Spatial immunophenotyping of PD1 and PDL1 in tumor cells and microenvironment of Nodal Follicular T Helper Ce l Lymphoma and Peripheral T Ce l Lymphoma , NOS

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Introduction

Peripheral T-cell lymphomas (PTCLs), represent a diverse group of malignancies derived from mature T cells [1]. According to the World Health Organization (WHO), this category encompasses several distinct entities, with peripheral T-cell lymphoma not otherwise specified (PTCL-NOS) being the most frequently diagnosed subtype [2-3], accounting for approximately 35% of T cell lymphomas in western countries [4]. Application of immunohistochemistry (IHC) plays a vital role in subtype classification and identification of nodal T-follicular helper (TFH) cell lymphoma subtype within PTCL [5]. Although IHC is a valuable tool for targeted treatment regimens like PD1 (programmed cell death 1) antibodies for PDL1expressing tumors, colocalizing signals using conventional IHC and visual scores is challenging [6-7].

Recently few studies have studied simultaneous quantification of markers in follicular T helper cell lymphoma and peripheral T cell Lymphoma, NOS and immune cell populations by Multiplex Immunofluorescence [8,9,10]. Only in a recent study of immunophenotyping of the PD-L1-positive cells in angioimmunoblastic T cell lymphoma found that PD-1 was exclusively in T-lymphocytes, whereas PD-L1 was revealed in the tumor microenvironment cells including macrophages Ref [11]

The PD-1/PD-L1 pathway is a potential prognostic and therapeutic biomarker for PTCL. Recent study showed Programmed Cell Death Protein 1/Programmed Cell Death Ligand-1 Axis activates Intracellular ERK Signaling in Tumor Cells which mediates poor prognosis in T-cell Lymphoma. [12]. In a study, PD-1 and PD-L1 on tumor cells and/or reactive immune cells expressions tended to be higher in AITLs than in PTCLs-NOS and was related to shorter overall survival in patients with AITL [13]. Recently, Geptanolimab (GB226), an anti-PD-1 antibody, demonstrated an objective response rate of 40.4% in relapsed/refractory PTCL. A subgroup analysis showed better response and survival in patients with PD-L1 ≥ 50% [Ref 14]. A combination of checkpoint inhibitors with other agents could be a promising option to enhance anti-tumor activity in T cell lymphoma [15].

In this study we have used multiplex immunofluorescence (mIF) in peripheral T cell lymphomas to identify detailed spatial analysis of immune markers, offering insights into checkpoint pathways (PD1/PDL1) and potential targets for immunotherapy.

