCR1* (NKp46),

confirming the results of a previous study reporting the presence of a cytotoxic $\gamma\delta$ T subset in EC (277).

Previous findings have suggested that, similarly to conventional CD8 T cells, PD-1 expression could identify tumor-reactive $\gamma\delta$ T cells. In line with this, we observed that tumor-infiltrating $\gamma\delta$ T cells express *ENTPD1* (CD39) and *CXCL13*, which has been shown to be expressed by tumor antigen-specific T cells (268,278,279). In accordance, $\gamma\delta$ TCR stimulation has been suggested to induce the upregulation of PD-1 on V δ 1 T cells (275). However, further investigations are needed to determine what the $\gamma\delta$ TCR recognizes and reacts to, in fact $\gamma\delta$ T cells do not recognize classic MHC molecules and, moreover, V δ 1 T cells have been proposed to be important in β_2 -microglobulin (β_2 M)-mutant tumors, excluding CD1 or MR1 as potential antigen-presenting molecules (117).

Remarkably, when we assessed the cytotoxic potential of $\gamma\delta$ T cells against autologous and heterologous tumors cells isolated from EC patients, we observed that tumor-infiltrating $\gamma\delta$ T cells, in particular V δ 1 T cells, are inhibited and are not able to degranulate. However, when we tried to unleash them with an antibody targeting PD-1 their degranulation capacity increased, confirming the role of PD-1 as IC in $\gamma\delta$ T cells. This is in line with previous results showing that the blockade of PD-L1, the ligands of PD-1, increased the killing activity of V δ 2⁺ TILs against autologous primary tumor cells (274) and partially rescued the cytokines production and the degranulation of V δ 1 T cells in the context of TCR stimulation (275). Interestingly, we observed that the response is not related to the disease stage, as we observed similar responses between early and advanced stages, suggesting the possibility of targeting PD-1 independently of the disease stage.

Considering the impact of targeting PD-1 on $\gamma\delta$ T cells we evaluated the clinical relevance of PD-1 in EC. Its expression is independent of disease stage, grading and histological subtypes, thus giving the rationale to inhibit it in any EC patient. In addition, the correlation of PD-1⁺ V δ 1 T cells with the tumor dimension evidenced its role in EC progression. Several studies reported that the phenotype of $\gamma\delta$ T cells can have a prognostic value, with a general association with a favorable prognosis (110). Similarly, in the UCEC cohort from TCGA, we observed that V δ 1 T cells are associated with a better OS, while V δ 2 T cells do not have any impact. Nevertheless, PD-1 expressed on both V δ 1 and V δ 2 T cells is associated with a good prognosis. This sheds light on finding correlations between the tumor and the circulation to identify possible biomarkers. For example, in colorectal liver metastasis intrahepatic CD69⁺ V δ 1 T cells could recirculate in the blood and are correlated

with the presence of a lower number of liver metastases as well as better OS (109). Analogously, in EC we found a correlation between tumor-infiltrating and circulating CD69⁺ V δ 1 T cells and most importantly between both V δ 1 T cells and PD-1⁺ V δ 1 T cells, confirming their prognostic role.

In summary, we assessed that $\gamma\delta$ T cells, especially the V δ 1 T subset, infiltrate EC tumor tissue and by expressing PD-1 are relevant in EC progression and prognosis. They retain their effector functions and upon PD-1 blockade they increase their cytotoxicity against primary EC tumor cells. These data highlight the possibility of targeting PD-1 on $\gamma\delta$ T cells for EC immunotherapy, so, we assessed the effect of ICIs against PD-1 on $\gamma\delta$ T cells *in vivo*. First of all, we observed that PD-1 blockade affects $\gamma\delta$ T cell effector functions, in fact, V δ 1 T cells showed the upregulation of pathways related to antigen processing and presentation, cell adhesion molecules, cytotoxicity and T cell receptor signaling upon anti-PD-1 treatment. This confirms the recently observed activation of $\gamma\delta$ T cells in melanoma patients treated with ICIs (280). Although the cells seem to return to their state before anti-PD-1 therapy over time, suggesting its effect is transient. Interestingly, in nonresponder patients we observed the upregulation of the PD-1 pathway. This is in line with an association observed between PD-1 expression on CD4 T cells and a worse clinical outcome after anti-PD-L1 treatment (281), however further investigations are needed to elucidate this. Remarkably, our data extends to EC the predictive role of V δ 1 T cells for the response to anti-PD-1 therapy, as just published for melanoma (275). In fact, comparing responders and nonresponders patients, a higher frequency of V δ 1 T cells has been observed in responder patients already before the therapy, due to their higher proliferative capacity compared with V δ 2 T cells.

