ESMO Translational Research Fellowship

(July 2019 – June 2021)

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FINAL REPORT

Host Institute: **Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland**
Mentor: **Prof. George Coukos and Dr. Denarda Dangaj**
Project title: **Characterization of stromal TILs specificity and activity in immune excluded epithelial ovarian cancer**

Home Institute: **Institute for Cancer Research and Cure (IRCCS), Candiolo, Turin, Italy**

Introduction

Epithelial ovarian cancer (EOC) is still the leading cause of death from gynaecological malignancies [1]. Despite documented evidence that the presence of tumor-infiltrating lymphocytes (TILs) in EOC correlates with overall survival (OS) [2] and that many EOCs possess immune circuits and express a wide range of tumor-associated antigens (TAAs), immunotherapy has obtained modest results so far [3-5]. Lack of response may in part be due to cancer-intrinsic intratumoral heterogeneity that associates with treatment resistance [6]. In addition, the heterogeneity that exists at the level of the tumor microenvironment (TME) is also likely to play a crucial role in shaping the response to immunotherapy [7].

Rationale and Aim

Solid tumors including EOC have been classically classified into three groups based on the geographical location of tumor infiltrating $CD8^+$ T cells: (1) the inflamed/infiltrated phenotype, which is infiltrated by CD8+ T cells in the tumour bed ; (2) the immune-excluded phenotype, which is infiltrated by CD8+ T cells in the tumour stroma/periphery and less in the tumour epithelium and (3) the immune desert phenotype, in which CD8+ T cells are absent or present in very low numbers with regard to both the tumor periphery/stroma and the tumor beds. [7]. These histologically established tumour-immune phenotypes provided a useful framework to profile immune contexture in solid tumours. However, it remains technically challenging to systematically define the immunophenotype of the TME of EOCs due to the highly heterogeneous and complex nature of its immune cell infiltration and their distribution. Moreover, it remains poorly understood how EOCs orchestrate their TMEs and understanding the crosstalk between the TIL compartments and the TME is an unmet need for ovarian cancer. Therefore, we aim to deeply characterize the TME leucocytes networks of EOC in order to identify the underlying mechanisms which sustain or limit T-cell inflammation.
Specific aims are as follow:
1) Describe $CD8^+$ TILs infiltration and the TME in EOC taking into account the intrinsic intra-tumoral heterogeneity (ITH)
2) Describe the evolution of $CD8^+$ TILs and leucocytes subpopulations from primary tumor to first recurrence
3) Characterize the TILs-antigen presenting cells (APC) niches ecosystem, their relevance for EOC TME and responses to chemo/immunotherapy
The ultimate goal is to identify the subset of patients who could derive the maximum benefit from immunotherapy.

Experimental design

Two big retrospective clinical cohorts including paired EOCs FFPE samples from primary tumor and first recurrence (UPENN [n=124] and IMCOL [n=56], Table 1 and 2, respectively) were characterized by multiplex immune-fluorescence (mIF).
Our UPENN cohort is comprised of 31 (50%) platinum-sensitive patients, 20 (32.2%) partially platinum sensitive and 11(17.8%) platinum resistant, while IMCOL cohort represents a fully platinum cohort with high rate of optimal primary

