Supplemental methods

Section I: MIFlowCyt Document

Section II: Cell Interaction Analysis

Section I: MIFlowCyt Document

1. Experiment Overview

1.1. Purpose: The goal of these studies was to evaluate lymphoid populations within renal cell carcinoma (RCC) patient tumors following radiation treatment as previously described (Singh et al. Clinical Cancer Research, 2017). Based on the findings of increased clonality of T cells in irradiated RCC (Chow et al. PNAS, 2020), we hypothesized that radiation increases levels of activated and proliferating $CD8^+$ T cells in tumor microenvironment.

1.2. Keywords: Renal Cell Carcinoma, High-dimensional flow cytometry, Lymphocytes, Radiation

1.3. Experiment Variables: Six nephrectomy only (control) RCC patient tumor samples were compared to five radiation treated patient tumors (nephrectomy four weeks post radiation).

1.4. Organization:

1.4.1: Name: Roswell Park Comprehensive Cancer Center

1.4.2: Address: Roswell Park Comprehensive Cancer Center, Elm and Carlton Streets, Buffalo, NY, 14263

1.5. Primary Contact:

1.5.1. Name: Jason Muhitch

1.5.2. Email Address: jason.muhitch@roswellpark.org

1.6. Date: The first tumor sample was collected on July 11, 2013. Final analysis of spectral flow cytometry CD8 data was completed in February 2023.

1.7. Conclusions: We identified increased levels of four distinct T cell populations in RCC patient tumor that had been treated with radiation therapy. Two of these populations showed increased levels of markers for exhaustion and proliferation.

1.8. Quality Control Measures: Antibodies used in the assay were tittered and saturating concentrations were used. Pilot experiments were performed prior to the main assay to validate the functionality of the all the antibodies in the panel using PBMCs and tumor samples. The Cytek Aurora instrument was QC daily using SpectraFlo® QC Beads (Cytek; Cat #N7-97355) to adjust laser performance based on baseline settings, laser delay, and to align height and area scaling factors for optimal signal resolution to ensure consistent performance was achieved daily.

2. Flow Sample/Specimen Details

2.1. Sample/Specimen Material Description : Renal cell carcinoma patient tumors with or without radiation treatment were resected, processed to single cell suspension, and cryopreserved. Preserved samples were thawed and used for flow cytometry analysis. Sample source, patient age, gender, phenotype, and treatment

Sl No	Sample Description	Source	Organism	Age	Gender	Phenotype Tumor Grade	Treatment
1	Single cell suspension	ccRCC tumors	*Homo sapiens*	53	F	3	None
2	Single cell suspension	ccRCC tumors	*Homo sapiens*	63	M	3A	None
3	Single cell suspension	ccRCC tumors	*Homo sapiens*	61	F	1B	None
4	Single cell suspension	ccRCC tumors	*Homo sapiens*	55	M	2B	None
5	Single cell suspension	ccRCC tumors	*Homo sapiens*	53	M	3	None
6	Single cell suspension	ccRCC tumors	*Homo sapiens*	70	M	1B	None
7	Single cell suspension	ccRCC tumors	*Homo sapiens*	53	M	2A	15Gy Radiation
8	Single cell suspension	ccRCC tumors	*Homo sapiens*	67	M	3A	15Gy Radiation
9	Single cell suspension	ccRCC tumors	*Homo sapiens*	67	M	3A	15Gy Radiation
10	Single cell suspension	ccRCC tumors	*Homo sapiens*	62	M	3A	15Gy Radiation
11	Single cell suspension	ccRCC tumors	*Homo sapiens*	57	F	2A	15Gy Radiation

ccRCC: clear cell Renal Cell Carcinoma

are described in the table.

2.2. Sample Characteristics: Renal cell carcinoma tumors are heavily infiltrated by immune cells. Evaluation of patient RCC tumors by mass cytometry has shown large cohorts of lymphocytes and myeloid populations.

2.3. Sample Treatment(s) Description: Patients received radiation dose (15Gy) as previously described (Singh et al. Clinical Cancer Research, 2017). Tumors were resected 4 weeks post radiation and processed to single cell suspension via enzymatic digestion prior to cryopreservation as previously described (Chow et al. PNAS, 2020). Single cell suspensions from non-radiated patient tumors were used as controls.

