Tumor-infiltrating T cells: Complete workflows allow faster and improved flow cytometric analysis of syngeneic mouse tumors

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Introduction

Immunotherapy approaches that engage T cells to attack tumors have proven clinical efficacy and tremendous potential in multiple cancers. Syngeneic mouse tumor models represent the gold standard to develop and analyze effects of immunotherapy, as they possess a fully competent immune repertoire. However, the phenotypic and functional analysis of tumor-infiltrating leukocytes (TILs) is technically challenging and labor intensive. The number of tumor-infiltrating T cells can be very low and small subpopulations might escape analysis as they get lost in the background noise. Importantly, tumor-infiltrating T cells are embedded in a cellular environment where antigen is abundant and surrounding cells express highly immunomodulatory molecules, such that unbiased cell-intrinsic functional characterization is hindered. When working with large cohort sizes, even immunophenotyping of TILs by flow cytometry is usually time consuming and data processing can be laborious. Therefore, it is fundamental to use effective tools to streamline the workflow and generate reliable data.

Material and methods

1 Complete workflows combining tissue storage, tissue dissociation, T cell isolation, and flow cytometric phenotyping

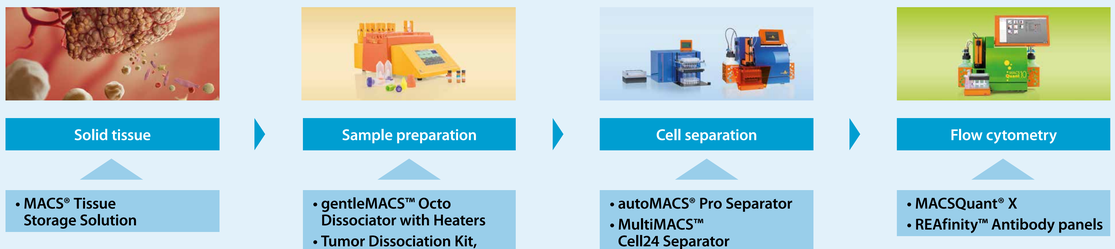

Figure 1

Panel 1			Panel 2		
Specificity	**Clone**	**Fluorochrome**	**Specificity**	**Clone**	**Fluorochrome**
CD8b	REA793	VioBlue®	CD8b	REA793	VioBlue
Viability dye		Viobility™ 405/520	Viability dye		Viobility 405/520
CD223 (Lag-3)	REA776	VioBright™ 515	CD103	REA789	VioBright 515
Anti-TIM-3	REA602	PE	CD183 (CXCR3)	REA724	PE
CD4	REA604	PE-Vio® 615	CD4	REA604	PE-Vio 615
CD137 (4-1BB)	REA936	PE-Vio 770	CD137 (4-1BB)	REA936	PE-Vio 770
CD279 (PD1)	REA802	APC	CD39	REA870	APC
CD44	REA664	APC-Vio 770	CD44	REA664	APC-Vio 770

Table 1

Results

1 Tissue storage solution maintained cell viability and phenotype up to 48 h after tissue collection

Tissues were dissociated freshly or stored in different solutions at the indicated temperatures for 48 h before dissociation. Graphs depict cell yield and viability. MACS® Tissue Storage Solution has been tested and validated on a variety of human and murine tissues including tumors, skin, heart, spleen, brain, and skeletal muscle. Results are shown for mouse lung tissue.

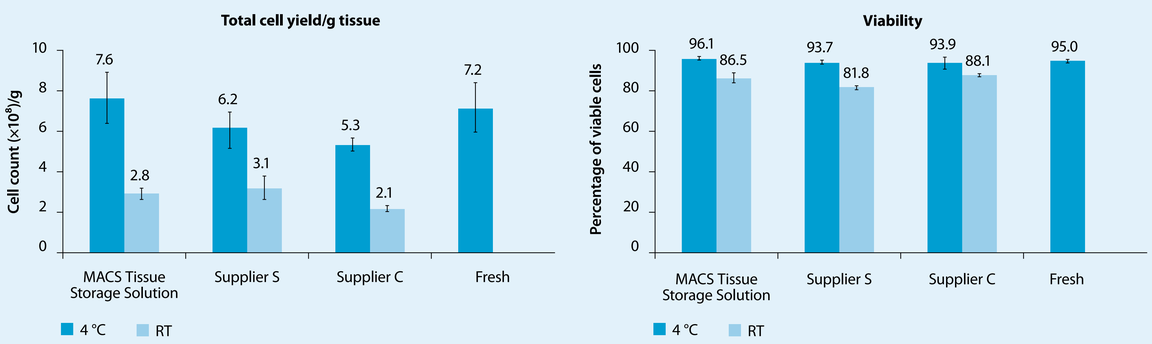

Figure 2

2 Optimal tumor dissociation was essential for analysis of critical tumor-infiltrating T cell subpopulations

B16-F10 tumors were collected and dissociated using the gentleMACS™ Octo Dissociator with Heaters in the presence (fig. 3A) or absence (fig. 3B) of Tumor Dissociation Kit enzymes. Cells were subsequently labeled with REAfinity™ Antibodies (table 1). Phenotypic analysis revealed that optimal enzymatic dissociation was essential for analysis of critical tumor-specific subpopulations such as PD1^{hi}TIM-3$^+$Lag-3$^+$CD39$^+$CD8$^+$ T cells present in B16-F10 tumors. Cells were gated on i) viable cells, ii) FSC/SSC, iii) single cells, iv) CD8$^+$ cells, and v) CD44$^+$ cells.

