ESMO Research Research Fellowship

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FINAL REPORT

Host Institute: **Cancer Research Center of Toulouse (CRCT, Inserm UMR 1037) and Institut Universitaire du Cancer de Toulouse-Oncopole**
Mentor: **Prof Maha Ayyoub**
Project title: **The role of tumor-specific CD8+ T cells in tumor evolution and response to immune checkpoint inhibitors in cervical cancer patients**
Home Institute: **Università degli Studi di Catania**

Introduction

Cervical cancer (CC) is the fourth most common female malignancy worldwide and results in over 300000 deaths every year [1]. High-risk subtypes of the human papilloma virus (HPV) are the cause of the disease in most cases. Despite significant decrease in mortality rates due to successful screening and HPV vaccination programs, for women with metastatic or recurrent disease, treatment options are scarce and prognosis remains poor. In the last decade, immunotherapy and especially immune checkpoint inhibitors (ICIs) have transformed cancer care and prognosis of several advanced stage solid tumors. More recently, PD-1 pathway inhibitors, a class of ICIs, have shown to improve survival in persistent, recurrent or metastatic CC patients and several lines of evidences point toward a strong rational behind this treatment strategy [2-8]. Unfortunately, primary and secondary resistance to ICIs is still a major clinical problem and the comprehension of the immunological mechanisms behind resistance is a central point for the development of effective immunotherapy strategies and the identification of predictive biomarkers.

Rationale and Aim

Antigen (Ag)-specific cytotoxic CD8+ T-cells are the most powerful effectors in the anticancer immune response and form the backbone of current successful cancer immunotherapies. The characterization of Ag-specific CD8+ T-cell exhaustion and responsiveness to ICIs is of paramount importance to understand primary and secondary resistance to ICIs as well as to guide the identification of predictive biomarkers to improve patient selection and immunotherapy strategies. Analysis of Ag-specificity of adoptively transferred tumor-infiltrating lymphocytes from CC patients responding to this therapy show that they contain T cells specific for three Ag families: cancer testis antigens (CTA), neoantigens and HPV antigens [8]. HPV-specific CD4+ and CD8+ T-cell responses have been previously assessed in CC patients, however no comprehensive assessment of immune checkpoint (IC) expression by these cells or by other Ag-specific CD8+ T cells, such CTA-specific, have been reported. Moreover, the mechanisms responsible for the instalment of CD8+ T-cell exhaustion and their relative contribution in regard of ICs expression are still unclear. In this context, the project allows the comparison of different Ag-specific responses in HPV+ CC and HNSCC potentially helping to shed light on the contribution of the tumor microenvironment characteristics, the tumor type and its carcinogenesis process or the Ag specificity itself in the development of CD8+ T-cell exhaustion. Expression of inhibitory ICs, such as PD-1, CTLA-4, TIGIT, and TIM-3, in tumor-infiltrating CD8+ T cells contributes to their functional exhaustion and has been recently shown, by our team, to be an indicator of spontaneous adaptive immune responses to tumors and a marker of response to ICIs [9,10]. In fact, tumor-specific and tumor infiltrating CD8+ T cells that concomitantly express PD-1, CTLA-4, TIGIT, and TIM-3 (defined as quadruple positive cells, QP) are endowed with high cytotoxic and functional potential and positively correlate with response and overall survival in ICI-treated squamous cell carcinoma of the head and neck (HNSCC) patients [9,10]. In

the same study, it has been demonstrated that circulating CTA-specific T cells in ovarian cancer express low levels of PD-1 and TIGIT but were CTLA-4 and TIM-3 negative. Importantly, these data imply that responsiveness to ICI is dependent upon a dual effect of PD-1/PD-L1 blockade: i) reversal of exhaustion of Ag-specific QP cells at the tumor site and ii) enhanced proliferation of PD-1+ circulating Ag-specific T cells that will replenish the tumor site.