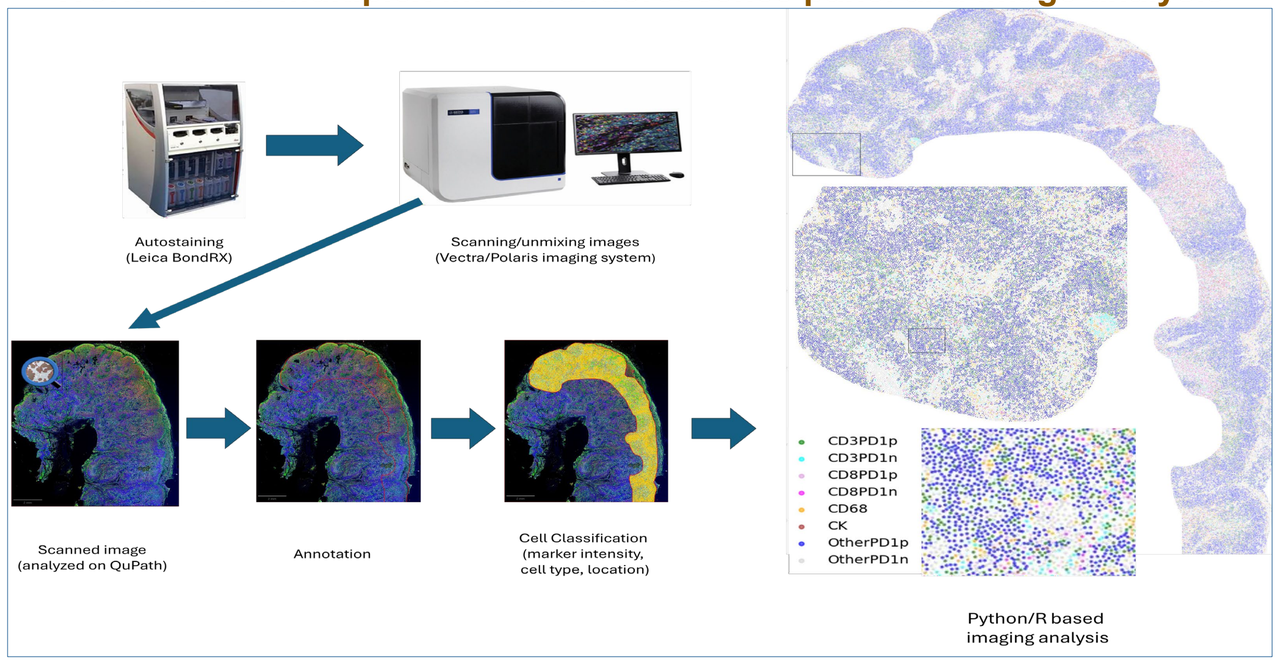
Design

Case selection: Retrospective cohorts were retrieved from archived samples of the Department of Pathology, UCDH. The cases were reviewed and confirmed of the diagnosis based on morphology and IHC between two board-certified hematopathologists 16 cases of peripheral T cell lymphoma including nodal follicular T Helper Cell Lymphoma were identified. All patient identifiers were removed, and the samples complied with the guidelines of our institutional review board.

Multiplex immunohistochemistry: For multiplex-immunohistochemistry, 4 microns tissue sections mounted on APEX BOND adhesive slide (Leica) were stained with Leica BondRX autostainer (Leica) by following pre-optimized immune panel (CD3, CD8, CD68, PD1, PDL1, panCK) (PMID: 33590360) [16]. Human tonsil sections were used as positive control for all markers, and the staining condition without primary antibodies was used as a negative control. Opal fluorophores (Akoya Biosciences) and Alexa750 (ThermoFisher) were assigned to each marker (CD3: Opal 480, PDL1: Opal520, CD8: Opal570, PD1: Opal620, panCK: Opal690, CD68: Alexa750). Stained slides were scanned with Vectra/Polaris multispectral imaging system (Akoya Biosciences).

Image acquisition and Data Analysis: Imaging analysis on pre-unmixed qptiff file was performed with QuPath(PMID: 29203879) [17]. The tissue images from our tissue sample and human tonsil were used to build AI- based cell classifier with cell specific markers. Regions of interest (ROI) were selected on IHC slide and equivalent area was annotated on QuPath to acquire cell segmentation data from the ROI. The cell segmentation data csv file was used to further analyze much detailed PD1 and PDL1 status on each cell. Python and R scripts were used to analyze and visualize each cell location.