surgery debulking (89.3%). First-line chemotherapy with three-weekly carboplatin-taxol regimen was consistent between the two cohorts. All haematoxylin and eosin (H&E)-stained cases were reviewed by a dedicated Pathologist to define the quality of tumor and stromal areas and exclude adjacent healthy tissue. Ventana Medical Systems and multispectral microscopy imaging (PerkinElmer Vectra 3.0™ technology) were used for the multiplex analysis. To identify and compare the phenotype of immune cell populations infiltrating both tumor and stromal islets we applied a mIF antibody panel staining for $CD8^+$, $CD11c^+$, $CD68^+$, $PD\text{-}1^+$, $PDL\text{-}1^+$, CK^+ and DAPI. Besides cell subset quantification we sought to identify $CD8^+$TILs-APC interacting micro neighborhoods or so called "CD8 TILs-APC niches". Immuno-phenotyping analysis was performed by digital pathology using the InForm software. We computed the number of positive cells of a given phenotype and normalized it by the tumor and stroma surface area (mm^2). We built in-house bioinformatic algorithms to automatically compute $CD8^+$ TIL infiltration by taking into consideration their infiltration intensity but also spatial distribution within the same tissue. In addition, we developed algorithmic methodologies for the computation of TILs-APC niches and the heterogeneity of their distribution in given neighborhoods.
Consecutive freshly-cut slides will be used to deeply characterise the $CD8^+$TILs-APC niches with the Hyperion Imaging System (®Fluidigm) which allows for simultaneous interrogation of 25 to 37 protein markers at subregional level while preserving the information in tissue architecture, cellular morphology and cell interactions.
Total DNA was extracted from FFPE selected samples using @Qiagen Isolation Kit for FFPE. A downstream library with customized-targeted genes panel (n=49) related to Homologous Recombination Deficiency (HRD) was built using ArcherDX VariantPlex and 8Mi reads were obtained per sample. Deep-sequencing analysis, including copy-number variants (CNV) and application of the sigma-3 signature will be applied [6].
To set up models of primary and recurrent peritoneal ovarian cancers we employed the *Brca1/2* isogenic ID8 cell lines (*Trp53$^{-/-}$Brca1/2$^{+/+}$*, *Trp53$^{-/-}$Brca1$^{-/-}$*, *Trp53$^{-/-}$Brca2$^{-/-}$*) [9-10]. We evaluated their *in-vivo* intraperitoneal tumor growth kinetics by Luciferase imaging, assessed their response to chemotherapy and characterized their immune infiltration and immune cell state comparatively, based on BRCA tumor mutational state. Mice were treated with weekly intraperitoneal (i.p) carboplatin (@Accord, 20mg/Kg) + Paclitaxel (@Labatec, 3mg/Kg) for 6 cycles mimicking EOC standard of care and sacrificed upon tumor growth. Tumor tissue as well as spleen and blood samples were harvested and immune infiltration is being analyzed by flow cytometry analysis (FACS) and chromogenic staining (IHC).

Results, Conclusions and Future Perspectives

Aim 1: Describe TILs infiltration and the TME in EOC taking into account the intrinsic intra-tumoral heterogeneity (ITH)

We first converted the well-established value of a mean of 5 intraepithelial(ie) CD8+/HPF [2] measured by conventional IHC to a cut-off equal to a mean of 21CD8+/mm2 quantified by mIF (Fig.1A). We validated this new mIF $CD8^+$ TIL cut-off according to OS in our two clinical cohorts. We demonstrated that patients with tumors infiltrated by >21cells/mm2 ie$CD8^+$ TILs had a better overall survival than those with tumors infiltrated <21cells/mm2 ie$CD8^+$ TILs, even when correcting for the residual tumor (R=0) at first-surgery (Fig1B).
While our new mIF-based cut-off performed well, we acknowledge that it still represents a mean value of CD8+ T cell infiltration and ignores the observed vast heterogeneity of CD8+ cells distribution across large tissue areas. To account for the intrinsic ITH of CD8+ T cell infiltration, we applied this new cut-off (21 CD8+ TILs/mm2) to small sub-regions of segmented tissues (or regions of interest, ROIs) acquired for both the epithelial and the tumor-related stroma compartments. We plotted the continuous distribution of intra-epithelial and stromal CD8+ infiltration within the same patient and established a new algorithm to define 4 new immune-categories in our two clinical cohorts: purely or homogenously inflamed, mixed or heterogeneously inflamed, excluded and desert ovarian cancers (Fig.1C-D). As shown in Figure 1E we demonstrated that patients with purely-inflamed and mixed-inflamed ovarian cancer tissues have a better and statistically significant OS compared to those with excluded and desert ovarian cancers (p=0.0214) (Fig.1E). To validate these results with applied our algorithm to a third external validation OC cohort (N=57). We obtained similar results and our algorithm separated long-term survivors (purely-inflamed and mixed-inflamed ovarian cancers) from patients with poor survival (excluded and desert ovarian cancers) (data not shown). This is to our knowledge the first algorithm with prognostic value that captures the heterogeneity of CD8+ TIL infiltration in EOC.
We then calculated the composition of our ovarian cancer cohorts in terms or CD8+ TIL inflammation. We found that the percentage of purely-inflamed samples varied from 39% to 17% in IMCOL and UPENN cohort respectively, the mixed-inflamed from 46% to 21%, 15% to 48% the excluded and no desert samples were present in IMCOL cohort versus a 14% in the UPENN series (fig.1D). We hypothesized that the difference in the percentage of inflamed and desert

