Exploring CD4+ T Helper Cell Differentiation in Tumor and Peripheral Blood of Clear Cell Renal Carcinoma Patient Using a 50-Plus-Parameter CyTOF Panel

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Introduction

CD4+ T helper (Th) cells are a diverse subset of T cells that orchestrate immune responses to acute/chronic infection and tumor growth and are involved in the pathogenesis of autoimmune diseases. Triggered by polarizing cytokines, Th cells differentiate into a range of heterogeneous populations with distinct expression patterns of surface receptors, cytokines and master transcription factors (TFs). Differentiated Th cells such as Th1, Th2, regulatory (Treg), follicular helper (Tfh) and Th17 play discrete roles in combating or promoting specific pathogens or tumors. Recent studies showed that the balance of positive and negative effects of Th cells can influence overall success of cancer immunotherapy. Thus, a deeper understanding of Th cell differentiation and functional states in cancer patients is required to harness the full potential of the immune system to sustain a durable, robust antitumor response.

In this study, we evaluated Th cell subsets in the tumor tissue and peripheral blood from a clear cell renal carcinoma (ccRCC) patient using a comprehensive 50-plus-parameter CyTOF™ panel. This panel includes a diverse array of phenotypic and functional markers identifying Th cell subsets as well as evaluating T cell memory, activation, proliferation, differentiation and exhaustion. Heterogeneous Th cell subsets with divergent composition and functions were observed in both tumor and blood of the ccRCC patient.

Unlike fluorescence-based cytometry, CyTOF technology has low signal spillover and no autofluorescence, and therefore spectral compensation and unmixing are not required. As a result, CyTOF technology enables in-depth single-cell analysis with exceptional resolution and fast panel design. Furthermore, antibody cocktails and stained samples can be frozen for later use and acquisition, enabling a streamlined and flexible workflow in translational and clinical research.

Materials and methods

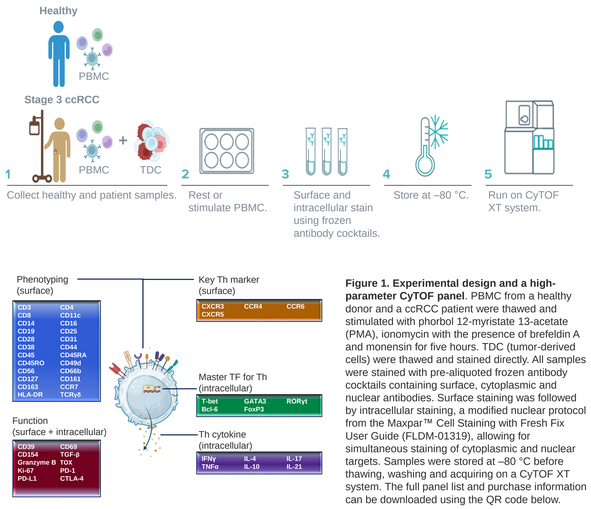

Figure 1. Experimental design and a high-parameter CyTOF panel. PBMC from a healthy donor and a ccRCC patient were thawed and stimulated with phorbol 12-myristate 13-acetate (PMA), ionomycin with the presence of brefeldin A and monensin for five hours. TDC (tumor-derived cells) were thawed and stained directly. All samples were stained with pre-aliquoted frozen antibody cocktails containing surface, cytoplasmic and nuclear antibodies. Surface staining was followed by intracellular staining, a modified nuclear protocol from the Maxpar™ Cell Staining with Fresh Fix User Guide (FLDM-01319), allowing for simultaneous staining of cytoplasmic and nuclear targets. Samples were stored at –80 °C before thawing, washing and acquiring on a CyTOF XT system. The full panel list and purchase information can be downloaded using the QR code below.

Conclusions

- Deep **single-cell profiling using a high-parameter CyTOF panel** unveiled an **immunosuppressive TME (tumor microenvironment)** composed of exhausted T cells, NK cells and suppressive macrophages
- Further **in-depth profiling** of **CD4+ Th cell subsets** from a ccRCC patient revealed Th17/Th1 dominant memory phenotypes in the PBMC sample whereas a Th1-polarized exhausted effector phenotype was found in the TDC sample
- **Exceptional signal resolution of TFs** disclosed a unique correlation between FoxP3 and Bcl-6 in TDC, implying the involvement of **Tfr (follicular regulatory T cells)** in the TME of ccRCC

Results

Deep cellular phenotyping with CyTOF technology reveal bias toward CD4+ T cell infiltration in the tumor microenvironment (TME)