The overall goal of this project is to decipher Ag-specific T-cell responses and exhaustion patterns in HPV+ CC. In parallel, the comparison of different Ag-specific CD8+ T-cell responses in CC vs HNSCC will help to shed light on the mechanisms guiding exhaustion instalment. Finally, these data will be instrumental to build a T-cell exhaustion signature that is predictive of disease extension and response to ICIs in CC.

AIM 1. Characterization of antigen-specific T-cell responses and exhaustion features in CC and HNSCC

Quantitative, transcriptomic and phenotypic characterization of circulating and tumor-infiltrating CD8+ T-cells specific for HPV and CTA antigens, in patients with premalignant lesions as well as treatment-naïve and ICI-treated HPV+ CC patients and HPV+ HNSCC. This approach will uncover potential Ag-specific, and/or site-specific regulatory mechanisms as well as the evolution of Ag-specific T-cells in the progression of the disease. Moreover, analysis of the quantitative evolution of Ag-specific CD8+ T-cells under ICI will allow for the comprehension of their contribution to ICI clinical efficacy.

AIM 2. Determine the prognostic and predictive value of CD8+ T-cell exhaustion signature in CC

Definition of an immunophenotype signature, in early stage vs locally advanced/metastatic CC specimens as well as in ICIs treated patients, to correlate the CD8+ T-cell exhaustion phenotype with clinical features and response to treatment.

Experimental design

The project has been implemented on a prospective cohorts of CC patients enrolled in two translational clinical protocols (MINER, NCT03514368 and DECIdE, NCT03958240) running across the Institut Universitaire du Cancer de Toulouse – Oncopole (IUCT-O), and the Cancer Research Center of Toulouse (CRCT). During the II year of this project, a prospective cohort of patients with a diagnosis of HNSCC, included in the above mentioned trials, has been integrated in the analysis. For each patient along with clinicopathological data, the following biological samples were collected: tumor infiltrating lymphocytes (TILs) and tumor biopsy at diagnosis, peripheral blood mononuclear cells (PBMCs) at diagnosis, at multiple time point during treatment with ICIs and at the time of progression.

AIM 1. Characterization of antigen-specific T-cell responses and exhaustion features in CC and HNSCC

For this purpose, fluorescent HLA class I/peptide multimers/tetramers and flow cytometry analysis will be used in order to characterize Ag-specific T cells, at diagnosis, in CIN3 as well as in early and late stage HPV+ CC and HPV+ HNSCC patients. The same technique will be used for the monitoring of responses in ICI treated patients. RNA-Seq technology will be used in order to comprehensively characterize Ag-specific T cells and tumor cells. This approach will uncover potential Ag-specific and /or site-specific regulatory mechanisms as well as the evolution of Ag-specific CD8+ T cells under ICI.

AIM 2. Determine the prognostic and predictive value of CD8+ T-cell exhaustion signature in CC

For this purpose, quantitative multiplex immunohistochemistry (qmIHC) will be used to quantify the signature in tumor biopsies obtained at diagnosis from early and late stage CC patients as well as prior to therapy from ICI-treated.

Results, Conclusions and Future Perspectives

Overall, 39 patients with a premalignant cervical cancer lesion (n = 6) or a CC diagnosis (n = 33) and 12 patients with p16+ HNSCC were included between January 2020 and April 2022. We first focused our effort on the HPV16+ CC (n = 13) and the p16+ HNSCC cohort. HPV+ tumors provide the opportunity to detect and characterize tumor-reactive CD8+ T cells adopting a defined set of virus-derived tumor-antigens. To do so, we focused on the known HPV oncogenes E5, E6 and E7, and on E2, which is involved in viral DNA maintenance. Potential CD8+ T cell epitopes derived from the HPV proteins and presented by a reference set of 27 human leukocyte antigens (HLA-A, B and C) covering 97% of the