samples observed between the 2 cohort may be related to the different enrichment in fully-platinum sensitive patients (Table 1 and 2) as a surrogate of BRCAness. Genome analysis, as described in methods section, is actually ongoing to correlate samples' immune-status with BRCA1/2 mutation and HRD.
To fully describe the TME captured by our mIF panel in each of our four novel ovarian cancer immuno-categories, we then plotted the cell densities for several phenotypes such as $CD8^{+}PD1^{+}$, $CD11c^{+}PD\text{-}L1^{+}$ and $CD68^{+}PD\text{-}L1^{+}$. We observed a clear up-regulation of CD8+PD1+, CD11c+PD-L1+ and CD68+PD-L1+ populations in tissue subregions (ROIs) of purely-inflamed samples (Fig.2A).

Aim 2: Characterize the TILs- APC niches ecosystem, their relevance for EOC TME and responses to chemo/immunotherapy.
We indirectly interrogated subregional neighborhoods networks by correlating mutually exclusive cell densities in all ROIs and observed that that purely-inflamed and platinum sensitive cases are enriched in homotypic niches ie-T cell- to-T-cell (i.e.CD8+/PD-1neg to CD8+/PD-1+) and macrophages-to-antigen-presenting cells (APC, i.e. CD11c to CD68+ expressing or not PD-L1+) but also heterotypic niches (Fig.2B).
By employing the *cell X- cell Y* positions we showed that it is possible to digitally reconstruct IF images and identify both homotypic and heterotopic TILs-APC niches embedded in tumor and stromal islets (examples shown in Fig.2 C-D). .
Using in-house established algorithms we computed the frequency of a given neighbor within 20um radius of a cell of interest (Fig. 3A). For example we set CD8+PD1+ as the "cell X-starting point" and calculated the PDL1 positive and negative APCs around this radius. We observed that that purely-inflamed samples exhibit higher number of TILs-APC interactions both in the intra-tumoral and stromal compartment (Fig.3B-C).
Moreover, a cohort of melanoma patients (N=40, 13 BRAF V600E mutated and 27 WT) treated with check-point inhibitors (N=20 responders and non-responders, respectively) are being analyzed at the current status of this report to validate the relevance of TILs-APC niches as a predictive biomarker for ICB treatment and potential prognostic value.
From mIF we could identify the subregions (ROIs) that were enriched in heterotypic and homotypic niches. Thus we were able to guide Hyperion imaging system to acquire images in selected ROIs and depict the real niches' ecosystem by tissue mass cytometry using a large panel of 25 markers) immune population (a representative example is shown in Fig.3D). Our future plan is to deeply characterise by Hyperion "mixed-inflamed" and "excluded" tissue regions. To capture the transcriptomic states of these regions and their mechanisms of TIL recruitment or exclusion, we will apply In Nanostring GeoMix already established in our immune landscape core facility.
To further validate results for the entire tissue slides we are employing tCYCIF which permits analysis of up to 40 leucocyte markers of patients comprising our 4 immuno-phenotypes (collaboration with Peter Sorger's Lab, Harvard University). This avenue aims at characterizing in our new immunophenotypes of ovarian cancers, more broadly the leucocyte and stromal networks that support, sustain or exclude CD8 T cell infiltration.
Furthermore, as our cohorts include some ovarian cancer cases with multiples sites at primary and secondary debulking we aim to perform single-cell analysis to further depict TILs and APC states and to capture ITH within the same patient.