Stage I HGSOC features an immunosuppressive microenvironment dominated by regulatory T cells

HGSOC is an extremely aggressive disease with a high mortality rate and a significant risk of relapse. The primary challenge in treating this disease is the delayed diagnosis, since many patients are asymptomatic or present non-specific symptoms in the early stages. Thus, acquiring more knowledge about the early stages of the disease could aid in its early diagnosis. However, most studies have focused on patients with advanced-stage OC, as they make up the majority of the patients, and there is a lack of knowledge regarding the initial steps of the disease. This could help also in understanding the processes that lead to the progression from early to advanced stage OC.

Our work provides a comprehensive atlas of the single-cell transcriptome of stage I HGSOC. This atlas offers detailed insights into the immune, stromal, and epithelial subsets and highlights their molecular signatures and intercellular communication patterns.

As already described in advanced OC (282) and other solid tumors (283–285), while the stromal component, comprising endothelial cells and fibroblasts, is similar among patients, the malignant epithelial cells exhibit a patient-specific expression pattern. Differently from advanced stages HGSOC where specialized CAF subpopulations with distinct functional markers have been identified (282), in early stages fibroblasts express only at model levels genes related to CAFs, such as *FAP*, and include both matrix CAFs-related metalloproteinases as *MMP11* and inflammatory CAFs-genes which comprise inflammatory cytokines as *IL6* (286,287). However, they constitute only a minority among CD45⁺ cells in early-stage HGSOC microenvironment, thus a larger cohort is essential to dissect their heterogeneity and to assess their role in HGSOC progression. On the other hand, epithelial cells constitute a huge proportion of CD45⁺ cells. Considering their patient-specific profile, they are characterized by a general common status of activation and stress-resistance response. Moreover, some cells exhibit selected features, thus indicating distinctive roles exerted by the various subpopulations of cancer cells, in particular there are clusters that resemble the fallopian origin, others that are involved in inflammation and immunoregulation processes, others in angiogenesis and others with EMT features. In addition, we observed that proliferating cells are important in the development of distinct cancer cells, in fact, pseudotime analysis revealed that they are the starting point for the differentiation of malignant cells into distinct branches. These branches represent the malignant progression of tumor cells, as specific clusters have been associated with a worse clinical outcome in advanced-stage OC patients. Therefore, in the early stages of HGSOC

the malignant epithelial cells are constitutively activated and exert various roles that are important in orchestrating the TME that could lead to the progression of this disease.

Accordingly, we observed a significant restructuring of the cellular composition within the TME, characterized by an abundance of CD4 Th2 and suppressive Tregs, TAMs, NK cells lacking cytotoxic potential, and an enrichment of exhausted CD8 T cells. This orchestrated immunosuppressive TME, with unresolved suppressive immune control mechanisms during the early stages of the disease, could contribute to disease progression. Consequently, these findings shed new light on the mechanisms of poorly understood transition from early to advanced stages in HGSOC.

Compared to the lymphoid compartment, the myeloid one constitutes only a minority in stage I HGSOC. Macrophages are characterized mainly by lipid-associated TAMs that proliferate and could further polarize into inflammatory and angiogenesis/inflammatory TAMs, while there are few TAMs without a lipid-associated profile. LA-TAMs have been already described in different solid tumors and they have been associated with the suppression of antitumor immune responses (251). Moreover, Angio-TAMs may promote tumor progression contributing to angiogenesis, while Inflam-TAMs might actively recruit and regulate the immune cells during the tumor-inflammatory responses (251). DCs are represented by the conventional cDC1, cDC2 and pDCs. As already described in other tumors (288), *LAMP3*⁺ DCs infiltrate also HGSOC already at early stages. These cells, defined as mature DCs and enriched in immunoregulatory molecules, could contribute to the immunosuppressive microenvironment (289).