2.4. Fluorescence Reagent(s) Description: Fluorescent reagent information (characteristic being measured, analyte, analyte detector, analyte reporter, clone, manufacturer, catalog number and dilution used) is described in the table.

Sl No	Characteristic being measured	Analyte	Analyte detector (antibody)	Analyte reporter (flurochrome)	Clone	Supplier	Catalog	Dillution
1	Cell surface protein	CCR5	Anti-CCR5	BUV395	2D7	BD	565224	1 in 50
2	Cell surface protein	Live/Dead	L/D Blue Stain	LIVE/DEAD™ Blue		ThermoFisher	L34962	1 in 500
3	Cell surface protein	CD31	Anti-CD31	BUV496	L1331.1	BD	749833	1 in 200
4	Cell surface protein	CD14	Anti-CD14	BUV563	МфР9	BD	741441	1 in 100
5	Cell surface protein	CD261	Anti-CD261	BUV615	S35-934	BD	752308	1 in 50
6	Cell surface protein	CD11c	Anti-CD11c	BUV661	B-ly6	BD	612967	1 in 200
7	Cell surface protein	CD56	Anti-CD56	BUV737	NCAM16.2	BD	612766	1 in 200
8	Cell surface protein	CD45RO	Anti-CD45RO	BUV805	UCHL1	BD	748367	1 in 100
9	Intracellular protein	CD68	Anti-CD68	BV421	Y1/82A	BD	564943	1 in 50
10	Cell surface protein	CD27	Anti-CD27	SB436	O323	ThermoFisher	62-0279-42	1 in 200
11	Cell surface protein	CD8	Anti-CD8	Pacific Blue	SK1	Biolegend	980906	1 in 200
12	Intracellular protein	KI67	Anti-KI67	BV480	B56	BD	566109	1 in 50
13	Cell surface protein	CD204	Anti-CD204	BV510	U23-56	BD	742439	1 in 50
14	Cell surface protein	CD4	Anti-CD4	Pacific Orange	RPA-T4	ThermoFisher	79-0049-42	1 in 100
15	Cell surface protein	CD28	Anti-CD28	BV605	CD28.2	Biolegend	302967	1 in 100
16	Cell surface protein	CXCR3	Anti-CXCR3	BV650	G025H7	Biolegend	353729	1 in 50
17	Cell surface protein	CCR6	Anti-CCR6	BV711	G034E3	Biolegend	353435	1 in 100
18	Cell surface protein	CCR4	Anti-CCR4	BV750	1G1	BD	746980	1 in 50
19	Cell surface protein	CCR7	Anti-CCR7	BV785	G043H7	Biolegend	353229	1 in 50
20	Cell surface protein	CD57	Anti-CD57	FITC	NK-1	BD	555619	1 in 200
21	Cell surface protein	CD3	Anti-CD3	Spark Blue 550	SK-7	Biolegend	344852	1 in 100
22	Cell surface protein	CD45	Anti-CD45	Nova Blue 610	2D1	ThermoFisher	H005T03B05	1 in 100
23	Cell surface protein	PD1	Anti-PD1	PerCP-Cy5.5	EH12.1	BD	561273	1 in 50
24	Cell surface protein	LAG3	Anti-LAG3	PerCP-eFluor710	3DS223H	ThermoFisher	46-2239-42	1 in 50
25	Intracellular protein	TOX	Anti-TOX	PE	REA473	Miltenyi	130-120-716	1 in 50
26	Cell surface protein	TIGIT	Anti-TIGIT	PE/Dazzle 594	A15153G	Biolegend	372716	1 in 50
27	Cell surface protein	CD25	Anti-CD25	PE-Fire/640	M-A251	Biolegend	356148	1 in 50
28	Cell surface protein	CD33	Anti-CD33	PE-Cy5	HIM3-4	ThermoFisher	15-0339-42	1 in 100
29	Cell surface protein	CD127	Anti-CD127	PE-Fire 700	A019D5	Biolegend	351366	1 in 100
30	Cell surface protein	CD253	Anti-CD253	PE Cy7	RIK-2	Biolegend	308216	1 in 50
31	Cell surface protein	TIM3	Anti-TIM3	APC	F38-2E2	ThermoFisher	62-3109-42	1 in 50
32	Cell surface protein	CD45RA	Anti-CD45RA	Spark NIR 685	HI100	Biolegend	304168	1 in 200
33	Cell surface protein	CX3CR1	Anti-CX3CR1	R718	G025H7	Biolegend	353730	1 in 50
34	Cell surface protein	CD38	Anti-CD38	APC-eFluor780	HIT2	ThermoFisher	47-0389-42	1 in 200
35	Cell surface protein	HLA-DR	Anti-HLADR	APC/Fire 810	L243	Biolegend	307674	1 in 100