Aim 3: Describe the temporal heterogeneity and evolution of TILs and leucocytes from primary to first recurrence surgery in ovarian cancer
To understand TILs evolution from primary tumor to first recurrence we represented a circus plot of evolution based on ieCD8+ of our immune-categories and showed that the CD8 evolution is indeed dynamic (Fig.3A). We observed 3 distinct patterns of intra-epithelial CD8+ infiltration: EOCs that have an increase, no changes or a decrease of TILs at recurrence confirming what has been already described in the context of neo-adjuvant studies in EOCs (*Nelson et al, Clin Cancer Research 2017*) and at first relapse (*Stanske et al., Neoplasia 2018*). Interestingly, out of the 4 categories only purely-inflamed EOCs showed the persistence of TILs-APC niches at recurrence as well as only fully-platinum sensitive (PS) ones while partially-platinum-sensitive and platinum-resistant (PR) showed lack of these interactions (Fig.3B-C). Moreover, as already showed in the previous report, only PS patients showed an increase in CD8+ in both the intra-epithelial and stromal compartment (Fig3D).
Thus, we hypothesize that TIL-APC niches maintain immune-inflammation over time, while their loss could affect the TME and consequently impact prognosis. To create models of primary and recurrent settings with known BRCA status, link tumor genetics with evolution of CD8+ TILs infiltration and dive deeply into transcriptomic phenotypes by scRNAseq and FACS, we treated C57BL6 ID8mice with the combination of carboplatin-taxol and evaluate tumor growth as displayed in Fig.4 (see also Methods section). After an initial response, both p53 and BRCA1mut subgroups showed

recurrence thus mimicking PROC (Fig.4E). At FACS analysis, BRCA1mut mice exhibit at recurrence a statistically significant higher number of CD8+ and CD11c+ in the spleen (Fig.4F), while no difference was observed in the tumor. IHC staining of CD8+ and CD11c+ on harvested tumor tissue is currently ongoing.
Interestingly, TIGIT and TIM-3 expression on both CD4+ and CD8+ was also higher in BRCA1 compared to p53, while no difference was noticed in relation to PD-1 (Fig.4G).

Conclusions and Future Perspectives:
Immune-checkpoint inhibitors failed to demonstrated activity in EOC so far and biomarker for patients' selection are urgently needed to guide next-generation of immunotherapy trials.
This is the largest study of primary and recurrent EOCs to date. Our results demonstrated CD8+ T-cell infiltration and spatial distribution in the TME are more on a continuum rather than discrete entities and therefore we propose a new immune-classification for EOC which takes into account patients' ITH, being "purely-inflamed patients" a minority. Moreover, we demonstrate that TILs-APC niches are enriched in the subset of "purely-inflamed" (17-30% in different cohorts) and maintain inflammation during evolution. This subgroup of patients could represent the ideal candidates for immune checkpoint blockade (ICB) therapy. Further analysis are needed to validate the above potential biomarker in other cancer types (i.e. melanoma) and its relevance for response to ICB.
Comparative analysis of the same tumors with divergent T cell infiltration from different areas or two different time points will allow us to make meaningful observations on potentially common mechanisms that drive T cell infiltration and phenotype at the steady state or post chemotherapy. Preliminary data on mouse model suggest that other check-point inhibitors beyond anti-PD-1 (i.e. TIM-3 and TIGIT) may warrant further investigation in BRCA1mut patients at recurrence.
We believe that elucidating how tumors orchestrate their microenvironments and how cell-cell interactions affect T cell infiltration will provide a framework for understanding the (lack of) response to immunotherapy in EOC and hopefully reflect in new insights of how pathogenic interactions can be reverted and a hope for making immunotherapies effective in this malignancy.

List of Publications and Presentations Resulting from the Translational Research Project

- **Intra-tumoral heterogeneity and evolution of TILs-APC niches in epithelial ovarian cancer.** *Pubblication expected by the end of the year*
- Facts and Hopes in Immunotherapy in Ovarian Cancer. *Clinical Cancer Research Review, publication expected by the end of the year.*

List of Publications and Presentations resulting from other projects during the fellowship period (if applicable)

- Low Dose Radiotherapy Reverses Tumor Immune Desertification and Resistance to Immunotherapy. Herrera F, Ronet C, Ochoa de Olza M, Barras D, Crespo I, Andreatta M, Corria-Osorio J, Spill A, Benedetti F, Genolet R, Orcurto A, Imbimbo M, **Ghisoni E**, Navarro Rodrigo B, Berthold D, Sarivalasis A, Zaman K, Duran R, Dromain C, Prior J, Schaefer N, Bourhis J, Dimopoulou G, Tsourti Z, Messemaker M, Smith T, Warren S, Foukas P, Rusakiewicz S, Pittet M, Zimmermann S, Sempoux C , Dafni U, Harari A, Kandalaft L, Carmona S, Dangaj D, Irving M, George Coukos. ***Cancer Discovery, in revision.***