population were predicted using the Immune Epitope Database, as previously described by Eberhardt and colleagues [11]. A total of 241 predicted 9–10 aminoacid-long peptides were commercially obtained. A peptide pool (Pool HPV) was generated containing all 241 peptides along with 23 minipools (MPs) containing each up to 22 peptides, with each peptides being represented in two MPs [Fig 1A]. To single out HPV-derived CD8+ T-cell epitopes, we cultured CD8+ T cells and CD14+ cells, isolated from PBMCs derived from CC and HNSCC patients, with the Pool HPV for 12 days. Then we tested the reactivity of expanded T cells with Pool HPV and MPs using intracellular cytokine staining (ICCS) [Fig 1B]. All single peptides shared by two MP were tested individually [Fig 1B-D]. Overall 37 different peptides have been identified (12 HPV E2 peptides, 12 HPV E5 peptides, 12 HPV E6 peptides, 3 HPV E7 peptides), in the 19 patients analysed so far [Figure 1E], with several epitopes being recognized by T cells from multiple patients. In order to predict peptide-HLA pairs for the entire cohort, patient- HLA-alleles were obtained by analysis of RNA-seq data on FFPE tumor samples or by HLA-typing using PBMC-derived DNA when tumor was not available. Preliminary data on an exploratory cohort of *ex vivo* HPV-specific circulating CD8+ T cells showed a different exhaustion profile in CC compared to HNSCC patients (data not shown). Based on these analyses ex vivo phenotyping of specific MHC class I tetramers is ongoing.

We analyzed the expression of CTAs from the RNA-seq data of patient's tumor, using the CTDatabase (www.cta.lncc.br), containing 276 listed CTAs. We excluded: i) CTAs expressed in healthy tissues other than the testis, ii) CTAs whose abnormalities are involved in diseases other than fertility or reproductive organ abnormalities and iii) CTAs expressed by almost all tumors. We also focused on CTAs that have been shown to be immunogenic in the literature. We decided to select CTAs with frank expression in some patients and absent in others and which are the most representative of the cohort, such as PRAME, KK-LC-1 (encoded by CT83), LAGE-1 (encoded by CTAG2), MAGEA3, MAGEA4, MAGEA6 and MAGEA12. Despite the absence of NY-ESO-1 expression (encoded by CTAG1B) in the RNA-seq data, we decided to retain this CTA due to the detection of a serological response in 1 patient with CC via ELISA (data not shown). CTA expression in each tumor sample is represented in Figure 2. The MAGE family is the most frequently expressed CTA in our cohort along with PRAME, followed by KKLC1 and NYESO1. The same strategy used for the definition of the HPV peptides has been used to generate pools, mini pools and single peptides of each of the mentioned CTA. PBMC-derived CD8+ T cell cultures with CTA derived peptides are ongoing. In addition, RNA-seq data are being analysed for tumor immune molecular signatures.

Regarding the *AIM 2* of this project, it is strictly dependent upon the information obtained through the AIM 1. No data are yet available.

We have set up an experimental strategy to dissect the role of HPV- and CTA-specific CD8+ T cells in CC and HNSCC at diagnosis and during treatment with ICIs. Our preliminary data suggest that a lower number of HPV-specific CD8+ T cells express PD-1, and potentially TIGIT, in CC compared to HNSCC. No conclusions can yet be drawn from these results. However, if these data are confirmed in the study cohort this could likely imply different immunophenotype/exhaustion profiles of HPV-specific CD8+ T cells in CC versus HNSCC. Indeed, the expression of different ICs in CD8+ T cells sharing the same HPV antigen specificity indicates that different stimuli (e.g. tumor microenvironment, HPV infection characteristics) would guide the immunophenotype/exhaustion of HPV-specific CD8+ T cells in CC vs HNSCC. Moreover, the integration in this project of the CTA-specific CD8+ T cell characterization allows shedding light on the role that different Ag families (viral vs CTA) play in the anticancer response and what is their exhaustion status in the same tumor type as well as across different tumor types. Altogether, the comprehension of these mechanisms may lead to a better tailoring of immunotherapy options.