The lymphocytic counterpart is composed by both the innate and the adaptive immunity. NK cells are enriched in the TME of early stage HGSOC. These cells resemble dNK cells being predominantly tissue-resident NK^{bright} cells expressing CD49a and CD103. However, they display low cytotoxicity and could contribute to angiogenesis as they are characterized by angiogenic-promoting factors. Similarly, even if the adaptive CD8 T cells exhibit a general status of activation, *GZMB*⁺ effector CD8 cells constitute only a minority. The main subpopulations are represented by *GZMK*⁺ cells characterized by low lytic potential, associated with tumor progression (250) and by tumor-specific T cells expressing CD39, but with an exhausted phenotype due to the expression of ICs. Therefore, the cytotoxic lymphocytes in the context of early-stage HGSOC exhibit a low cytotoxic potential and an exhausted phenotype, likely contributing to HGSOC progression.

The dominant population in the immune microenvironment is represented by CD4 TILs. These cells display a tissue-associated activating phenotype and among them we

could distinguish helper T cells, constituted by both Th1 and Th2, and importantly the suppressive Tregs which represent a huge proportion among CD4 TILs. To confirm whether consistent Treg infiltration is a common feature of stage I HGSOC we will perform immunohistochemistry on a larger cohort of patients assessing both CD4 and FOXP3 expression. This would support the notion that a property of early-stage tumors could favor Treg accumulation in the TME.

Unsupervised clustering revealed Treg heterogeneity in stage I HGSOC, which has been resolved into five main subsets that differ from the naïve ones through various stages of activation and effector immunosuppressive potential. A majority of Tregs in stage I HGSOC exhibit higher levels of effector markers and *FOXP3* expression compared to blood Tregs, resulting in an inhibitory outcome from cell-cell interactions. Among the identified subsets, *FOXP3^{high}* Tregs demonstrated the highest expression of *IL2RA* and *CTLA4* (290,291), key mediators of Treg inhibitory function controlled by *FOXP3*, accounting for its superior suppressive capacity. *FOXP3^{high}* Treg cells are also found to be present in a majority of human solid tumors (292). Similarly, highly suppressive *FOXP3^{high}* Tregs have been identified in advanced stages OC and linked to a worse prognosis (293). Our study showed evidence of their role in supporting HGSOC during early growth. Moreover, we identified that the *FOXP3^{high}* subset predominantly expresses *LAYN*, a marker that defines highly TCR-activated Tregs (294) and associates with poor prognosis in OC. The less abundant *MKI67^{high}* Treg subset identified in stage I HGSOC was characterized by high expression of proliferation-associated genes. This indicates their significance *in vivo*, despite their limited number, as they likely contribute to the sustained expansion and activity of Tregs. Furthermore, we identified a sizable fraction of *FOXP3⁺* Tregs whose suppressive activity may be conferred by a combination of cytotoxic activity, secretion of suppressive *TGFB1*, and, similar to the *FOXP3^{high}* subset, to markers such as *IL2RA* and cell-contact-dependent suppressive mechanisms including expression of *CTLA4*. Although conventional CD4 T cells may also express *FOXP3* transiently without exhibiting suppressive activity, here, we found that all *FOXP3*-expressing Tregs show a thymic origin by expressing *IKZF2* (Helios), a TF that stabilizes the inhibitory activity of Tregs, indicating that in early stage HGSOC their differentiation originates from naïve Tregs. Accordingly, we also observed a lack of tissue-resident Tregs in healthy ovarian stroma, excluding Tregs origin from the ovarian tissue.

In addition to the resolved suppressive Treg cell subpopulations, our study also identified the ex-Treg phenotype, which seems to arise from transcriptional program

instability due to the loss of *FOXP3* expression. Certain pro-inflammatory signals, such as IFN- γ , can cause a rapid loss of suppressor activity and reprogram the Tregs into a pro-inflammatory phenotype (237,266,295–298). Our findings highlight that the presence of an instability program in Tregs assembles into a specific differentiation pathway, termed Path-II. Trajectory differentiation analysis revealed that, on one hand, *FOXP3*^{high} cells primarily follow Path-I, ending with the *MKI67*^{high} proliferating Treg subset. Conversely, *FOXP3*⁺ and ex-Tregs contribute to Path-II. Upon exploring the TFs associated with Path-I or Path-II, we found that *FOXP3* acts as the main regulator of Path-I, while Path-II displays a high expression of IFN-regulatory factor 8 (*IRF8*), a downstream target of IFN- γ signaling (299). Intriguingly, *IRF8* expression was commonly observed among the top TFs in *FOXP3*⁺ and ex-Treg subsets, suggesting its involvement in promoting Path-II Treg profile. Moreover, the expression of both *IFNG* and its receptors *IFNGR1/2*, known to be involved in Treg destabilization (266), was detected across cell-cell interactions specifically within the *FOXP3*⁺ subset. These data suggest IFN- γ as a possible driver for Treg instability in stage I HGSOC, as already observed in melanoma and head and neck squamous cell carcinoma (266). However, we cannot exclude that other factors may also contribute to Path-II profiling, such as *NOTCH1*-signaling, which results to be part of the *FOXP3*⁺ Treg cell-cell communication pattern and has been implicated in Treg dysregulation (300).