- Cell-autonomous Inflammation of BRCA1-deficient Ovarian Cancers Drives both Tumor-intrinsic Immunoreactivity and Immune Resistance through STING. Bruand M, Barras D, Mina M, **Ghisoni E**, Morotti M, Lanitis E, Fahr N, Desbuisson M, Zhang H, Chong C, Chee S, Tsianou T, Dorier J, Stevenson BJ, Iseli C, Ronet C, Bobisse S, Genolet R, Walton J, Bassani-Sternberg M, Kandalaft LE, Ren B, McNeish I, Swisher E, Harari A, Delorenzi M, Ciriello G, Irving M, Rusakiewicz S, Foukas PG, Martinon F, Dangaj D. and Coukos G.. ***Cell Reports, in press.***

- Late-onset and long-lasting immune-related adverse events from immune checkpoint-inhibitors: An overlooked aspect in immunotherapy. Ghisoni E, Wicky A, Bouchaab H, Imbimbo M, Delyon J, Gautron Moura B, Gérard CL, Latifyan S, Özdemir BC, Caikovski M, Pradervand S, Tavazzi E, Gatta R, Marandino L, Valabrega G, Aglietta

M, Obeid M, Homicsko K, Mederos Alfonso NN, Zimmermann S, Coukos G, Peters S, Cuendet MA, Di Maio M, Michielin O. ***Eur J Cancer. 2021*** **May;149:153-164.**

- Ovarian Cancer Immunotherapy: Turning up the Heat. Ghisoni E, Imbimbo M, Zimmermann S, Valabrega, G. **Int J Mol Sci 2019 Jun 15;20(12):2927.**

Posters:

- In-depth immune and molecular profiling of melanoma patients receiving adoptive T-cell therapy reveals biomarkers of efficacy in ATATIL study. Orcurto A, Chiffelle J, **Ghisoni E,** Barras D, Crespo I, Navarro Rodrigo B, Ochoa de Olza M, Imbimbo M, Rusakiewicz S, Tissot S, Gannon P, Dafni U, Zimmermann S, Kandalaft LE, Michielin O, Bassani-Sternberg M, Dangaj D, Trueb L, Harari A and Coukos G. **Journal of Clinical Oncology 2021 39:15_suppl, 2533-2533. ASCO 2021 Abstr 2533.**

Selection of Courses and Workshops Attended During the Fellowship

- ESMO Gynecological Cancer 2021 Virtual, 25-26 June 2021
- Ludwig-Oxford Symposium on Cancer Early Detection and Epigenetics, 28-29 April 2021
- ESMO Immuno-Oncology Congress, 11-14 December 2019, Geneva, Switzerland (poster presenter)
- ESMO Advanced Course on Lung Cancer in Immunotherapy, 3-4th July 2019, Zürich, Switzerland

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All my colleagues and the staff of the Immuno-Oncology Service at CHUV.

Personal Statement

I would like to thank ESMO for this great opportunity, which has already become a great success, personally and professionally, and which will continue with several ongoing research projects and my MD-PhD track. The experience I gained will sustainably impact my future career as medical oncology and clinician scientist.

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Table 1 and 2. IMCOL and UPENN cohorts' baseline patients' characteristics.

Patient number (N)	**28**
Median age at diagnosis, years (range)	58 (33-72)
Histological subtype	
High grade serous	24 (85,7%)
Endometrioid	2 (7,1%)
Clear cell	2 (7,1%)
Staging (FIGO)	
IC	2 (7,1%)
IIB	3 (10,7%)
IIIA	1 (3,5%)
IIIB	3 (10,7%)
IIIC	13 (46,4%)
IV	4 (14,3%)
unknown	2 (7,1%)
Residual tumor at PDS	
R=0	25 (89,3%)
R=1	3 (10,7%)
Residual tumor at 2nd surgery	
R=0	22 (78,5%)
R=1	3 (10,7%)
unknown	3 (10,7%)
Median PFS1, months (range)	23 (13-99)
Median PFS2, months (range)	19 (4-38)
Adjuvant chemoterapy	
Carboplatin-taxol	28 (100%)
BRCA status	
gBRCA1-2 mut	4 (14,2%)
gBRCA wt	14 (50%)
uknown	10 (35,8%)