The project will proceed in order to complete the following objectives:

- Completion of the identification of the immunodominant HPV- and CTA-epitopes and phenotyping of ex vivo specific T cells from PBMC and TIL using fluorescent HLA/peptide multimers. Matched analysis of tumor infiltrating Ag-specific CD8+ T cells will also be performed to characterize in depth these cells.
- RNA-seq data analysis to identify gene expression signatures of the tumor microenvironment potentially associated with specific CD8+ phenotypes.
- Gene-expression profile, immunophenotype and functional characterization will be performed on Ag-specific

CD8+T cells isolated and cloned from PBMCs of CC and HNSCC patients

- The data obtained above will be used to define an exhaustion signature in tumor-specific T cells that could be predictive of disease extension and of response to ICIs in CC patients

The fellow has been recently recruited as attending physician in the Phase I Trial Unit of the Institute Universitaire du Cancer de Toulouse-Oncopole. She also continues to be integrated as a researcher in the team directed by her ESMO mentor, Prof Maha Ayyoub, and will be responsible for the advancement of this project by mentoring and co-supervising a PhD student focusing on this work.

List of Publications and Presentations Resulting from the Translational Research Project "The role of tumor-specific CD8+ T cells in tumor evolution and response to immune checkpoint inhibitors in cervical cancer patients"

In view of the strong immunological background and translational nature of this project as well as the interconnection of *in vitro* and *in silico* experimental set up, no data have been submitted yet for publication. We plan to provide more robust data in the upcoming months and submit them for publication.

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

- Ricciuti B, Wang X, Alessi JV, Rizvi H, Mahadevan NR, Li YY, Polio A, Lindsay J, Umeton R, Sinha R, Vokes NI, Recondo G, Lamberti G, Lawrence M, Vaz VR, **Leonardi GC**, et al. Association of High Tumor Mutation Burden in Non-Small Cell Lung Cancers With Increased Immune Infiltration and Improved Clinical Outcomes of PD-L1 Blockade Across PD-L1 Expression Levels. JAMA Oncol. 2022 Aug 1;8(8):1160-1168. doi: 10.1001/jamaoncol.2022.1981. Erratum in: JAMA Oncol. 2022;8(11):1702.
- Karaca Atabay E, Mecca C, Wang Q, Ambrogio C, Mota I, Prokoph N, Mura G, Martinengo C, Patrucco E, **Leonardi GC**, et al. Tyrosine phosphatases regulate resistance to ALK inhibitors in ALK+ anaplastic large cell lymphoma. Blood. 2022;139(5):717-731
- Vivarelli S, Falzone L, **Leonardi GC**, et al. Novel insights on gut microbiota manipulation and immune checkpoint inhibition in cancer (Review). Int J Oncol. 2021;59(3):75.
- Mota I, Patrucco E, Mastini C, Mahadevan NR, Thai TC, **Leonardi GC** et al. Vaccination restores defective T cell priming to create effective ALK+ lung cancer immunotherapy in tandem with immune checkpoint and tyrosine kinase inhibition. Under review in Nature Cancer
- Cheong TC, Wang Q, Jang A, **Leonardi GC** et al. Genome-wide identification of oncogenic fusions by functional translocation sequencing. Under review in Nature

Selection of Courses and Workshops Attended During the Fellowship

ESMO Annnual Meeting 2022

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Personal Statement (not mandatory)

The ESMO Research Fellowship represents an exceptional opportunity to develop clinical and scientific expertise by spending a training period abroad. It also represents a great time for building a research and professional network. I would like to thank the ESMO Committee for giving me the opportunity to gain fundamental skills and cultivate collaborations in an innovative scientific field that has helped me advance my professional career.

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11. Eberhardt CS., et al. Nature (2021). Doi.org/10.1038/s41586-021-03862-z

SIGNATURES

Award Recipient full name	***Signature and Date***
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Research Mentor full name	***Signature and Date***
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