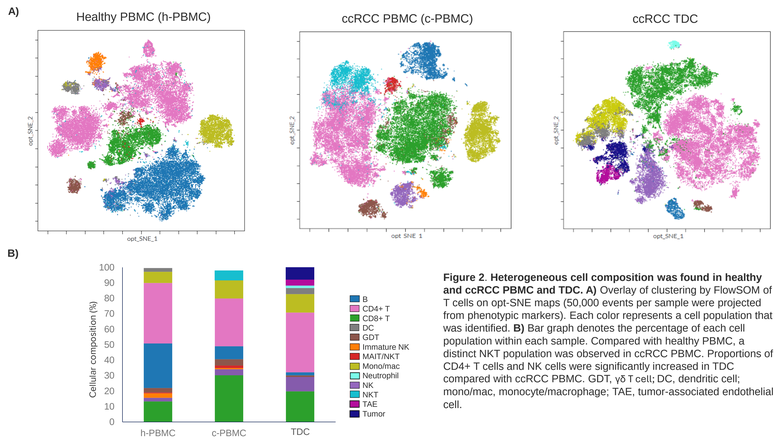

Figure 2. Heterogeneous cell composition was found in healthy and ccRCC PBMC and TDC. A) Overlay of clustering by FlowSOM of T cells on opt-SNE maps (50,000 events per sample were projected from phenotypic markers). Each color represents a cell population that was identified. **B)** Bar graph denotes the percentage of each cell population within each sample. Compared with healthy PBMC, a distinct NKT population was observed in ccRCC PBMC. Proportions of CD4+ T cells and NK cells were significantly increased in TDC compared with ccRCC PBMC. GDT, γδ T cell; DC, dendritic cell; mono/mac, monocyte/macrophage; TAE, tumor-associated endothelial cell.

Comprehensive single-cell profiling unveils an immunosuppressive TME composed of activated and exhausted T cells, NK cells and suppressive macrophages

Figure 3. Single-cell profiling revealed that T cells and NK cells became highly activated and exhausted, while macrophages became suppressive in the TME. A) CD45+ cells from blood and tumor of the ccRCC patient (50,000 events each) were projected into opt-SNE space using all phenotypic and functional markers. Hierarchical clustering heat map denotes transformed median expression of functional makers in PBMC and TDC across all identified cell populations. TCM, central memory T cell; TEMRA, terminally differentiated effector memory T cell; Teff, effector T cell. Colored boxes indicate three TDC clusters and their PBMC counterparts – activated and exhausted CD4+T/NKT cluster (red), suppressive macrophage cluster (green) and activated and exhausted NK cluster (blue). **B)** Biaxial plots show the upregulation of PD-1 and CD69 in MAIT/NKT; increased granzyme B and CD38 expression in NK cells; and elevated expression of granzyme B and TOX in CD8+ T cells, indicating activated and exhausted effector phenotype of T cells and NK cells in the TME. **C)** Expressions of key surface and nuclear functional markers were overlaid onto macrophage cluster of the opt-SNE coordinates. Upregulation of PD-L1 and CD39 combined with downregulation of Ki-67 and TGF-β suggest an immunosuppressive phenotype of macrophages in the TME.

In-depth profiling of CD4+ T helper cells shows dominant Th1 polarization in the TME with activated and exhausted effector phenotypes