Patient number (N)	**62**
Median age at diagnosis, years (range)	54 (19-81)
Histological subtype	
High grade serous	53 (85,6%)
Endometrioid	2 (3,2%)
Clear cell	4 (6,4%)
Serous borderline	3 (4,8%)
Staging (FIGO)	
IA	1 (1,6%)
IC	1 (1,6%)
IIB	4 (6,4%)
IIIA	2 (3,2%)
IIIB	3 (4,8%)
IIIC	44 (71,1%)
IV	7 (11,3%)
Residual tumor at PDS	
R=0	36 (58,1%)
R=1	26 (41,9%)
Residual tumor at 2nd surgery	
R=0	41 (66,1%)
R=1	21 (33,9%)
Median PFS1, months (range)	10 (3-84)
Platinum-sensitive	31 (50%)
Partially platinum-sensitive	20 (32,2%)
Platinum resistant	11 (17,8%)
Median PFS2, months (range)	10 (2-246)
Adjuvant chemotherapy	
None	2 (3,2%)
Platinum-based doublet	57 (92%)
unkonown	3 (4,8%)
BRCA status	
gBRCA mut	1 (1,6%)
gBRCA wt	1 (1,6%)
uknown	60 (96,8%)

Fig 1. New Immune classification of EOC.

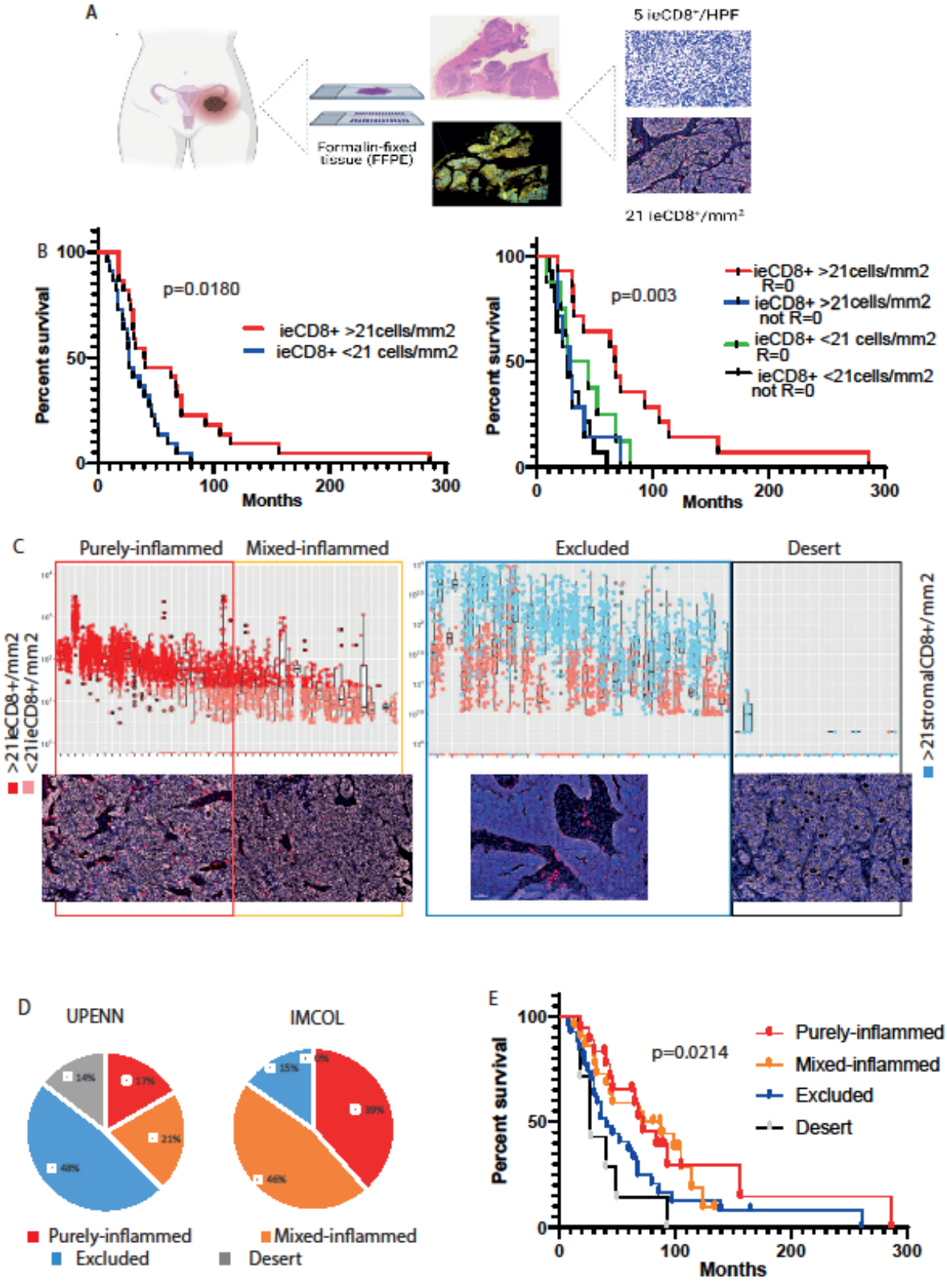

Fig. 2. TME and TILs-APC description across different immune-categories in EOC.

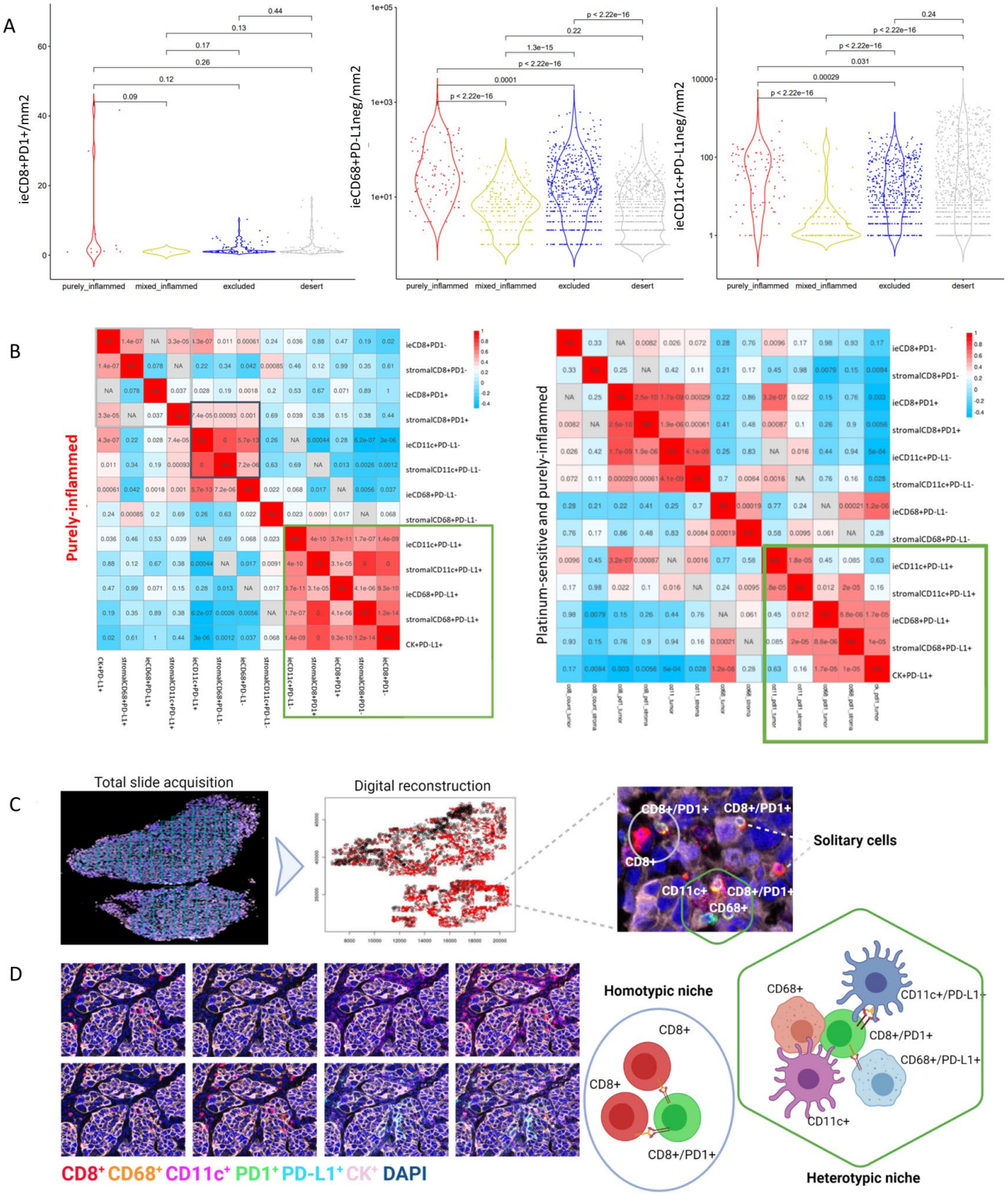

Fig. 3. Niches' ecosystem and TILs-APC networks.