The instability of Tregs in stage I HGSOC leads to the high expression of *CXCL13*, a chemokine responsible for the recruitment of CXCR5-expressing immune cells, such as naïve B cells and T follicular helper (Tfh) cells (301). In HGSOC elevated *CXCL13* expression correlates with prolonged patients' survival and initial formation of TLS, an ectopic lymphoid aggregate recruiting and coordinating antitumor response of cellular and humoral immunity. The TLS formation in OC was associated with *CXCL13*-producing CD4 T cells (239). Our study reveals that the dominant producer of *CXCL13* among CD4 T cells originates from ex-Tregs, thereby supporting the notion that Treg instability contributes to immune boosting in HGSOC.

Our findings underscore the dual lineage tracing of Tregs in stage I HGSOC, wherein they can exert either suppressive or enhancing effects on tumor immunity, thus impacting cancer prognosis. Moreover, our results emphasize the therapeutic significance of selectively targeting suppressive Tregs to induce their specific differentiation fate. While systemic depletion of Tregs offers the advantage of being easily monitored through the quantification of circulating Tregs, its clinical benefits have been modest or limited, coupled with a high risk to evoke autoimmunity. In contrast, local antibody delivery shows less

adverse effects (302–304). Alternatively, targeting the differentiation fate of Tregs represents another promising therapeutic avenue. However, further studies investigating the complete array of molecular mechanisms and differentially expressed transcription factors are warranted to achieve a more comprehensive understanding of the dual-path differentiation process.

The limitations of our study include the small sample size, which requires validation from a larger clinically heterogeneous TCGA cohort of advanced stages OC, and the lack of scTCR analysis, which could yield meaningful insights into TCR overlapping between distinctively developed Treg subpopulations. Nonetheless, our unbiased single-cell approach has significantly enriched our understanding of Treg cell heterogeneity, function, and differentiation. This has led to the discovery of previously unrecognized dual pathways of Tregs polarization in stage I HGSOC, offering promising avenues for further exploration of Tregs in HGSOC disease and providing valuable insights for potential therapeutic interventions.

Future plans

Endometrial cancer

To confirm the association of PD-1 with tumor-reactive $\gamma\delta$ T cells, we will analyze the TCR sequencing of FACS-sorted PD-1⁺ and PD-1⁻ tumor-infiltrating V δ 1 T cells to assess if specific clones are associated with PD-1. Moreover, to identify the factors that could induce or maintain the expression of PD-1 we will perform further analysis on the scRNA-seq dataset through *SCENIC* tool and *in vitro* experiments using an organotypic model which mimics the TME to investigate its impact on $\gamma\delta$ T cells. In particular, in collaboration with the Unit of Gynecological Oncology Research of European Institute of Oncology directed by Dr. Ugo Cavallaro, we plan to establish an organotypic model, which is a model based on the tri-culture of fibroblasts, mesothelial cells and tumor cells, already used for ovarian cancer (305). Fibroblasts and mesothelial cells will be isolated from the omentum, while tumor cells will be isolated as described in the Materials and Methods section “Isolation of primary tumor cells from EC tumor tissues”. Then, the organotypic model in which the tumor cells are co-cultured with fibroblasts and mesothelial cells will be established in order to then assess the expression of PD-1 and other ICs as well as the proliferative capacity and the anti-tumor activity of $\gamma\delta$ T cells.

The limit of our co-culture experiment setting was that we could not assess the spatial localization of the cells, to overcome this we plan to assess the $\gamma\delta$ T cells response to PD-1 blockade by taking advantage of an ex vivo tumor fragment platform, already used to dissect the response to PD-1 blockade (306), through which small pieces of EC tumor tissue will be directly treated with an anti-PD-1 mAb and then the response of $\gamma\delta$ T cells will be evaluated by imaging.