3. Instrument Details

3.1. Instrument Manufacturer: Cytek Biosciences

3.2. Instrument Model: Cytek™ Aurora

3.3.1. Instrument Configuration and Settings:

3.3.1.1. Flow cells: Clean Flow Cell, 12x75 mm polystyrene and polypropylene tubes

3.3.1.2. Fluidics Information: Sample flow rates – Low (15µL/min), Medium (30µL/min), High (60µL/min).

3.3.2. Light Source(s)

3.3.2.1. Light source type: Lasers

3.3.2.2. Light source excitatory wavelength: 335 nm, 405 nm, 488 nm, 561 nm, 640 nm

3.3.2.3. Light source power at excitatory wavelength: 335 nm (20 mW), 405 nm (100 mW), 488 nm (50 mW), 561 nm (50 mW), 640 nm (80 mw)

3.3.2.4. Light source beam: Flat-Top laser beam profile with narrow vertical beam height optimized for small particle detection.

3.3.3. Excitation optics configuration:

Ultraviolet detector module: 16 channels unevenly spaced bandwidth from 365-829 nm.

Violet detector module: 16 channels unevenly spaced bandwidth from 420-829 nm.

Blue detector module: 14 channels unevenly spaced bandwidth from 498-829 nm.

Yellow-Green detector module: 10 channels unevenly spaced bandwidth from 567-829 nm.

Red detector module: 8 channels unevenly spaced bandwidth from 652-829 nm.

3.3.4. Optical Filters:

FSC: high-performance semiconductor detector with 488nm bandpass filter
SSC: two high-performance semiconductor detectors with 405nm and 488nm bandpass filters

3.3.5. Optical detectors: Proprietary high sensitivity Coarse Wavelength Division Multiplexing (CWDM) semiconductor array per laser.

3.4 Other Information:

POWER: 100-140 VAC, 15A or 200-250 VAC, 10A
HEAT DISSIPATION: 500 W with all solid-state lasers
TEMPERATURE: 15–28°C
HUMIDITY: 20%-85% relative non-condensing
AIR FILTERING: No excessive dust or smoke
LIGHTING: No special requirements

4. Data Analysis Details

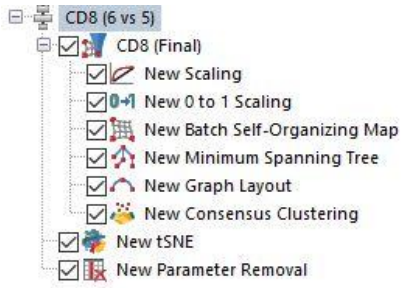
4.1. List-mode Data File: Both Raw and Unmixed FCS files for 11 patients will be available for download at ImmPort under access code SDY1998 post acceptance.

4.2. Compensation Details: Live spectral unmixing was performed using SPECTROFLO SOFTWARE during sample acquisition. A second compensation step was performed using FCS Express 7. Compensation matrix is provided with the FCS datafiles.

4.3. Data Transformation Details:

4.3.1. Purpose of Data Transformation: FlowSOM clustering was used to performed unsupervised clustering for the purpose of identifying subsets within CD8+ cells. tSNE analysis (dimensionality reduction) was done to visualize these clusters on a 2D plot. Both analyses were performed using the pipeline feature of FCS Express 7.