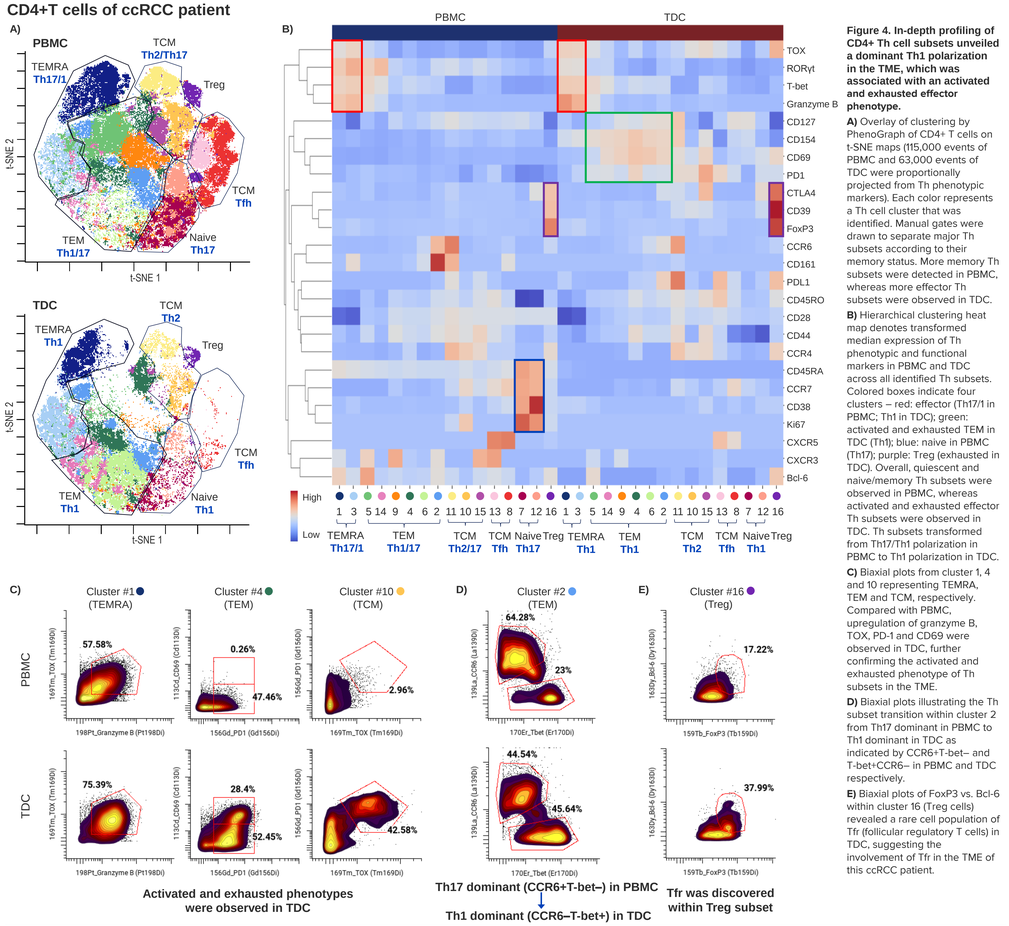

Figure 4. In-depth profiling of CD4+ Th cell subsets unveiled a dominant Th1 polarization in the TME, which was associated with an activated and exhausted effector phenotype.

A) Overlay of clustering by PhenoGraph of CD4+ T cells on t-SNE maps (115,000 events of PBMC and 63,000 events of TDC were proportionally projected from Th phenotypic markers). Each color represents a Th cell cluster that was identified. Manual gates were drawn to separate major Th subsets according to their memory status. More memory Th subsets were detected in PBMC, whereas more effector Th subsets were observed in TDC.

B) Hierarchical clustering heat map denotes transformed median expression of Th phenotypic and functional markers in PBMC and TDC across all identified Th subsets. Colored boxes indicate four clusters – red: effector (Th17/1 in PBMC; Th1 in TDC); green: activated and exhausted TEM in TDC (Th1); blue: naive in PBMC (Th17); purple: Treg (exhausted in TDC). Overall, quiescent and naive/memory Th subsets were observed in PBMC, whereas activated and exhausted effector Th subsets were observed in TDC. Th subsets transformed from Th17/Th1 polarization in PBMC to Th1 polarization in TDC.

C) Biaxial plots from cluster 1, 4 and 10 representing TEMRA, TEM and TCM, respectively. Compared with PBMC, upregulation of granzyme B, TOX, PD-1 and CD69 were observed in TDC, further confirming the activated and exhausted phenotype of Th subsets in the TME.

D) Biaxial plots illustrating the Th subset transition within cluster 2 from Th17 dominant in PBMC to Th1 dominant in TDC as indicated by CCR6+T-bet– and T-bet+CCR6– in PBMC and TDC respectively.

E) Biaxial plots of FoxP3 vs. Bcl-6 within cluster 16 (Treg cells) revealed a rare cell population of Tfr (follicular regulatory T cells) in TDC, suggesting the involvement of Tfr in the TME of this ccRCC patient.

Diverse and interrelated cytokine profiling of Th cells confirms Th17/Th1 polarization in ccRCC PBMC

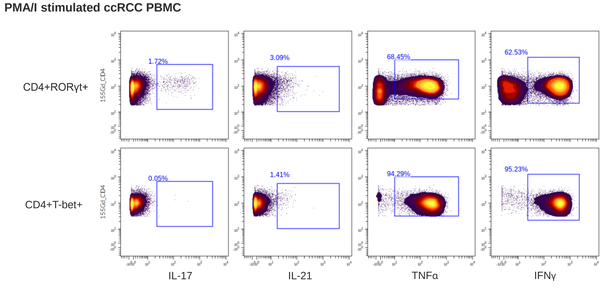

Figure 5. Cytokine functional profiling within CD4+ Th subsets confirmed the Th17/Th1-dominant phenotype in ccRCC PBMC.
To further confirm the Th polarization profiling, PBMC from the ccRCC patient were stimulated with PMA and ionomycin (PMA/I) for five hours. Th17-associated cytokines Th17 and Th21 and Th1-associated cytokines IFNγ and TNFα were evaluated within CD4+RORγt+ and CD4+T-bet+ cell subsets, respectively. As expected, nearly all CD4+T-bet+ cells produced IFNγ and TNFα, but not IL-17. While CD4+RORγt+ cells produced IL-17 exclusively, a substantial amount of these cells produced IFNγ and TNFα as well. The diverse and interrelated cytokine profiling observed is in line with Figure 4B, in which multiple Th subsets of PBMC expressed both RORγt and T-bet, supporting the Th17/Th1 polarization in ccRCC PBMC.

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