Ovarian cancer

To support the expression of *CXCL13* by CD4 T cells, we are going to perform the co-detection of *CXCL13* and CD4 through RNAscope technique in a larger cohort of stage I HGSOC patients, to confirm that this is a common mechanism of early stage TME. Moreover, immunofluorescence assessing also CD20 expression will indicate the possible association with the formation of TLS.

Acknowledgments

First of all, I would like to thank all the patients enrolled in the study.

I am immensely grateful to Professor Domenico Mavilio for his guidance and to Joanna Mikulak, my supervisor, for her invaluable support throughout this project. I am thankful for their help with data interpretation and thesis corrections. I am particularly grateful to all members, past and present, of the Laboratory of Clinical and Experimental Immunology, especially Paolo Marzano, Sara Terzoli, and Giampaolo Martiniello, who performed the scRNA-seq analyses.

Thanks to the fellowships provided by the University of Milan and the Associazione Italiana per la Ricerca sul Cancro (AIRC), which made this work possible.

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Appendix

List of publications

- 1) Valentina Cazzetta, Elena Bruni, Sara Terzoli, Claudia Carena, Sara Franzese, Rocco Piazza, Paolo Marzano, Matteo Donadon, Guido Torzilli, Matteo Cimino, Matteo Simonelli, Lorenzo Bello, Anna Villa, Likai Tan, Sarina Ravens, Immo Prinz, Domenico Supino, Federico S. Colombo, Enrico Lugli, Emanuela Marcenaro, Eric Vivier, Silvia Della Bella, Joanna Mikulak and Domenico Mavilio. **NKG2A expression identifies a subset of human V δ 2 T cells exerting the highest antitumor effector functions.** *Cell Reports*. 2021. doi: 10.1016/j.celrep.2021.109871.
- 2) Elena Bruni, Matteo Cimino, Matteo Donadon, Roberta Carriero, Sara Terzoli, Rocco Piazza, Sarina Ravens, Immo Prinz, Valentina Cazzetta, Paolo Marzano, Paolo Kunderfranco, Clelia Peano, Cristiana Soldani, Barbara Franceschini, Federico S. Colombo, Cecilia Garlanda, Alberto Mantovani, Guido Torzilli, Joanna Mikulak and Domenico Mavilio. **Intrahepatic CD69 V δ 1 T cells re-circulate in the blood of metastatic colorectal cancer patients and limit tumor progression.** *Journal for ImmunoTherapy of Cancer*. 2022. doi: 10.1136/jitc-2022-004579.
- 3) Valentina Cazzetta, Delphine Depierreux, Francesco Colucci, Joanna Mikulak and Domenico Mavilio. **NKG2A Immune Checkpoint in V δ 2 T Cells - Emerging Application in Cancer Immunotherapy.** *Cancers*. 2023. doi: 10.3390/cancers15041264.
- 4) Elin Bernson, Oisín Huhn, Veronika Karlsson, Delia Hawkes, Maria Lycke, Valentina Cazzetta, Joanna Mikulak, James Hall, Anna M. Piskorz, Rosalba Portuesi, Domenico Vitobello, Barbara Fiamengo, Gabriele Siesto, Amir Horowitz, Hormas Ghadially, Domenico Mavilio, James D. Brenton, Karin Sundfeldt and Francesco Colucci. **Identification of Tissue-Resident Natural Killer and T Lymphocytes with Anti-Tumor Properties in Ascites of Ovarian Cancer Patients.** *Cancers*. 2023. doi: 10.3390/cancers15133362.

Dissemination of results

Preliminary results of this project were presented as oral presentation and posters in the conferences listed below:

1. Poster presentation at SIICA International Retreat of PhD Students in Immunology 2022

"Targeting $\gamma\delta$ T cells for immunotherapy of endometrial cancer"

Valentina Cazzetta, Rosalba Portuesi, Domenico Vitobello, Joanna Mikulak and Domenico Mavilio

15/09/2022-17/09/2022 L'Aquila, Italy

2. Oral and poster presentation at Immunology 2023

"Targeting $\gamma\delta$ T cells for immunotherapy of endometrial cancer"

Valentina Cazzetta, Rosalba Portuesi, Domenico Vitobello, Joanna Mikulak and Domenico Mavilio