4.3.2. Data Transformation Description: The steps used in the pipeline are described below.

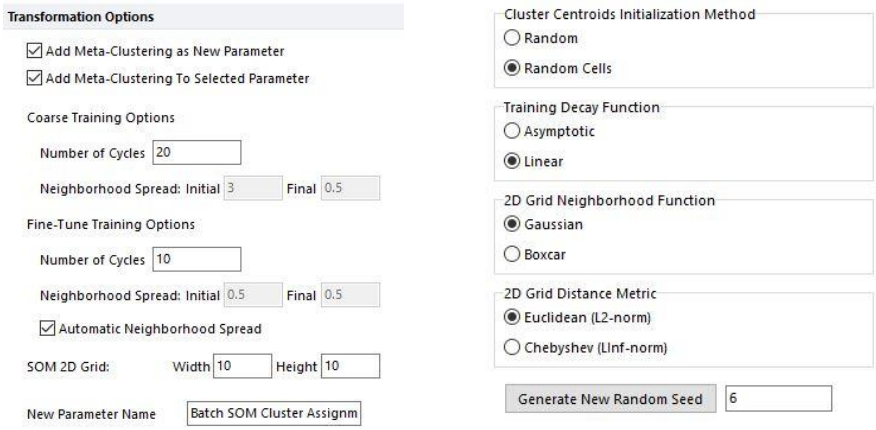


FlowSOM: To identify CD8+ subsets, all CD8+ cells from each unmixed patient data files were first exported out as new FCS files. Gating strategy is highlighted in supplementary figure 2a. 10000 events from each CD8+ data files were then concatenated (using FCS Express) to generate one FCS file with all patient data combined. This file was then used to perform FlowSOM and tSNE analyses.

FlowSOM hierarchical clustering to identify subpopulations was performed using CCR4, CCR5, CCR6, CCR7, CD25, CD27, CD28, CD38, CD45-RA, CD45-RO, CD56, CD57, CD127, CXCR3, CX3CR1, HLA-DR, KI67, LAG3, PD-1, TIGIT, TIM3, and TOX markers.

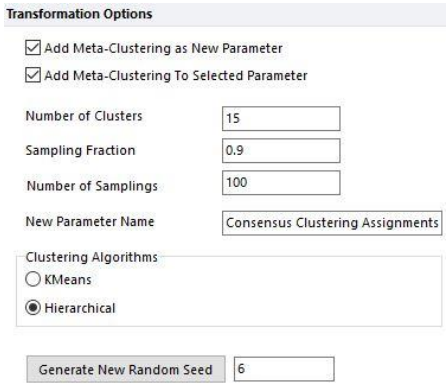
New Scaling: The specified parameters were scaled using the new scaling step. Additional 0-1 scaling step was performed to visualize the data on one scale.

Batch Self-Organizing Map: 0 to 1 scaled parameter for each marker were used to run the batch self-organizing map step. The following settings were used:

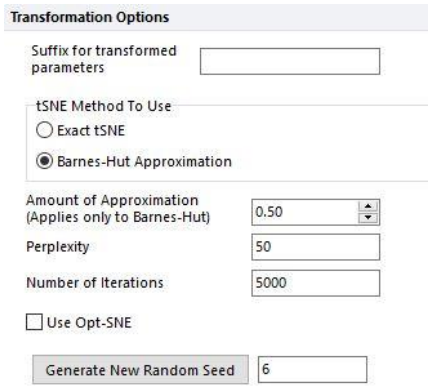


Minimum spanning tree and **Graph Layout** were generated with default software set up.

Consensus clustering was performed with following settings:



tSNE: 0 to 1 scaled **values** of specified parameters were used to generate tSNE plots. Following settings were used to generate the plots:



4.3.3. Other relevant data: All spectral analyses was performed using FCS Express7 software package (De 517 Novo Software, version 7.08.0018)

4.4. Gating (Data Filtering) Details:

4.4.1. Gate Description: Gating strategy to export out $CD8^+$ T cell events from all patient files is shown in supplemental figure 2a.

4.4.2. Gate Statistics: Frequencies for each sub-cluster for each patient is highlighted in supplemental table 4.

4.4.3. Gate Boundaries: Gates for different clusters on tSNE plots were generated using consensus clustering assignments identified via FlowSOM.

Section II: Cell interaction analysis

Relevant target cell genes for responses of interest were identified from established pathways and relevant publications (KEGG 04612, Antigen processing and presentation; KEGG 04210, Apoptosis; KEGG 05140, Leishmaniasis; Dror et al. 2007)[35]; second, potential ligands from source cells were identified by a ranked by Pearson correlation calculated based on cross referencing top-expressed source cell genes with a ligand-target-matrix[20]; potential target cell receptors were identified by cross-referencing top-expressed target cell genes with a ligand-receptor-network[20] of established physical interactors of predicted source cell ligands.