Workflow for multiplex immunofluorescence panel and image analysis

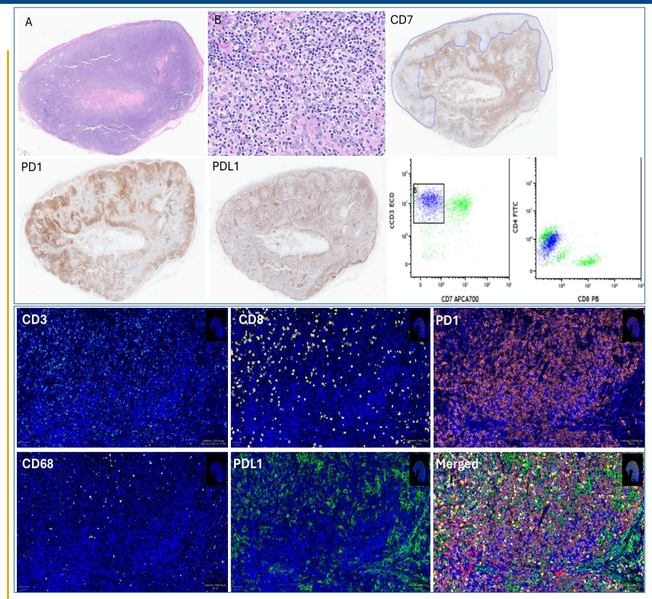


Results

A case of Angioimmunoblastic T Cell Lymphoma was selected with distinct immunophenotype lacking surface CD3 on flow cytometry. Tumor rich area was annotated based on the morphology, and immunohistochemistry of lymphoma (CD3dim+, CD7-, CD4+.CD8-) as an aid to differentiate lymphoma cells from reactive T cells in tumor microenvironment. High expression of PDL1 >50% was noted with strong and diffuse staining for PD1 on tumor cells (Top Panel). Images of single antibodies were merged to evaluate expression of multiple antibodies on the tumor cells and their microenvironment. In the tumor rich area, PDL1 staining pattern did not match CD68 and was most compatible with PD1 which was confirmed by merged images (Bottom Panel). Quantification analysis of the entire lymph node and annotated area revealed similar findings. Percentage of PDL1+ cells was calculated on T cells and CD8 positive T cells with and without PD1 as well as on histiocytes. Percentage of other PDL1+ cells with expression of PD1 was also measured. In this study we found that 71.07% of the histiocytes expressed PDL1. Considering Lymphoma is positive for PD1 and CD4; 49.9% of T cells in tumor microenvironment (CD3/PD1n) expressed PDL1 including subset of CD8+ T cells . PDL1 and PD1 was also expressed in 18.38% of CD8+ T cells in tumor microenvironment (Table 1).

Classification	PDL1+ Counts	PDL1- Counts	PDL1+ (%)
CD3PD1n	4458	4475	49.9
CD3PD1p	7848	32226	19.58
CD68	16905	6883	71.07
CD8PD1n	5532	6763	44.99
CD8PD1p	2418	10740	18.38
Other PD1n	43453	52720	45.18
Other PD1p	30675	113816	21.23

Table 1: Quantitative multiparametric analysis of cellular components



Top panel: A. HnE image showing an axillary lymph node involved by a T cell lymphoma B. Higher magnification image (40X) showing the tumor cells are medium to large with vesicular chromatin. CD7 immunohistochemistry (IHC) shows the tumor cells are negative for CD7, mapped on the image. PD1 IHC is positive on the tumor cells. PDL1 IHC shows subset positivity on the tumor cells. Flowcytometry images showing the tumor cells are positive for CD3 (cytoplasmic), dim subset CD4 positive and negative for CD7 and CD8.
Bottom panel: Immunofluorescence images. CD3, PD1 and subset PDL1 highlighting the tumor cells which are negative for CD8 and CD68. Merged image showing the spatial relationship of all the stained cells.

Summary and Conclusions

Multiplex immunofluorescence enabled quantification and analysis of cellular components within lymphoma. Although heterogeneity of the tumor and overlapping immunophenotype between lymphoma cells and background reactive T cells make distinction difficult; the case study of angioimmunoblastic T cell lymphoma provided insight into the expression of PDL1 in the lymphoma cells and its microenvironment. In this study we found that PD1 and PDL1 are expressed in subset of macrophages and reactive T cells in tumor microenvironment. In this PD1+ lymphoma, we noted that there are 19.58% of T cells that express PD1 and PDL1 which are not CD8 suggesting that they may express CD4. In addition , there are about 21.23% cells that are positive for PD1 but are not T cells or macrophages. We hypothesize these may represent the lymphoma cells either with dim or negative CD3 expression in this lymphoma distinctly negative for surface CD3. The study of our cohort cases will provide additional insight by optimal characterization of lymphoma cells among their microenvironment. Spatial proximity analysis will evaluate PDL 1 + cells within heterogenous environment of PTCLs . Outcomes will enhance our current knowledge in the expression of PDL1 in PTCLs which can be further support check-point inhibitors as targeted therapy in PTCLs.

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