11/05/2023-15/05/2023 Washington DC, USA

3. Poster presentation at XIV National Congress SIICA 2023

"Targeting $\gamma\delta$ T cells for immunotherapy of endometrial cancer"

Valentina Cazzetta, Paolo Marzano, Rosalba Portuesi, Domenico Vitobello, Joanna Mikulak and Domenico Mavilio

22/05/2023-25/05/2023 Verona, Italy

4. Poster presentation at 12 Milano Meets Immunology (MMI) 2023

"Targeting $\gamma\delta$ T cells for immunotherapy of endometrial cancer"

Valentina Cazzetta, Paolo Marzano, Rosalba Portuesi, Domenico Vitobello, Joanna Mikulak and Domenico Mavilio

13/07/2023 Milano, Italy

5. Poster presentation at 7 BioMeTra Workshop

"Targeting $\gamma\delta$ T cells for immunotherapy of endometrial cancer"

Valentina Cazzetta, Paolo Marzano, Rosalba Portuesi, Domenico Vitobello, Joanna Mikulak and Domenico Mavilio

19/09/2023 Segrate, Italy

In the future months, I will present part of the data of this project as poster presentation at the 6th International Conference on Translational Immunology.

Moreover, being a recipient of the AIRC fellowship from March 2023, I will present the aim of the association to students at a high school. On this occasion, I will explain my PhD project and the results obtained.

Lay Summary

Gynecologic malignancies are a group of solid tumors which include endometrial cancer (EC) and ovarian cancer (OC). Patients with advanced/recurrent disease have poor outcomes and respond poorly to current management treatments. Thus, it is important the development of novel therapeutic approaches. In this context, remarkable progresses have been made in immunotherapy. To date, the efficacy is not satisfactory, thus a detailed understanding of the biology and interactions between cancer and the immune system is essential for the recognition and development of new therapeutic strategies.

In EC unconventional $\gamma\delta$ T cells infiltrate EC lesions. These cells maintain their functional activity, however, within the tumor, they exhibit increased expression of PD-1. Crucially, their activity can be enhanced by PD-1 blockade and their presence and specific activation have been associated with the response to anti-PD-1 therapy. These data indicate the feasibility of targeting $\gamma\delta$ T cells to enhance current immunotherapeutic strategies for EC.

On the other hand, within the OC lesions, we detected high enrichment of CD4 regulatory T cells (Tregs) displaying heterogeneous profiles and promoting tumor progression. These findings highlight the pivotal role of Tregs in the early establishment of an immunosuppressive tumor microenvironment in OC and the potential of manipulating Tregs as a promising target for therapeutic interventions.

Le neoplasie ginecologiche sono un gruppo di tumori solidi che comprendono il cancro dell'endometrio e il cancro dell'ovaio. I pazienti con malattia avanzata/ricorrente hanno prognosi negativa e rispondono scarsamente agli attuali trattamenti. Pertanto, è importante lo sviluppo di nuovi approcci terapeutici. In questo contesto sono stati compiuti notevoli progressi nel campo dell'immunoterapia. Ad oggi l'efficacia non è soddisfacente, quindi una comprensione dettagliata della biologia e delle interazioni tra cancro e sistema immunitario è essenziale per il riconoscimento e lo sviluppo di nuove strategie terapeutiche.

Nel cancro dell'endometrio le cellule T $\gamma\delta$ non convenzionali infiltrano la lesione. Queste cellule mantengono la loro attività funzionale, tuttavia, all'interno del tumore, mostrano una maggiore espressione di PD-1. La loro attività può essere potenziata dal blocco del PD-1 e la loro presenza e attivazione specifica sono state associate alla risposta alla terapia anti-PD-1. Questi dati indicano la possibilità di utilizzare le cellule T $\gamma\delta$ per migliorare le attuali strategie immunoterapeutiche per il cancro dell'endometrio.

D'altra parte, all'interno delle lesioni di cancro dell'ovaio, abbiamo rilevato un elevato arricchimento di cellule T regolatorie CD4 (Treg) che mostrano profili eterogenei e promuovono la progressione del tumore. Questi risultati evidenziano il ruolo chiave delle Treg nella creazione precoce di un microambiente tumorale immunosoppressivo nel cancro dell'ovaio e la possibilità di manipolare le Treg come bersaglio promettente per interventi terapeutici.