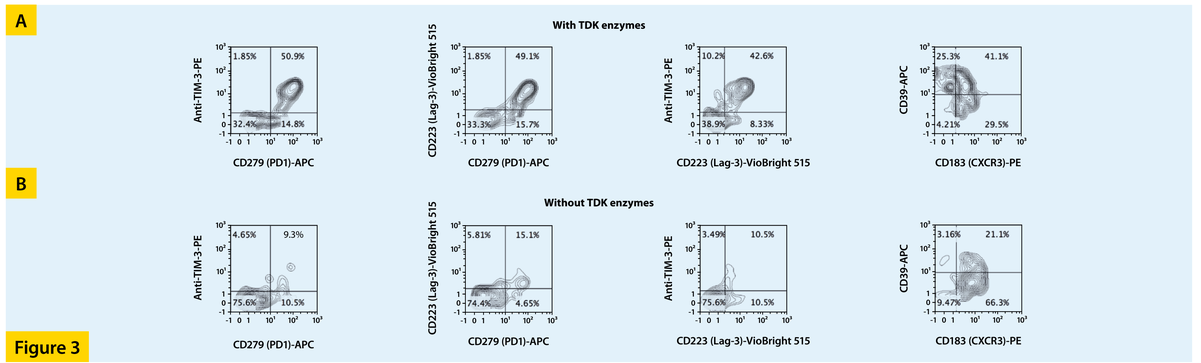

Figure 3

3 Magnetic isolation of tumor-infiltrating T cells greatly reduced time of analysis

We have developed new CD4$^+$, CD8$^+$, and pan T cell–specific reagents for magnetic cell isolation by MACS Technology directly from dissociated tumor tissue. We used optimized panels of fluorochrome-conjugated REAfinity Antibodies to phenotypically characterize tumor-infiltrating T cells (table 1). Bulk tumor cells (left) or magnetically isolated CD8$^+$ T cells (right) from B16-OVA tumors were labeled and analyzed by flow cytometry using the MACSQuant® Analyzer 10. Cells were gated on i) viable cells, ii) FSC/SSC, iii) single cells. Magnetic cell isolation resulted in purities above 80%, which represents an up to 500-fold enrichment of the target cell population, dramatically decreasing time of analysis (table 2).

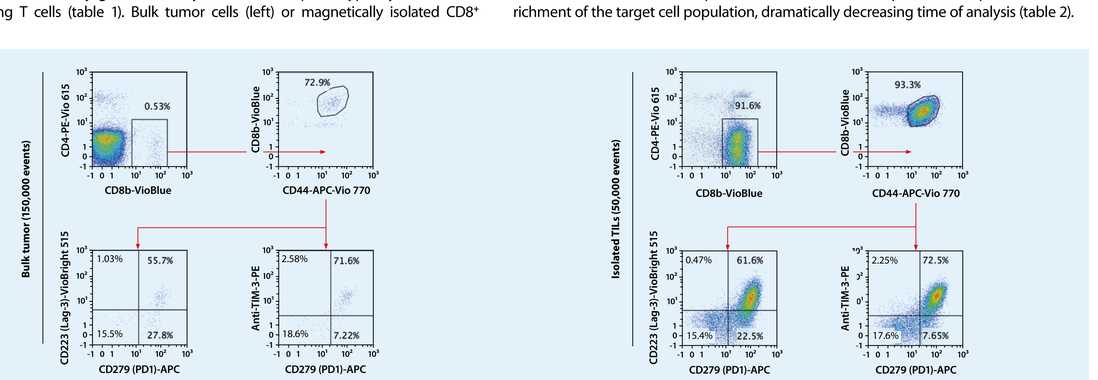

Figure 4

Cell type	Cells to analyze	Frequency	Events to collect	Flow cytometry time/sample*	Total flow cytometry time**
CD4$^+$ T cells					
Bulk	5,000	0.18%	7.96×10^6	66.3 min	>22 h
Isolated***	5,000	92.4%	5.41×10^4	0.5 min	~20 min
CD8$^+$ T cells					
Bulk	5,000	0.96%	2.80×10^6	23.3 min	>7.5 h
Isolated***	5,000	80.5%	4.37×10^4	0.4 min	~17 min
T cells					
Bulk	10,000	6.24%	8.13×10^5	6.8 min	>2.4 h
Isolated***	10,000	84.9%	3.24×10^4	0.3 min	~16 min

* Flow rate: 2,000 events/s
** Considering 20 samples. Includes automated mixing and rinsing between samples on the MACSQuant X
*** Isolation using CD8 (TIL), CD4 (TIL), or CD4/CD8 (TIL) MicroBeads, respectively

Table 2

Conclusion

We have optimized workflows that include standardized processing of tumor samples, newly developed tools for (semi-)automated magnetic isolation of tumor-infiltrating T cells, and automated flow cytometric analysis. These workflows greatly reduce experimental time (table 3) and allow the performance of more complex experimental setups. We believe that the use of these innovative tools and workflows can significantly increase the quality of the data obtained in immuno-oncology and immunotherapy research.

Workflow step	Traditional protocol	Time required (for 20 samples)	Optimized protocol	Time required (for 20 samples)
Tissue resection	Resection performed on the same day	100 min	Resection performed the day before; samples preserved overnight in Tissue Storage Solution	100 min
Sample preparation	Manual dissociation, Enzymatic digestion	105 min	Fully automated tissue dissociation, Cell counting	61 min
Cell isolation	Density gradient centrifugation, Cell counting	120 min	Automated TIL enrichment	32 min
Cell analysis	Flow cytometry of bulk tumor cells	160–1220 min	Flow cytometry of enriched TILs	30 min
		Σ 485–1545 min*		**Σ 123 min****

* Total time required to complete the entire workflow in one day; all processing steps need to be done in a row.
** Total time required to perform sample preparation, cell isolation, and cell analysis in one day; tissue resection can be done on a different day.

Table 3