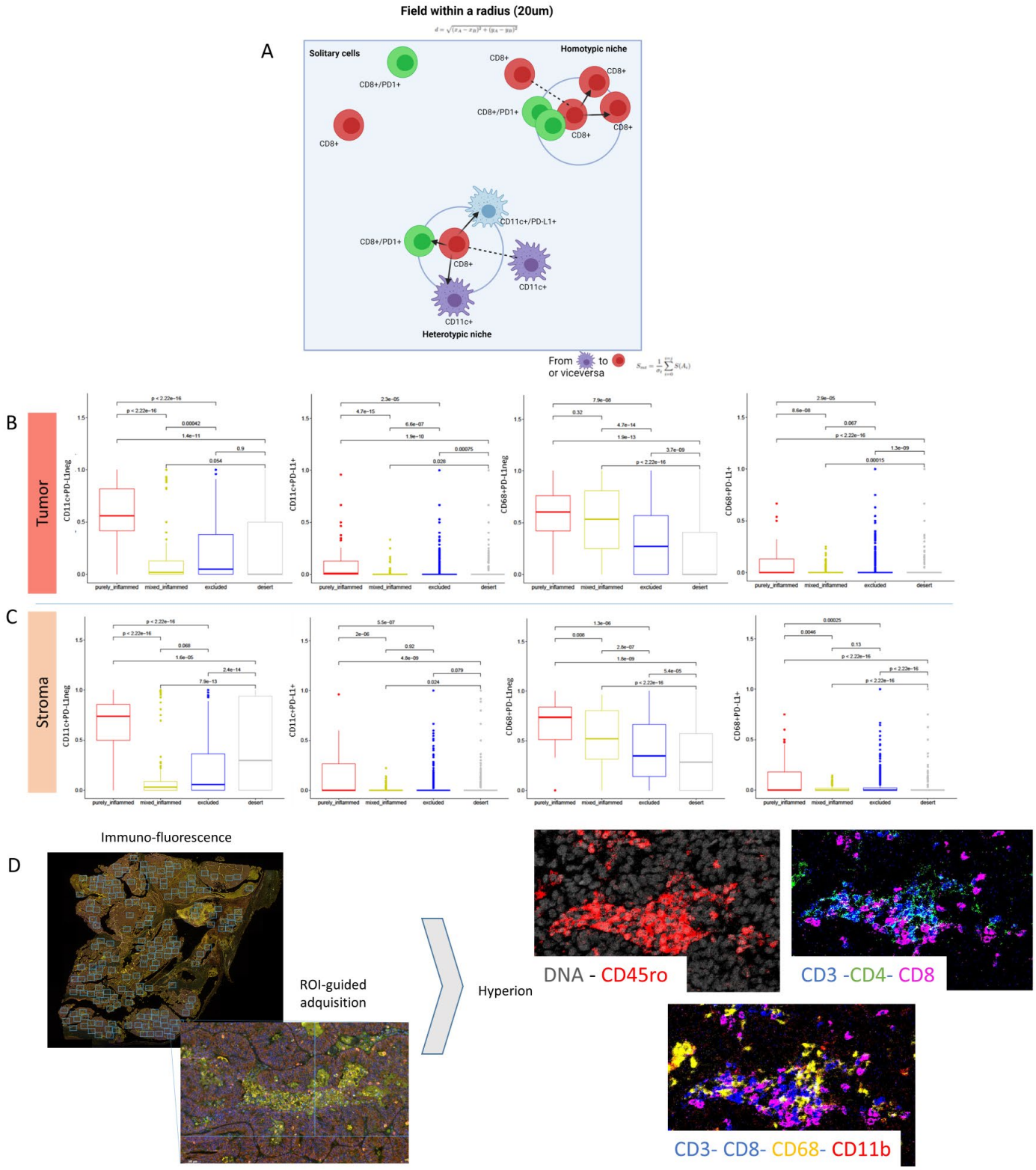

Fig. 4. TILs-APC evolution in EOC.